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La valorisation du lactosérum par fermentation : description et facettes d'une nouvelle technologie.

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Résumé

Le lactosérum est une ressource disponible en très grande quantité et ses protéines présentent des avantages nutritifs, technologiques et commerciaux indéniables. Une nouvelle technologie de fermentation fut développée afin d'exploiter les pleins potentiels de cette ressource. Cette plate-forme technologique permet de produire une nouvelle gamme d'actifs biologiques aux applications très variées, sous la forme de matrices protéiques malléables (MPM).

Les MPM sont composés d'environ 80% d'eau, 8% de protéines, 6% de minéraux (dont 1,8 % de calcium), 5 % de glucides, 1% de gras et $6X10^{11}$ bactéries par 100g. Ces matrices permettent d'allier des fonctions technologiques (épaississantes, émulsifiantes et texturisantes), aux fonctions nutritives et santés. Elles permettent la formulation de produits légers (yogourt, mayonnaise, beurre) et de produits santé (modulation immunitaire et amélioration du bilan lipidique). Un brevet, en dépôt international, est aussi présenté pour la protection du procédé de production, du produit (MPM) et des applications de cette nouvelle technologie.

De nouvelles souches bactériennes furent isolées de grains de kéfir pour le développement de cette technologie. Ces souches appartiennent à l'espèce *Lactobacillus kefiranofaciens*. Parmi celle-ci, la souche R2C2 représente la souche la plus robuste jamais décrite de cette espèce et la souche INIX est surproductrice d'exopolysaccharide. Quatre nouvelles souches de cette espèce furent décrites et comparées aux données actuelles de la littérature. L'appartenance aux sous-espèces *kefiranofaciens* et *kefirgranum* est discutée, des amorces d'ADN spécifiques à l'espèce furent développées à partir du gène de l'ARN ribosomiale 16S et l'appartenance de cette espèce au grand groupe des acidophilus souligne son caractère probiotique qui fut démontré *in-vitro* et *in-vivo*. Un brevet, en dépôt international, est aussi présenté pour la protection des aspects probiotiques de ces souches.

Outre les composantes et les applications de cette technologie, un axe exploratoire de production d'enzymes naturelles des streptomycètes fut aussi développé dans le but éventuel d'utiliser ces enzymes pour la production de nouveaux MPM. Les travaux réalisés ont mené à la purification et à la caractérisation sommaire d'une nouvelle enzyme (SLP25). Ces travaux ont aussi démontré que la forte relation entre l'activité des protéases et l'adaptation écologique des organismes en fait un groupe d'un grand intérêt pour l'appréciation des processus évolutifs. L'analyse phylogénétique de cette nouvelle enzyme a permis de proposer la formation d'une sous-famille et de démontrer que SLP25 est en fait un exemple d'échange latéral d'un gène d'un procaryote à un eucaryote.

L'avenir de cette nouvelle technologie de valorisation du lactosérum et de ses produits est discuté en conclusion. Un article, deux manuscrits déposés, ainsi que deux brevets, sont présenté dans la section « Autres contributions ». Un premier manuscrit présente les effets anti-inflammatoires de *L. kefiranofaciens*. Un article de revue introduit la notion de « Lactoceutique » comme produit d'alimentation fonctionnelle ou nutraceutique d'origine laitier et un autre manuscrit présente les résultats de l'utilisation des MPM comme application cosméceutique. Les deux brevets portent sur l'utilisation des EPS de *L. kefiranofaciens* pour la formulation de médicament; un décrit son utilisation pour la formulation en générale et un autre décrit une application particulière pour le traitement du cancer. L'invention permet de solubiliser et d'améliorer l'effet de certains médicaments soit par synergie ou par augmentation de l'absorption.

Bonne lecture

Eric Simard

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Avant-propos

Ces travaux prennent origine d'un passage direct de la maîtrise en ressource renouvelable de l'Université du Québec à Chicoutimi. Afin de permettre de bien comprendre la démarche poursuivie, une brève explication des travaux effectués durant la maîtrise sera fournie et le projet sera présenté en continuité avec l'ensemble des activités de recherches reliées. Le projet de maîtrise portait sur le développement d'une nouvelle technologie de valorisation du lactosérum. Il visait à démontrer qu'il est possible de récupérer les protéines du lactosérum, suite à une fermentation avec une culture mixte de levures et de bactéries lactiques, en ajustant le pH et en récupérant les agglomérats produits par centrifugation. L'ensemble des recherches et activités reliées au projet « Lactosérum » avec la Fromagerie Boivin, émane d'une démarche entièrement originale. Le programme de recherche développé de 1995 à 1999 portait sur l'utilisation des grains de kéfir pour permettre la récupération des protéines solubles du lactosérum (maîtrise année 1998-1999). L'élaboration du projet de recherche, la recherche de subvention et de financement, la réalisation d'une importante revue de littérature, la coordination des chercheurs et du personnel impliqué ainsi que la réalisation des expérimentations furent toutes des tâches réalisées par l'étudiant avant et durant l'année à la maîtrise.

La culture des grains de kéfir en lactosérum demande une adaptation particulière pour que celle-ci soit productive (augmentation de la masse des grains). Les grains de kéfir constituent un microhabitat complexe structuré principalement par un exopolysaccharide bactérien, le kéfiran. Les grains sont de taille variable, d'environ 1 millimètre à 1 centimètre, et ressemblent à l'extrémité d'un chou-fleur. Une microflore s'y distribue de façon non uniforme selon la taille des grains et la préférence des microorganismes pour la disponibilité de l'oxygène. Les interactions entre les différentes populations microbiennes sont nombreuses allant de la protection de leur habitat par la production d'agents antimicrobiens, à la production de facteurs de croissance bénéfiques à différentes populations.

Les travaux réalisés ont porté sur la fermentation du lactosérum par des grains de kéfir sur des périodes de 48 heures à des volumes de 250 ml à 200 L. Ces travaux ont permis de stabiliser les proportions des différentes populations bactériennes de façon à obtenir une croissance des grains constante et appropriée à la récupération des protéines du lactosérum. Plus la croissance des grains est rapide, plus la proportion des protéines récupérées est importante. La croissance des grains est fortement dépendante de la proportion des populations de levures et de bactéries lactiques.

Les conditions développées (secret industriel) permettaient la récupération de 50% des protéines du lactosérum avec une augmentation de 50% de la masse des grains de kéfir à toutes les 48 heures. Toutefois, cette période de fermentation demeure trop longue pour une application industrielle de récupération des protéines. De plus, les caractéristiques du produit obtenu ne sont pas suffisamment constantes dues aux variations des proportions des populations de bactéries et de levures du consortium utilisé. La conservation du produit est aussi problématique du à la production de CO_2 par les levures et les bactéries hétérofermentaires. Cette première version de la technologie demeure intéressante pour la production et la purification de kéfiran à grande échelle et à faible coût.

Une nouvelle version de la technologie a permis de diminuer les temps de fermentation et d'améliorer la constance et la conservation du produit. L'hypothèse élaborée pour y arriver fut que « les microorganismes responsables de la bonne récupération des protéines peuvent être isolés du consortium et utilisés de façon séparée ». Étant donné la corrélation positive entre la croissance des grains et la récupération des protéines, une attention particulière fut portée aux bactéries principalement responsables de la croissance des grains : *Lactobacillus kefiranofaciens.* Quatre souches furent isolées des grains en croissance. Il s'avère que l'utilisation de cette seule espèce permet de régler les problèmes précédents. Les travaux présentés dans cette thèse constituent la suite de ces développements.

Remerciements

Cette thèse représente l'aboutissement de mes travaux de recherche sur le lactosérum, durant lesquels le soutien de nombreuses personnes a été essentiel. L'élaboration de concepts originaux par de nouvelles façons de voir les choses demande de la créativité et du courage. Je voudrais remercier particulièrement les personnes qui ont cru suffisamment en ma créativité pour s'y engager pleinement et celles qui m'ont appuyé et soutenu pour me donner le courage nécessaire pour concrétiser mes visions.

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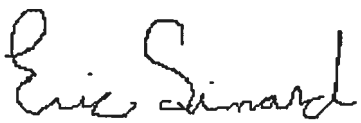
On génère toujours plus de
questions que de réponses.
La qualité d'un grand
chercheur réside à la fois
dans sa capacité à percevoir
les questions et dans sa
capacité à orienter les
développements permettant
des avancés pertinentes.



Revue de littérature

Chapitre 1 : le lactosérum

Le lactosérum provient de la fabrication du fromage. Pour chaque kilogramme de fromage fabriqué à partir de 10 litres de lait, 9 litres de lactosérum sont produits. Ce lactosérum contient de 6 à 7 % de solides totaux, composés à 70 % de lactose (environ cinquante grammes par litre) et 12 % de protéines solubles du lait qui n'ont pas précipitées durant la fabrication du fromage (Meyrath et *al.*, 1979) (tableau 1 et 2). La figure 1 présente les utilisations et volumes mondiaux de lactosérum produit et le tableau 3 présente la production annuelle des 10 principaux producteurs de lactosérum.

1.1 Caractéristiques des différents lactosérums

Les caractéristiques du lactosérum dépendent du type de fromage produit. La concentration en lactose et en protéines de ces lactosérums demeure sensiblement uniforme. La concentration des caséines résiduelles (les fines), des globules de gras et de certains minéraux (principalement les ions calcium, citrate, phosphate et magnésium) varient en fonction des variables de production (Morr et Ha, 1993). La majorité du lactosérum produit au Québec est un lactosérum de cheddar doux. Les tableaux 1 et 2 présentent la composition caractéristique de différents lactosérums.

Les sous-produits de transformation du lactosérum, à partir de l'utilisation des membranes d'ultrafiltration, sont nommés perméats. Ils contiennent une certaine quantité des différentes constituantes du lactosérum, sans les globules de gras et sans les molécules protéiques de haut poids moléculaire ou agrégées, en quantité variable selon la porosité de la membrane utilisée et l'origine du lactosérum (Pearce, 1992).

Tableau 1 : Composition de différents lactosérums doux.

Lactosérums doux				
Constituants	Emmental	Edam/St-Paulin	Camembert	Cheddar
Liquide				
Solides (%)	6.5	5.0	6.5	6.1 – 6.6
pH	6.7	6.5	6.1	6.1
Solides (%)				
Lactose	76	75	75	74 - 81
Protéines	13.5	13.5	13.0	12.8 – 15.2
Cendre	8.0	8.5	9.0	7.6 – 9.2
Acide lactique	1.8	2.0	2.2	2.0
Gras	1.0	1.0	1.0	1.0
Minéraux :				
Calcium	0.6	0.65	0.7	0,61 – 0.78
Phosphore	0.6	0.65	0.7	0.76
Chlorure de sodium	2.5	2.5	2.5	2.5

Adapté de Pearce, 1992.

Tableau 2 : Composition de différents lactosérums acides.

Lactosérums acides				
Constituants	D'acide lactique	D'acide chlorhydrique	D'acide sulfurique	Présure
Liquide				
Solides (%)	6.0	5.8 – 6.1	6.0 – 6.3	6.4 – 6.7
pH	4.0	4.6	4.6	6.6
Solides (%)				
Lactose	65.5	70 - 76	68 - 74	75 - 80
Protéines	12	9.9 – 12.8	9.9 – 11.7	13.8 – 15.5
Cendre	12	11.6 – 19.4	12 - 13	7.0 – 8.0
Acide lactique	10	ND	ND	ND
Gras	0.5	ND	ND	ND
Minéraux :				0,6
Calcium	1.9	2.0 – 2.4	2.0 – 2.4	0.7 – 0.8
Phosphore	1.5	2.8 – 3.2	2.8 – 3.2	1.0 – 1.4
Chlorure de sodium	2.5	7.5	2.5	2.5

Adapté de Pearce, 1992.

Tableau 3 : Production annuelle des principaux producteurs mondiaux de lactosérum).

Compagnie	**Volume annuel estimé de lactosérum liquide (milliard de litre)**
Lactalis/Lactosérum	5.0
Borculo-Domo	4.2
Kraft	>4.0
Leprino	>4.0
Arla Foods	4.0
DFA	>3.0
DMV	3.0
Glanbia	3.0
Fonterra	>2.5
Land O'Lakes	2.5

(Nielsen et Affertsholt, 2003).

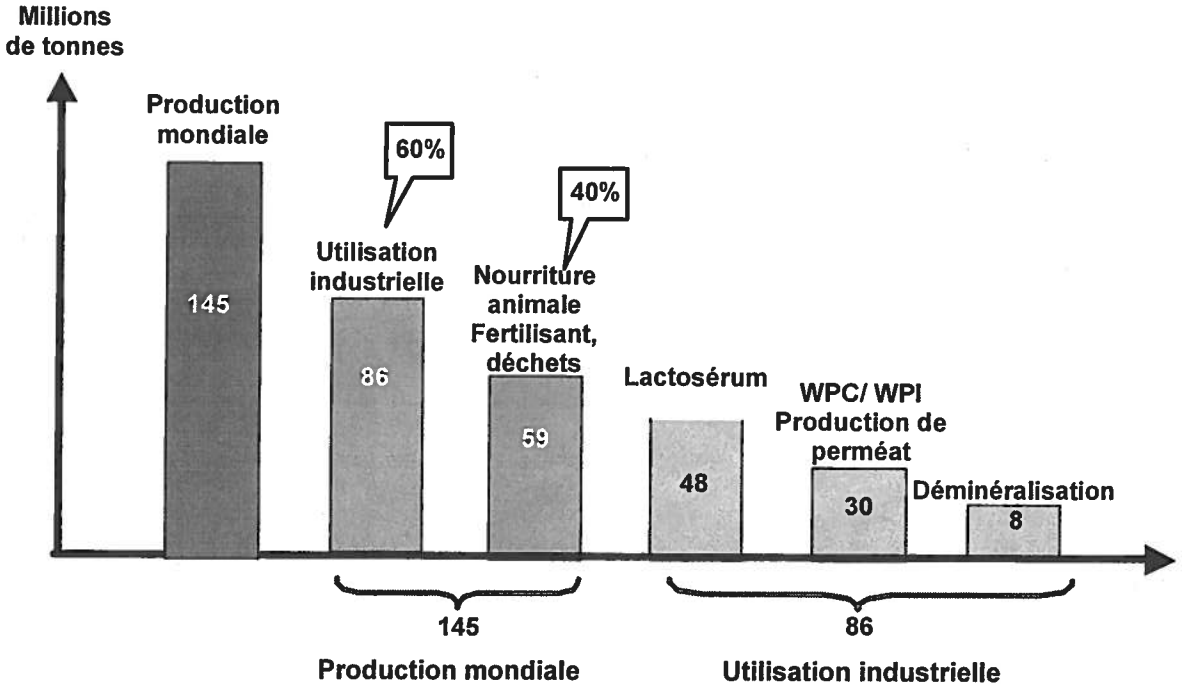


Figure 1 : Volumes et utilisations du lactosérum à l'échelle mondial. (Nielsen et Affertsholt, 2003).

1.2 Les procédés de valorisation du lactosérum.

Le lactosérum possède une charge polluante très élevée, de l'ordre de 35 000 à 70 000 unités de DBO5 (CQVB, 1993). La charge polluante acceptée d'un effluent de fromagerie est d'environ 30 unités de DBO5. La production d'une petite fromagerie (environ 7 millions de litres par année) équivaut à la charge polluante d'une ville de 13 000 habitants. C'est pourquoi il est très coûteux de traiter le lactosérum comme un rejet.

Les possibilités de valorisation biotechnologique du lactosérum sont nombreuses (Gonzàlez Siso, 1996 ; Blenford, 1996 ; Puranik et Kanawjia, 1995; Etzel, 2004; Ekici et *al.*, 2005; Vasala et *al.*, 2005; Briczinski et Roberts, 2002; Athanasiadis et *al.*, 2002; Rimada et *al.*, 2001). La figure 2 résume les différents procédés et traitements utilisés pour la valorisation du lactosérum et de ses sous-produits. Par contre, les technologies existantes sont très coûteuses et demandent des volumes élevés pour être rentables. Il s'agit principalement de l'ultrafiltration, de la microfiltration, du séchage par atomisation, de l'osmose inverse, de la séparation par chromatographie et de la production de protéines d'origine unicellulaire (POU) (Rolland, 1993 ; Mann, 1986 ; Smoragiewicz et *al.*, 1986 ; CQVB, 1993 ; Sienkiewicz, 1990, Tyagi et *al.*, 1991; Paquin, 2004; Etzel, 2004).

Les petites et moyennes fromageries, qui produisent moins de 150 000 litres de lactosérum par jour, ne disposent pas des équipements nécessaires à la fabrication d'ingrédients pour l'alimentation humaine. Leurs volumes de production étant trop faibles pour assurer la rentabilité d'une telle valorisation. Bien que le lactose (40 - 50 g / L) soit prédominant dans le lactosérum, par sa valeur nutritionnelle, les composantes ayant le plus de valeur sont les protéines (Kleine et *al.*, 1995; Paquin, 2004). Ces protéines possèdent une valeur nutritionnelle élevée étant donné leur composition en acides aminés essentiels et leurs propriétés fonctionnelles intéressantes (solubilité, propriétés moussantes, émulsifiantes et gélifiantes) pour la formulation de produits alimentaires destinés à l'alimentation humaine.

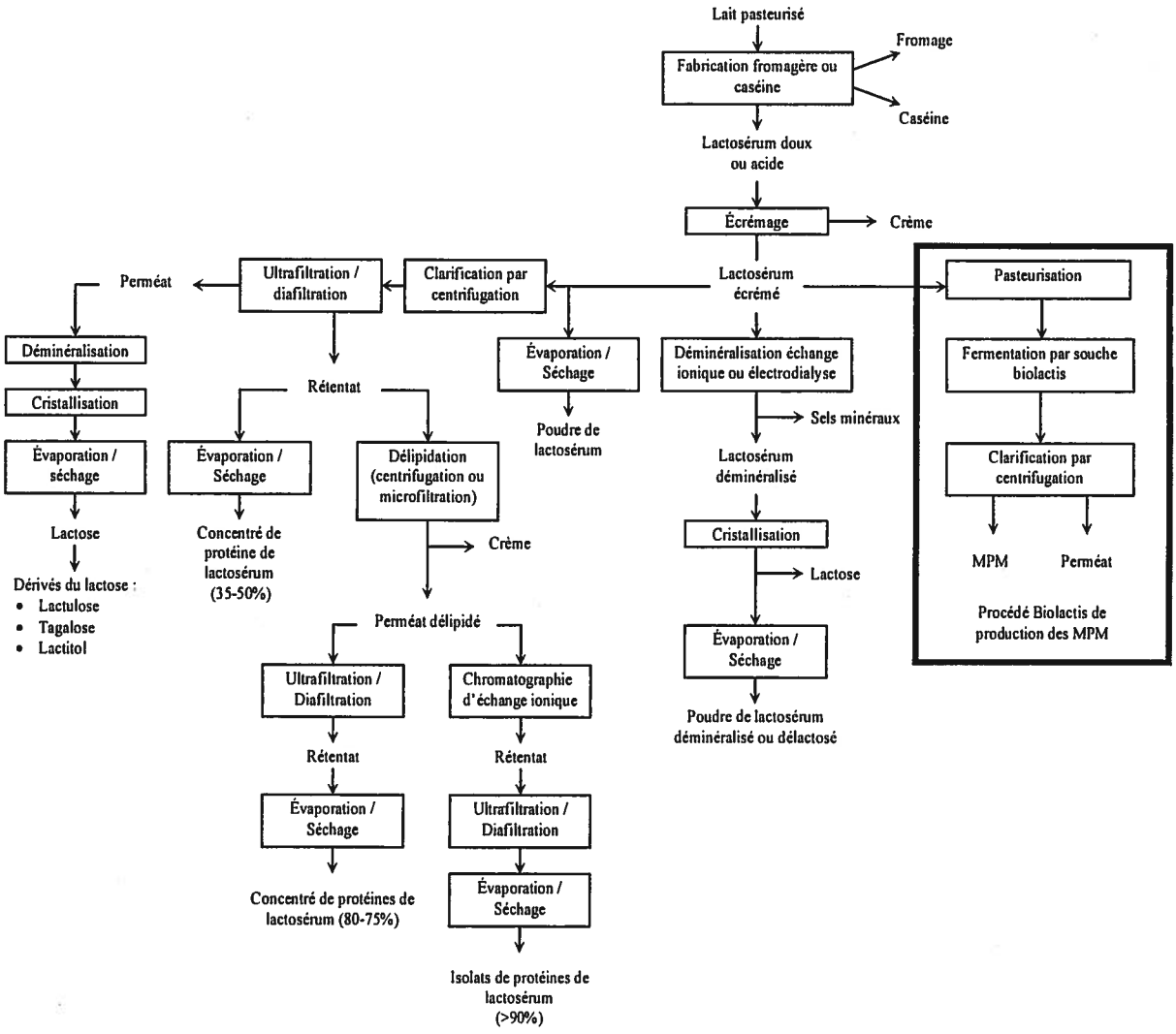


Figure 2 : Procédés et traitements utilisés pour la valorisation du lactosérum et de ses sous-produits. Adapté de Paquin, 2004.

Les principaux procédés permettant de concentrer les protéines du lactosérum sont la production de complexe métaphosphate – protéines, la filtration sur gel de Séphadex et le fractionnement par ultrafiltration ou microfiltration. Les concentrés proprement dit (WPC : "whey protein concentrated") contiennent habituellement de 35 à 50 % de protéines fractionnées par ultrafiltration (pouvant aller jusqu'à 80%). Les isolats de protéines de lactosérum (WPI : "whey protein isolated") contiennent plus de 90 % de protéines et sont obtenus par absorption d'échange d'ions ou par microfiltration (Huffman et Harper, 1999; Morr et *al.*, 1993; Paquin, 2004). Les tableaux 4 et 5 résument la composition chimique et protéique, respectivement, de WPC et WPI commerciaux.

Certains procédés permettent d'obtenir des fractions enrichies en protéines comme la β-lactoglobuline, l'α-lactalbumine, les immunoglobulines, la lactoferrine, la lactoperoxidase ou d'autres protéines présentent sous forme de traces. Les différences au niveau de leur masse moléculaire, de leur concentration ou de leur point isoélectrique (tableau 6) permettent leur séparation. Les procédés industriels utilisés peuvent être séparés en quatre grandes catégories : la précipitation sélective, la filtration sur membrane, l'absorption sélection et l'élution sélective (Etzel, 2004). Ces produits de fractionnement trouvent des applications aussi variées que l'alimentation, les substances antimicrobiennes, les préparations préservatives, pharmaceutiques, cosmétiques et médicales (Regester et Belford, 1999; Huffman et Harper, 1999; Lemieux et *al.*, 2004; Marshall, 2004; Etzel, 2004; Ha et *al.*, 2003; Walzem et *al.*, 2002).

1.3 Les protéines du lactosérum

Les protéines du lactosérum peuvent être séparées en deux groupes : les protéines majeures et les protéines mineures. Parmi les protéines majeures, on retrouve la β-lactoglobuline et l'α-lactalbumine (environ 3,2 et 1.2 g/L respectivement, 65 à 80 % des protéines). Les protéines mineures sont les immunoglobulines, l'albumine bovine sérique, la lactoferrine, la lactoperoxydase, la phosphatase alcaline, la catalase, etc. (Cayot et Lorient, 1998; Lefebvre et *al.*, 1990). Le tableau 7 présente les propriétés chimiques et physico-chimiques des principales protéines du lactosérum.

Tableau 4 : Composition chimique de WPC et WPI.

Composantes	WPC	WPI
Humidité	4.14 – 6.01	2.4 – 5.57
Protéine	72.0 – 76.6	88.6 – 92.7
Azote non protéique	0.93 – 4.56	0.29 – 0.34
Lactose	2.13 – 5.75	0.42 – 0.46
Lipides totaux	3.30 – 7.38	0.39 – 0.67
Phospholipides	0.80 – 1.54	0.11 – 0.31
Minéraux	2.52 – 6.04	1.37 – 2.15
Sodium	0.15 – 1.71	0.36 – 0.42
Potassium	0.07 – 0.46	0.10 – 0.16
Calcium	0.23 – 1.05	0.20 – 0.24
Magnésium	0.02 – 0.40	0.02 – 0.03
Phosphore	0.20 – 1.30	0.05

Adapté de Morr et Ha, 1993.

Tableau 5 : Composition protéique de WPC et WPI commerciaux (%).

Composantes	WPC	WPI
Immunoglobulines	3.8 – 15.4	5.9 – 7.5
Albumine bovine sérique	5.8 – 19.6	7.2 – 10.9
β - lactoglobuline	40.4 – 76.9	67.6 – 74.8
α - lactalbumine	14.5 – 24.8	8.3 – 17.5

Adapté de Morr et Ha, 1993.

Tableau 6 : Propriétés physiques des protéines laitières.

Protéine	Masse moléculaire (kg/mol)	Concentration (g/L)	pI
β - lactoglobuline	18	3.2	5.4
α - lactalbumine	14	1.2	4.4
Albumine bovine sérique	66	0.4	5.1
Immunoglobulines	150	0.7	5-8
Lactoferrine	77	0.1	7.9
Lactoperoxidase	78	0.03	9.6
κ-Caséine	19	3.3	5.8
α-Caséine	24	9.3	5.2
β-Caséine	24	13	4.9/5.3
Glycomacropeptide	8.6	1.5	<3.8

Adapté de Etzel, 2004.

Tableau 7 : Propriétés chimiques et physico-chimiques des principales protéines du lactosérum.

	β - Lg*	α - La*	BSA*	Ig*
Point isoélectrique	5.2	4.2 – 4.5	4.7 – 4.9	5.5 – 8.3
Concentration (g/L)	2 - 4	1.0 – 1.5	0.1 - 0.4	0.4 – 1
Concentration (%, P/P)	56 - 60	18 - 24	6 - 12	6 – 12
Masse moléculaire (kDa)	18, 3	14, 2	69, 0	150 - 1000
Hydrophobicité (kcal / résidu)	1075	1020	995	Variable
Acides aminés / molécule	162	123	582	Variable
Résidus non polaires / molécule	54	44	163	Variable
Résidus cystéines / molécule	5	8	35	Variable
Ponts disulfures / molécule	2	4	17	Variable
Groupement sulfhydrile libre	1	0	1	Variable

Adapté de Morr et Ha, 1993 ; Cayot et Lorient, 1998.
* Abréviation : β - Lg; β-lactoglobuline, α - La; α-lactalbumine, BSA; albumine bovine sérique, Ig; immunoglobuline.

La β-lactoglobuline et l'α-lactalbumine sont de type globulaire. La β-lactoglobuline possède un haut niveau de structuration avec une très forte proportion de feuillets β hydrophobes (43 à 50 % des résidus d'acides aminés). Il existe un compactage βαβ (une hélice α prise en sandwich entre deux feuillets β) qui, combiné à la richesse en feuillets β, assurerait l'existence d'un cœur hydrophobe au sein de la β-lactoglobuline. Cette richesse en feuillets β hydrophobes serait à l'origine d'interactions entre les protéines qui conduisent à l'agrégation ou à la formation de gels thermotropiques (Cayot et Lorient, 1998 ; Otte et *al.*, 1997; Havea et *al.*, 2004 ; Kontopidis et *al.*, 2004).

Cette protéine à de fortes propriétés gélifiantes utilisées pour la production de gels protéiques (Macrae et *al.*, 1993). Ces gels sont des structures complexes, fermes, formées par un réseau tridimensionnel d'agglomérats qui renferment une grande quantité d'eau (7 à 12 % de protéines pour environ 80% d'eau). Leur composition en sel et en composés protéiques et non protéiques affecte leur texture, les caractéristiques sensorielles et l'acceptabilité de différents produits alimentaires dans lesquels ils sont utilisés (Ju et Kilara, 1998). Leur formation résulte d'un processus d'agglomération qui dépend des facteurs décrits à la partie 1.5.

Pour ce qui est de l'α-lactalbumine, il s'agit d'une métalloprotéine dont la structure est fortement ordonnée par des ponts disulfures et un cation. Le degré d'organisation de sa structure secondaire est assez faible en comparaison avec la β-lactoglobuline. La structure tridimensionnelle de cette protéine est toutefois bien rigide. Elle possède deux sites de fixation du calcium, un site pour le zinc, quatre ponts disulfures et une poche hydrophobe. Elle ressemble de près au lysozyme, provenant probablement du même ancêtre génétique (Cayot et Lorient, 1998). L'α-lactalbumine n'aurait qu'un rôle mineur dans les interactions d'agglomération des protéines du lactosérum. Seule en solution, elle ne forme aucun agrégat lorsqu'elle est dénaturée thermiquement. Cette protéine agglomère toutefois bien avec la β-lactoglobuline et l'albumine bovine pour former des coagrégats lors de la dénaturation thermique du mélange (Hines et Foegeding, 1993). Elle présente aussi de forts potentiels pour la formulation d'aliments non-allergènes pour enfant (Macrae et *al.*, 1993).

1.4 L'agglomération des protéines du lactosérum

Le déploiement (déstructuration ou dénaturation) des protéines en solution (particulièrement la β-lactoglobuline) produit une agglomération majeure des protéines du lactosérum. Cette déstructuration affecte la solubilité des protéines à différents pH (Britten et *al.*, 1994; Abascal et *al.*, 2004). C'est sur ce principe que s'appuient les procédés de dénaturation thermique permettant d'agglomérer et de récupérer les protéines du lactosérum (Hadziyev, 1987 ; Cayot et Lorient, 1998). Le terme dénaturation thermique désigne la rupture d'interactions de faible énergie qui assurent l'existence des structures secondaires à quaternaires; ces ruptures de liens étant suivies de réarrangements aléatoires de zones protéiques entre elles au cours du refroidissement. Les protéines agglomèrent sous l'action des interactions hydrophobes, des liens ioniques, des nouveaux ponts disulfures et hydrogènes. Les principales forces responsables des agglomérations sont les interactions hydrophobes (Cayot et Lorient, 1998). Après précipitation et dépôt, les protéines sont récupérées par décantation ou centrifugation, puis lavées de l'excès de sel, récupérées par centrifugation ou filtration avant le séchage, pour être finalement moulues et empaquetées (Macrae et *al.*, 1993). Les protéines ont cependant perdu leurs propriétés fonctionnelles d'origine.

Ces technologies sont utilisées pour ajouter des protéines de lactosérum au fromage durant la production (Dybing et *al.*, 1998). Il y a un autre moyen, énergiquement moins dispendieux, de déstructurer les protéines : l'hydrolyse enzymatique. La force principalement responsable de l'agglomération après l'hydrolyse enzymatique demeure les interactions hydrophobes (Otte et *al.*, 1997).

Certains groupes de recherche ont travaillé sur les processus d'agglomération résultant de l'hydrolyse des protéines du lactosérum (Caessens et *al.*, 1999 a, b; Ju et *al.*, 1995, 1996, 1997, 1998a, 1998b, 1998c ; Huang et *al.*, 1999; Madsen et *al.*, 1997 ; Otte et *al.*, 1996a, 1996b, 1997, 1998 ; Sato et *al.*, 1995; Smyth et FitzGerald, 1999; Spellman et *al.*, 2005; Doucet et *al.*, 2003a; Doucet et *al.*, 2003b; Burke et *al.*, 2002; Bertrand et *al.*, 2002; Barros et *al.*, 2002). Ces travaux ont tous été réalisés dans le but de développer des gels de protéines de lactosérum, présentant de nouvelles propriétés fonctionnelles. Il fut démontré que la β-lactoglobuline native n'est pas un constituant majeur des agglomérats. Ceux-ci sont plutôt formés d'un grand nombre de peptides ayant un poids moléculaire variant de 2000 à 6000 Da environ (Otte et *al.*, 1997). La formation d'agglomérats en solution peut toutefois empêcher l'enzyme d'atteindre des sites particuliers d'hydrolyse et ainsi protéger une certaine partie des polypeptides contre l'hydrolyse (Otte et *al.*, 1997). Pour ce qui est des autres protéines, même si l'α-lactalbumine ne joue qu'un rôle mineur dans la formation des agglomérats, cette protéine agglomère toutefois bien avec la β-lactoglobuline et l'albumine bovine pour former des coagrégats (Ju et Kilara, 1998).

1.5 Facteurs affectant l'agglomération

Beaucoup d'études ont porté sur les facteurs affectant l'agglomération des protéines du lactosérum (Boye et *al.*, 1995 ; Britten et *al.*, 1994; Foegeding et *al.*, 1992 ; Hines et Foegeding, 1993 ; Hongsprabhas et *al.*, 1997 ; Jeyarajah et *al.*, 1994 ; Ju et *al.*, 1998; Matsudomi et *al.*, 1991 ; Xiong et *al.*, 1993 ; Oldfield et *al.*, 2005; Eissa et *al.*, 2005; Spellman et *al.*, 2005; Huppertz et *al.*, 2004; Abascal et *al.*, 2004; Havea et *al.*, 2004; Alting et *al.*, 2004; Doucet et *al.*, 2003a; Doucet et *al.*, 2003b; Alting et *al.*, 2003; Weinbreck et *al.*, 2003; Burke et *al.*, 2002; Havea et *al.*, 2002). Parmi ces facteurs on note principalement : le

taux d'hydrolyse (approche enzymatique), le pH, la présence de sels, l'utilisation d'un floculant, la température (dénaturation), la concentration et la composition de la solution en protéines (quantité et proportion des différentes protéines).

1.5.1 L'hydrolyse enzymatique

L'effet de l'hydrolyse enzymatique est variable selon le type d'enzyme utilisé et le taux d'hydrolyse atteint (Caessens et *al.*, 1999). L'hydrolyse enzymatique peut diminuer l'allergènicité de ces protéines, améliorer leurs propriétés fonctionnelles ou encore, produire des saveurs intéressantes (Schmidt et Poll, 1991). La β-lactoglobuline, et dans une moindre mesure les autres protéines du lactosérum, sont particulièrement difficiles à hydrolyser enzymatiquement (par différents enzymes protéolytiques) (Meyrath et *al.*, 1979 ; Cayot et Lorient, 1998; Madsen et Qvist, 1997). La structure globulaire de ces protéines expliquerait cette résistance à l'hydrolyse (Madsen et Qvist, 1997). Les travaux de Pallavicini et ses collaborateurs (1988), sont les seuls travaux, cités dans la littérature, ayant porté sur l'utilisation d'une protéase pour la récupération des protéines du lactosérum. Ils ont évalué la possibilité de récupérer les protéines du lactosérum en utilisant la subtilisine Carlsberg (Alcalase), une protéase présentant une efficacité très élevée pour l'hydrolyse des protéines du lactosérum (Mutilangi et *al.*, 1995). Après une heure d'hydrolyse, 2400 et 800 ppm de Ca^{++} ajouté au lactosérum acide et doux respectivement, 20 % et 28 % des protéines furent récupérées. Il n'est cependant pas possible d'augmenter ces rendements par une hydrolyse prolongée avec la subtilisine Carlsberg (Mutilangi et *al.*, 1995).

Pour que la récupération des protéines de lactosérum par cette voie (enzymatique) soit optimale, l'enzyme impliquée doit être la plus spécifique possible afin de diminuer les pertes associées aux peptides qui ne participeront pas aux agglomérations. Ainsi, des préparations industrielles d'enzymes présentant une activité exopeptidique élevée (clivant les protéines aux extrémités) produisent des hydrolysats ayant subit un taux d'hydrolyse élevé, mais ayant un contenu en peptides hydrophobes bas (Smyth et FitzGerald, 1998). À ce niveau, la subtilisine Carlsberg qui est une endopeptidase, n'est pas suffisamment spécifique. Elle produit un grand nombre de peptides qui ne participent pas à l'agglomération.

Schmidt et Poll (1991), ont étudié l'hydrolyse de l'α-lactalbumine et de la β-lactoglobuline par différents enzymes protéolytiques (6 protéases à sérine dont une subtilisine de *Bacillus subtilis*, 2 à cystéine dont la papaïne, 2 à acide aspartique dont la pepsine et 4 métalloprotéases dont la pronase). Certaines de ces solutions sont des mélanges de protéases présentant différentes spécificités. Par exemple, la pronase contient un mélange d'enzymes protéolytiques produites par *Streptomyces griseus*. Parmi les enzymes utilisées, la subtilisine de *B. subtilis,* la protéinase K et la pronase ont donné des résultats intéressants où la totalité des protéines natives ont été affectées par l'hydrolyse et où la subtilisine et la pronase ont produit des peptides de haut poids moléculaire en début d'hydrolyse (30 minutes à 2 heures). L'hydrolyse par la protéinase K fut trop rapide pour distinguer ces gros fragments. Le choix de l'enzyme est donc primordial.

Deux phénomènes peuvent expliquer les résultats décevants obtenus par l'équipe de Pallavicini avec la subtilisine Carlsberg: une spécificité insuffisante de l'enzyme, ce qui augmente la proportion de petits fragments produits qui ne participent pas à l'agglomération, et une proportion insuffisante de la β-lactoglobuline qui est affectée par l'enzyme à faible taux d'hydrolyse. La première hypothèse est appuyée par le fait que les subtilisines sont peu spécifiques (Barrett et *al.,* 1998). Une information vient appuyer la deuxième hypothèse; une augmentation du taux initial de réaction par une augmentation du ratio enzyme / substrat, augmente le taux d'hydrolyse maximal qu'il est possible d'atteindre et ce, spécialement avec l'enzyme Carlsberg (Mutilangi et *al.,* 1995). La subtilisine Carlsberg hydrolyserait les protéines selon une voie rapprochée du modèle « one by one » plutôt que par le modèle « zipper ». Le modèle « one by one » est caractérisé par une première étape d'hydrolyse du substrat sous sa forme native suivie de sa dégradation complète (hydrolyse subséquente) avant qu'une seconde molécule de substrat ne soit affectée par l'hydrolyse. Le modèle « zipper » est la dégradation simultanée de toutes les molécules de substrat où l'on ne retrouve plus de substrat sous sa forme native après le début de la réaction. Il existe ainsi une multitude de modèles entre ces deux modèles qui dépendent de l'enzyme et du substrat utilisé (Demay, 1993).

Il est logique de croire que pour un substrat difficile à hydrolyser (par exemple la β-lactoglobuline), les peptides libérés, suite aux premiers sites de coupe d'une endopeptidase comme la subtilisine Carlsberg, seront plus faciles à hydrolyser que la protéine sous sa forme native. Ceci favoriserait ainsi une voie globale d'hydrolyse se rapprochant du modèle « one by one » ce qui expliquerait les résultats de Mutilangi et ses collaborateurs (1995) pour lesquels une augmentation du taux initial de réaction par une augmentation du ratio enzyme / substrat, augmente le taux d'hydrolyse maximal qu'il est possible d'atteindre. Une telle voie d'hydrolyse globale fut démontrée pour l'hydrolyse de l'albumine bovine sérique (BSA) par la subtilisine Carlsberg (Demay, 1993). Il faut aussi considérer que certains peptides résiduels de l'hydrolyse de la β-lactoglobuline produisent une inhibition de l'hydrolyse chez l'enzyme Carlsberg (Mutilangi et *al.*, 1995).

Malgré le grand nombre d'études portant sur l'hydrolyse des protéines du lactosérum, une seule enzyme est réellement connue pour ses propriétés particulières d'agglomération : la protéase BLP produite par *Bacillus licheniformis* (Ju et *al.*, 1995, 1996, 1997, 1998a, 1998b ; Huang et *al.*, 1999; Madsen et Qvist, 1997 ; Mutilangi et *al.*, 1995 ; Otte et *al.*, 1995, 1996a, 1996b, 1997). Il s'agit d'une protéase extraite de la préparation industrielle de l'Alcalase (la préparation industrielle de l'enzyme Carlsberg) produite par *B. licheniformis* (Breddam et Meldal., 1992; Svendsen et Breddam, 1992). Cette enzyme fait partie des endopeptidases à acide glutamique de type I (glutamyl endopeptidase I; Barrett et *al.*, 1998).

Cette subtilisine produit au moins 25 peptides différents à partir de la β-lactoglobuline (une protéine de 18 300 Da), ce qui correspond à sa possibilité de clivage (Madsen et *al.*, 1997). L'efficacité de l'enzyme BLP pour l'hydrolyse des protéines du lactosérum et la formation d'agglomérats, provient de sa spécificité pour deux acides aminés chargés négativement (les acides glutamique et aspartique). Ces résidus acides hydrophiles doivent occuper des positions de surface sur la β-lactoglobuline ce qui les rend accessibles pour l'hydrolyse. Cette enzyme présente aussi d'autres spécificités que celles pour les résidus d'acide glutamique et aspartique. (Madsen et *al.*, 1997). Les travaux récents de Spellman et ses collaborateurs (2005), ont démontré que l'activité d'agglomération des protéines du

lactosérum de la préparation enzymatique « Alcalase » de *Bacillus licheniformis* proviendrait de sa spécificité pour les résidus chargés positivement.

Afin de développer des propriétés fonctionnelles toujours plus intéressantes, Otte et ses collaborateurs (1998), ont proposé un clivage chimique de la β-lactoglobuline B, suffisamment spécifique pour ne produire que quelques gros fragments de la protéine. Théoriquement, c'est ainsi qu'il faudrait procéder, par voie enzymatique, pour optimiser l'agglomération des protéines au maximum. Les fragments de haut poids moléculaire produits permettraient une récupération optimale des protéines sans perte associée aux peptides ne participant pas à l'agglomération. Pour la production des MPM, il s'agirait d'une augmentation des rendements en protéines.

Sato et ses collaborateurs (1995), ont présenté des résultats très intéressants au sujet d'une protéase produite par *Streptomyces griseus.* Cette protéase a produit un gel protéique à partir d'une solution d'isolats de protéines de lactosérum (WPI) partiellement dénaturés thermiquement, plus rapidement que les autres protéases testées dans cette étude (trypsine, papaïne, pepsine et pronase). Ce qui est des plus intéressants, c'est que ce gel fut produit à un taux d'hydrolyse inférieur aux autres gels et que pour un même temps d'incubation et un taux d'hydrolyse similaire, il est plus ferme. Les tableaux 8 et 9 ci-dessous présentent ces principaux résultats. Contrairement aux affirmations de Otte et ses collaborateurs (1995), il semble qu'il n'y ait pas seulement la subtilisine Carlsberg qui soit capable d'augmenter la capacité des protéines du lactosérum à former des gels plus fermes aux environs d'un pH neutre. Les protéines utilisées dans ces essais avaient toutefois été dénaturées thermiquement.

Plusieurs protéases de *S. griseus* sont connues : la protéinase A, B et la trypsine de *S .griseus* (Czapinska et Otlewski, 1999), la protéinase E, et une protéase spécifique pour les résidus d'acide glutamique (Barrett et *al.,* 1998). Étant donné que l'efficacité de l'enzyme BLP envers la β-lactoglobuline peut provenir de sa spécificité pour l'acide glutamique, cette enzyme de *S. griseus* pourrait être intéressante. Il est important de préciser que cette protéase de *S. griseus* ne présente aucune homologie avec l'enzyme Carlsberg.

1.5.2 Le pH

Le pH de la solution agit à deux niveaux différents : l'efficacité enzymatique et le pH d'agglomération. Le pH aura un effet sur la structure et la charge de l'enzyme, mais aussi sur la structure et la charge du substrat (Pelmont, 1995). La β-lactoglobuline est une protéine très fortement repliée par ses interactions hydrophobes. Le pH aura un effet sur ces charges de surface et sur ses interactions avec les différents sels en solution, ce qui influencera son niveau de repliement et son potentiel zêta (Cayot et Lorient, 1998). Ainsi, à différents pH, la protéine sera plus ou moins facile à hydrolyser peu importe l'enzyme utilisée.

Pour ce qui est de l'agglomération, les gels thermiques présentent une agrégation maximale pour des valeurs de pH entre 3.5 et 5.5 (généralement 4.2) quand la charge de la molécule est minimale (près du point isoélectrique de ces protéines; tableaux 8 et 9). Les travaux de Otte et ses collaborateurs (1996) ont démontré que ce pH optimal d'agglomération diminue en importance et qu'un second maximum apparaît suite à l'hydrolyse par la subtilisine BLP. Ce second maximum se déplace vers pH 7 avec l'augmentation du taux d'hydrolyse. Nos travaux sur l'agglomération des protéines du lactosérum (résultats non publiés) ont démontré une amélioration des rendements en récupération des protéines pour des pH aux environs de 7 – 7.5. Cette valeur de pH est de plus très appropriée pour l'utilisation des subtilisines (Barrett et al., 1998).

Tableau 8 : Degrés d'hydrolyse atteints par des solutions d'isolats de protéines de lactosérum (WPI) partiellement dénaturées thermiquement, hydrolysées par la trypsine, la papaïne, la pronase, la protéase type XXI et la pepsine à 37 °C, à leur point de gélation.

Enzymes utilisées	Temps d'incubation (heure)	Degré d'hydrolyse (%)	pH
Trypsine	8	27.1	6.37
Papaïne	2	23.1	6.50
Pronase	4	28.2	6.46
Protéase type XXI	**1,3**	**15.6**	6.40
Pepsine	8*	0.0	6.97

Adapté de Sato et *al.*, 1995.
*Temps maximal., aucune hydrolyse, aucun gel.

Tableau 9 : Dureté des gels produits par des solutions d'isolats de protéines de lactosérum (WPI) partiellement dénaturées thermiquement, hydrolysées par la trypsine, la papaïne, la pronase, la protéase type XXI à 37 oC.

Enzyme utilisée	Temps d'incubation (heure)	Degré d'hydrolyse (%)	Dureté du gel (g)
Trypsine	15	29,4	128.7
Papaïne	15	47,6	256.0
Pronase	15	39,4	286.7
Protéase	15	38,5	**362.3**

Adapté de Sato et *al.*, 1995.

1.5.3 La présence de sels

Les travaux de Otte et ses collaborateurs (1997) ont montré que lorsque la force ionique de la solution est élevée (chlorure de sodium 1M), les interactions hydrophobes et les ponts hydrogènes jouent un rôle majeur dans la formation des agglomérats de la β-lactoglobuline (comme dans le cas du lactosérum). À plus faible force ionique, ce sont les attractions électrostatiques qui sont les principales forces d'agglomération. Une forte force ionique tend à diminuer la répulsion électrostatique entre les protéines en neutralisant les

groupements ionisables par des ions mobiles (Boye et *al.*, 1995). Les protéines forment alors plus facilement des agrégats à partir des interactions hydrophobes. La force ionique de la solution est donc un facteur primordial à considérer. Havea et ses collaborateurs (2002), ont démontré que la composition minérale des concentrés de protéines de lactosérum industriels (WPC) joue un rôle majeur dans le comportement d'agglomération des protéines.

La présence de calcium est aussi particulièrement importante. Il agit à la fois sur la structure de la protéine, sur l'activité enzymatique et sur la formation d'agglomérats. Les ions calcium en solution induisent des changements structuraux légers chez la β-lactoglobuline qui augmente son hydrophobicité (Jeyarajah et Allen, 1994). Il est possible de provoquer la gélification de la β-lactoglobuline en solution en ajoutant une certaine quantité d'ions calcium. Comme pour la thermodénaturation, il semble que ce soient les interactions hydrophobes qui assurent un rôle prédominant dans le gel, le calcium n'est que l'initiateur en déstructurant les protéines. Ces ions bivalents renforcent le gel par la suite en formant des ponts calciques (Jeyarajah et Allen, 1994; Cayot et Lorient, 1998). Schmidt et Poll (1991), ont démontré que la présence de 10 mM de Ca^{2+} induit un changement de conformation suffisant pour rendre la β-lactoglobuline sensible à l'hydrolyse par la trypsine (pratiquement aucune hydrolyse détectée sans l'ajout de Ca^{2+}). Ce même ajout a rendu l'α-lactalbumine insensible à l'hydrolyse par la pancréatine. Les ions calcium augmentent l'activité enzymatique de certaines protéases, dont les subtilisines. Ces protéases possèdent généralement deux sites de fixation du calcium (de forte et faible affinité) et la fixation du calcium à chacun de ces sites augmente leur activité protéolytique (Heslot, 1996).

Le calcium forme également des doubles ponts avec les protéines en solution, ce qui a pour effet de favoriser la mise en place d'interactions hydrophobes et de les solidifier par la suite (Cayot et Lorient, 1998). Dybing et ses collaborateurs (1998), ont étudié l'effet de liaison de différents sels sur les protéines du lactosérum. Le chlorure de calcium et le pyrophosphate tétrasodique ($Na_4P_2O_7$) ont été retenus pour leur capacité à améliorer la formation d'agglomérats. L'effet du phosphate sur l'agglomération est complémentaire à celui du calcium. Le phosphate produit des ponts protéine – phosphate - protéine à cause de ses groupements anioniques, ou encore, des ponts couplés au calcium : phosphate – calcium -

protéine (Ellinger, 1980). Les possibilités de liaison sont donc plus nombreuses en présence des deux sels.

1.5.4 Les complexes protéines – polysaccharides

La gélation de solution aqueuse de protéines et de polysaccharides a reçu dernièrement une attention particulière de la communauté scientifique. Ces gels présentent des propriétés commerciales intéressantes pour la formulation de nouveaux produits à texture spécifique comme les ingrédients faibles en gras. Les mélanges de biopolymères (comme les agrégats protéiques et les polysaccharides) permettent d'obtenir une plus grande diversité de textures, de propriétés fonctionnelles et de stabilité que les biopolymères purs (Dumay et al., 1999). Les propriétés d'hydratation (solubilité, viscosité), structurantes (agrégation, gélation) et de surface (moussantes, émulsifiantes) sont utilisées dans une vaste gamme de domaines. Parmi ceux-ci, on note la purification de macromolécules, la microencapsulation, la formulation alimentaire (remplacement de gras, agents texturants), la synthèse de biomatériaux (films d'emballage comestibles, greffes artificielles), etc. Une importante revue de littérature fut produite sur le sujet (Schmitt et *al.*, 1998).

Le comportement d'une solution aqueuse contenant deux types de biopolymères joue un rôle important sur la fonctionnalité du mélange résultant. Dans la plupart des cas, la solution mixte de polymères est instable et une séparation de phases peut se réaliser de deux façons, produisant deux types de mélanges différents. Les interactions entre les deux polymères peuvent être répulsives et produire un système en deux phases où chacune des phases est enrichie en un polymère. Si les interactions sont attractives, le système peut présenter deux phases où les polymères seront concentrés dans l'une des phases (la phase concentrée). Le phénomène de concentration résulte d'interactions non spécifiques (électrostatiques, de van der Waals, hydrophobiques, ponts hydrogènes) entre les polymères qui mènent à la formation d'un complexe soluble ou insoluble (Schmitt et *al.*, 1998). Dumay et ses collaborateurs (1999), ont étudié le premier type de système formé par la β-lactoglobuline dénaturée sous pression et thermiquement, avec une solution d'alginate de sodium et de pectine méthoxylée. Dans ce type de système, les caractéristiques du complexe de polymères formé dépendent de l'incompatibilité thermodynamique.

Le deuxième système est plus susceptible d'affecter l'agglomération des protéines du lactosérum puisque le second polymère agit comme un floculant. Les floculants favorisent l'association des particules colloïdales libres en solution pour former des agglomérats. Un polysaccharide floculant bien connu pour son action sur les protéines du lactosérum est la kappa-carragénine (" κ-carrageenan ") (Dybing et *al.,* 1998; Weinbreck et *al.,* 2004). Les interactions protéines-polysaccharides sont spécifiques au type de polysaccharide utilisé et ils ne sont pas tous des floculants protéiques. Nos travaux antérieurs (résultats non publiés) ont démontré qu'un polymère bactérien a un effet marqué sur la formation des agglomérats (le kéfiran des grains de kéfir). Suite à la dénaturation des protéines, le polymère participe à l'agglomération de façon à former des coagglomérats plus gros favorisant la précipitation. Le complexe résultant possède à la fois les caractéristiques d'un polymère de polysaccharide (propriétés d'hydratation) et celles d'un polymère protéique des protéines du lactosérum (structurantes, émulsifiantes, moussantes, etc.). Le complexe est très stable et a permis la formulation de sauces type mayonnaise ou vinaigrette sans œuf, avec la moitié de la matière grasse, présentant la même texture et un goût similaire (résultats non publiés). Plusieurs autres applications ont aussi été envisagées.

La formation de ces complexes sera aussi affectée par la composition de la solution (polymères, sels, lipides, etc.), par les caractéristiques physico-chimiques de celle-ci (température, pression, pH) et par les traitements mécaniques de récupération des agglomérats (agitation, forces de cisaillement, etc.) (Dumay et *al.,* 1999; Dybing et *al.,* 1998; Schmitt et *al.,* 1998; Walkenstrom et *al.,* 1999). De nombreuses études récentes portent sur les différents types de polymères pouvant interagir avec les protéines de lactosérum et les conditions de ces interactions. On retrouve entre autres le galactomannan (Monteiro et al., 2005), différents amidons (Ye et al., 2004), la gomme arabique (Weinbreck et al., 2004B; Weinbreck et al., 2003) et la carragénine (Weinbreck et al., 2004A).

1.5.5 La concentration et la composition de la solution en protéines

Les travaux de Verheul et Roefs (1998), ont démontré que les protéines ne participent pas à la formation d'agrégats dans les mêmes proportions que celles de la solution. En

fonction du temps, la concentration de l'α-lactalbumine, des immunoglobulines et de l'albumine bovine sérique libres diminue plus rapidement que la concentration de la β-lactoglobuline libre. Ainsi, bien que la composition protéique de la solution était de 78 % de β-lactoglobuline et de 22 % des trois autres protéines (solution de WPI), au point de gélation (formation d'un gel par la solution), la β-lactoglobuline ne constituait que 45 % des agrégats (à pH 6,8). De plus, la formation de gels de β-lactoglobuline pure nécessite une quantité moindre d'agrégats protéiques en solution que la formation d'un gel protéique de WPI (à pH 6,8). La β-lactoglobuline forme donc plus facilement le réseau protéique structurant le gel ce qui résulte en un gel dit plus perméable, ayant une structure moins compacte.

Il fut démontré que l'α-lactalbumine ne forme pas d'agrégats à des pH supérieurs à 7 lors de la formation de gels à partir de WPC (Cayot et Lorient, 1998). La β-lactoglobuline forme des agrégats à pH acide ou alcalin ce qui démontre que la formation des gels dépend principalement, particulièrement à pH alcalin, des agrégats formés par la β-lactoglobuline. Les travaux de Matsudomi et ses collaborateurs (1991) ont cependant démontré que la concentration de protéines nécessaires pour la formation d'un gel à pH 8, après 15 minutes à 90 C, est plus basse pour la BSA que pour la β-lactoglobuline (4 et 5 % respectivement).

Hines et Foegeding (1993) ont déterminé que la constante d'agrégation du second ordre de la dénaturation de l'α-lactalbumine, de la β-lactoglobuline et de la BSA chauffées seules en solution à 80 C se présente dans l'ordre : BSA >> β-lactoglobuline > α-lactalbumine. La constante d'agrégation de l'α-lactalbumine augmente lorsqu'elle est chauffée en présence de la β-lactoglobuline, ce qui suggère une coagrégation. Ils ont démontré que l'α-lactalbumine seule en solution ne forme aucun agrégat lorsqu'elle est dénaturée thermiquement. Cette protéine agglomère toutefois bien avec la β-lactoglobuline et l'albumine bovine sérique (BSA) pour former des coagrégats lors de la dénaturation thermique du mélange. De plus, les qualités rhéologiques de la solution sont dépendantes des ratios entre la β-lactoglobuline et l'albumine bovine sérique. Ceci démontre bien que la formation d'agrégats est dépendante de la nature et de la proportion des constituantes protéiques.

1.5.6 La température

Comme mentionné auparavant, la dénaturation thermique des protéines du lactosérum engendre une agglomération de celles-ci. Cette agglomération se réalise en deux phases. La dénaturation des protéines native est suivie d'interactions protéines – protéines qui résultent en un réseau tridimensionnel formant le gel (Boye et al., 1995). La concentration initiale en protéines est un facteur significatif qui détermine la grosseur et/ou la forme des polymères formés durant la première phase (protéines – protéines). La température et la durée du chauffage sont des facteurs affectant la formation du réseau tridimensionnel durant la seconde phase (Vardhanabhuti et Foegeding, 1999; Verheul et Roefs, 1998).

Il est nécessaire de différencier dénaturation réversible de dénaturation irréversible. La dénaturation réversible permet un retour à la structure initiale de la protéine si la température est ramenée à sa valeur initiale. La dénaturation irréversible provoque une modification permanente de la structure de la protéine. Dong Chen et ses collaborateurs (1998), ont démontré qu'en considérant que la dénaturation est réversible jusqu'à un certain point et que dans le lactosérum plusieurs types de protéines coexistent, la réaction de dénaturation de la β-lactoglobuline en solution ne peut être considérée comme la réaction simple d'une seule étape généralement décrite dans la littérature. Cette réaction de dénaturation est aussi influencée par les différents facteurs énumérés précédemment comme le pH, la présence de sels, la présence de floculants, la concentration et la composition en protéines de la solution et on peut même ajouter la présence de glucides (comme le lactose), le temps de montée de la température, le type de variant génétique des protéines et la présence de lipides (Boye et *al.*, 1995; Britten et Giroux, 1994; Cayot et Lorient, 1998; Foegeding, et *al.*, 1992; Hines et Foegeding, 1993; Hoffmann et van Mil, 1999; Hongspabhas et Barbut, 1997; Matsudomi et *al.*, 1991; Verheul et Roefs, 1998; Xiong et *al.*, 1993; Havea et *al.*, 2002; Bertrand et *al.*, 2002; Havea et *al.*, 2004; Oldfield et *al.*, 2005).

Pour les protéines de lactosérum prises individuellement, l'ordre de dénaturation irréversible est le suivant : immunoglobulines > sérum albumine bovine > β-lactoglobuline > α-lactalbumine (ce qui va dans le même sens que la constante d'agrégation du second ordre

de Hines et Foegeding (1993)). La température de dénaturation de ces protéines est respectivement de : 72, 64, 78 et de 62 °C. L'irréversibilité n'est atteinte qu'à 100 °C pour l'α-lactalbumine et à 70 °C pour la β-lactoglobuline. L'α-lactalbumine est donc beaucoup plus résistante à une dénaturation thermique permanente (Cayot et Lorient, 1998). Pour une solution de lactosérum, suite à un chauffage à 95 °C durant 5 minutes à pH 4,5, la totalité de la β-lactoglobuline et de l'albumine sérique bovine précipite. Une fraction de l'α-lactalbumine subsiste dans le surnageant (Cayot et Lorient, 1998).

Les protéines dénaturées peuvent être récupérées à partir d'un « solids-ejecting clarifier ». Cette méthode permet de récupérer de 90 à 95 % des protéines coagulées (Morr, 1993). En absence de calcium et à pH inférieur à 6, aucune précipitation n'apparaît même au voisinage du pI (Cayot et Lorient, 1998). Entre les pH 4,5 et 7, en présence ou en absence de calcium, une solution d'α-lactalbumine seule en solution ne précipite pas. La présence de la β-lactoglobuline conduit à la formation de coprécipités. La précipitation thermo-induite des protéines du lactosérum dans cette gamme de pH est due, au moins en partie, à la présence de la β-lactoglobuline et à sa grande richesse en feuillets β hydrophobes (Cayot et Lorient, 1998).

1.6 La β-lactoglobuline

1.6.1 Structure primaire

La β-lactoglobuline est constituée de 162 acides aminés (tableau 10), elle comporte 2 ponts disulfures ainsi qu'une fonction thiol sur le résidu cystéine 119 ou 121 (Morr et *al.*, 1993). La distribution de la fonction SH entre les résidus Cys 121 et Cys 119 est de 1 : 1. Il existe présentement 7 variants génétiques de la β-lactoglobuline, mais les plus fréquemment rencontrés sont les variants A et B avec une fréquence respective de 46 à 50 % et de 50 à 54 % pour les vaches Holstein – Frisonne (Cayot et Lorient, 1998). Ces variants diffèrent par deux acides aminés : l'asparagine 64 pour le variant A est changée par une glycine dans le variant B et la valine 118 est changé pour une alanine chez ces mêmes variants (Qin et *al.*, 1999). À des pH près de la neutralité, la charge du variant A est donc plus négative que celle

du variant B, ce qui permet de les séparer sous leur forme native par électrophorèse ou par chromatographie d'échange d'ions (Cayot et Lorient, 1998).

1.6.2 Structure secondaire et tertiaire

La β-lactoglobuline possède un haut niveau de structuration, avec une très forte proportion de feuillets β hydrophobes (43 à 50 % des résidus d'acides aminés), 10 à 15 % de la séquence en hélices α et 15 à 20 % en courbures β (Cayot et Lorient, 1998). Elle appartient à la super famille des lipocalines. Les membres de cette famille présentent une structure de huit brins β antiparallèles formant une cavité centrale qui a pour fonction de lier différents types de molécules hydrophobes (figure 3 A et B) (Qin et *al.*, 1999). Pour la β-lactoglobuline, l'hélice 130 – 140 qui se situe entre les feuillets 115 – 124 et 145 –150 forme un compactage renforcé par les ponts disulfures, qui combiné à la richesse en feuilletsβ, assure la création du cœur hydrophobe (Cayot et Lorient, 1998; Chen et *al.*, 1993). Cette structure spatiale très serrée rend la β-lactoglobuline peu accessible pour l'hydrolyse enzymatique.

Tableau 10 : Composition en acides aminés de la β-lactoglobuline A et B.

Résidus	Nombre	%	Résidus	Nombre	%
Hydrophobes			Hydrophiles		
Alanine	15 (16*)	9,2	Neutres		
Cystéine	5	3.1	Asparagine	5	3.1
Glycine	3 (4)	1.9	Glutamine	9	5.6
Isoleucine	9	5.6	Serine	7	4.3
Leucine	21	13.0	Thréonine	8	4.9
Méthionine	4	2.5	Tyrosine	5	3.1
Phénylalanine	4	2.5	Basique		
Proline	8	4.9	Arginine	3	1.9
Tryptophane	2	1.2	Histidine	2	1.2
Valine	10 (9)	6.2	Lysine	15	9.2
			Acides		
			Aspartate	11 (10)	6.8
			Glutamate	16	9.9
TOTAL	81 (82)	50	TOTAL	81 (80)	50

* Composition du variant B.
Morr et al., 1993.

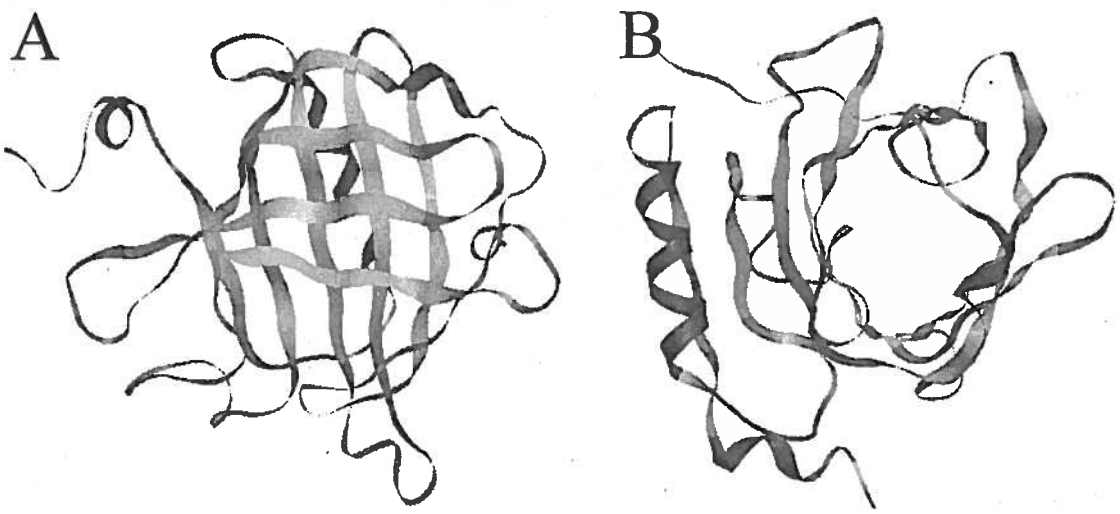


Figure 3 : Structure tertiaire de la β-lactoglobuline bovine; A de côté, B de face. PDB (protein data bank) : 1DV9, 2000.

Sur ses 10 brins β, 8 se retrouvent en deux feuillets antiparallèles presque perpendiculaires (figure 3 A). Le premier est formé des séquences 145 – 150, 115 – 124, 102 – 109, 89 – 97 et 80 – 84 ; et le second des séquences 62 – 76, 47 – 58 et 39 – 44. Le segment 16 à 27 peut être placé avec une des deux plaques et peut permettre une certaine flexibilité de la molécule selon les conditions du milieu. De plus, la boucle formée par le deuxième pont disulfure serait peu stable et flexible (Cayot et Lorient, 1998). Il n'existerait pas de différences importantes entre les formes tridimensionnelles des variants A et B, mais la substitution V118A du variant B réduirait cinq contacts hydrophobes de cette région et en diminuerait légèrement sa rigidité. Cela a pour conséquence de diminuer quelque peu la stabilité thermique du variant B comparativement à celle du variant A. Ces différences structurales furent étudiées en détail par le groupe de Qin et ses collaborateurs (1999).

Le monomère de β-lactoglobuline à l'apparence d'un calice à l'intérieur hydrophobe. Sa très forte ressemblance avec la protéine transporteuse de rétinol (vitamine A) a attiré l'attention sur une possible fonction de cette protéine pour le transport et / ou l'adsorption de cette vitamine ou de certains acides gras des lipides, au niveau du tube digestif. La capacité de la β-lactoglobuline à fixer le rétinol permet même de la récupérer par adsorption sélective sur une colonne greffée de rétinol. Cette capacité à lier différentes substances, comme entre

autres le cholestérol et la vitamine D2 fut revue récemment par Kontopidis et ses collaborateurs (2004) en fonction de son rôle physiologique. De plus, la β-lactoglobuline résiste fortement à l'hydrolyse par les enzymes digestives comme la pepsine et la chymotrypsine. La protéolyse par ces enzymes libère surtout des petits peptides et très peu de gros peptides en début de réaction (Cayot et Lorient, 1998).

Finalement, la β-lactoglobuline apparaît en solution sous forme de dimère, structure non covalente, à pH neutre, où les calices formés par sa structure tridimensionnelle s'emboîtent l'un dans l'autre. La β-lactoglobuline A forme même des octamères à pH plus acide, autour de son point isoélectrique (pI de 5,1 et optimum de l'association octa-unitaire à 4,65) (Qin et *al.*, 1999; Cayot et Lorient, 1998).

Chapitre 2 : les enzymes protéolytiques

Les enzymes protéolytiques, qui hydrolysent les liens peptidiques, sont appelées les peptidases. Les peptidases comprennent deux types d'enzymes : les exopeptidases qui hydrolysent séquentiellement les acides aminés aux extrémités N ou C terminales des protéines et les endopeptidases qui hydrolysent des liens peptidiques à l'intérieur de la chaîne d'acides aminés. Les endopeptidases sont aussi appelées protéinases (Pelmont, 1995).

2.1 Classification

Les peptidases sont classifiées selon leur mécanisme catalytique en cinq catégories : les peptidases à sérine, les peptidases à thréonine, les peptidases à cystéine, les peptidases à aspartate et les métallopeptidases. Les peptidases à sérine, thréonine et cystéine diffèrent de façon catalytique des deux autres groupes de peptidases; elles agissent comme des transférases. Chez ces peptidases (Ser/Thr/Cys), l'attaque nucléophile, brisant le lien peptidique, provient d'un acide aminé et mène à la formation d'un intermédiaire covalent ; l'acyl – enzyme. Pour les deux autres groupes, l'attaque nucléophile qui provient d'une molécule d'eau activée ne mène pas à la formation de l'acyl – enzyme. Les peptidases où cette attaque nucléophile est produite par le groupement hydroxyle d'un résidu sérine sont

nommées les peptidases à sérine (Barrett et *al.*, 1998; Rawling et Barrett, 1994; Sarath et al., 1989).

2.2 Les peptidases à sérine

Barrett et ses collaborateurs (1998), ont classé les peptidases à sérine en huit clans incluant les peptidases à thréonine (Clan TA) dû à leur forte similarité réactionnelle (tableau 11). Ces peptidases, réalisent une vaste gamme de réactions d'hydrolyse peptidique dépendantes de leur très grande diversité de spécificité. L'étude de ces enzymes depuis les 40 dernières années a permis d'accumuler beaucoup d'informations sur la séquence en nucléotides de leur gène, sur leur séquence en acides aminés, sur leurs structures secondaire et tertiaire, et sur les données cinétiques et thermodynamiques de réaction envers leurs substrats et leurs inhibiteurs (Czapinska et Otlewski, 1999). Pour les subtilases (Clan SB, famille S8), les chymotrypsines (Clan SA) et les carboxypeptidases (Clan SC), la même triade du site catalytique est présente (Asp/His/Ser), mais leur structure tridimensionnelle très différente est issue d'une évolution convergente. L'orientation géométrique des résidus catalytiques fut conservée. De plus, les groupements adjacents qui permettent de stabiliser les états de transition sont aussi disposés de façon similaire (Perona et Craik, 1995).

La triade catalytique de ces peptidases catalyse l'hydrolyse des liaisons peptidiques en deux étapes. La première étape de la réaction (figure 4) est la formation d'un intermédiaire, l'acyl-enzyme, entre le substrat et le résidu de sérine de la triade catalytique. La formation de cet intermédiaire covalent passe par un état tétraédral de transition, chargé négativement, durant lequel le lien peptidique est clivé. La deuxième étape (figure 5), la déacylation, est l'hydrolyse de l'acyl-enzyme par l'attaque d'une molécule d'eau qui libère le peptide et régénère le groupement hydroxyle de la sérine de la triade (Barrett et *al.*, 1998; Dunn, 1989). Ce mécanisme est capable d'accélérer l'hydrolyse des liens peptidiques par un facteur de plus de 10 relativement à la réaction non catalysée (Perona et Craik, 1995). Il est le même pour les trois clans de protéases à sérine présentant la triade Asp/His/Ser (Barrett et *al.*, 1998). Ces deux étapes peuvent être divisées en cinq sous étapes qui seront décrites de façon approfondie pour les subtilisines afin de permettre une meilleure description des interactions substrat – protéine.

2.3 Les subtilisines.

Le nom subtilisine provient de *Bacillus subtilis*, d'où la première enzyme du genre fut isolée. La première enzyme connue fut la subtilisine Carlsberg de *Bacillus licheniformis* (subtilisine A, subtilopeptidase A ou Alcalase Novo ™). Plusieurs enzymes de ce groupe sont très bien décrites: la subtilisine Carlsberg, BPN de *Bacillus amyloliquefaciens* (Nagarse ™, subtilisine B, subtilopeptidase B et C, de Novo), la subtilisine 147 de *Bacillus lentus* (Espérase ™), la subtilisine PB92 de *Bacillus alcalophilus* (Maxacal ™ et la subtilisine 309, un variant N85S; la Savinase ™), la subtilisine E de *Bacillus subtilis* (subtilisine I168), et autres. (Barrett et *al.*, 1998).

Tableau 11 : Les résidus catalytiques, le repliement et le nombre de famille des différents clans de peptidases à sérine.

Clan	Résidu (s) catalytique (s)	Repliement	Nombre de famille	Exemple
SA	His, Asp, Ser	Double tonneau β	9	Trypsine
SB	Asp, His, Ser	Feuillet β parallèle	1	Subtilisine
SC	Ser, Asp, His	α/β hydrolase	6	Carboxypeptidase C
SE	Ser, Lys	Hélice α, β sandwich	3	Pénicilline liaison 5
SF	Ser, Lys (His)	Tonneau β simple	4	TSP protéase
SH	His, Ser, His	Entièrement β	1	Cytomégalovirus
TA	Thr	α, β, α, β sandwich	3	Protéasome
Autre	----	------	8	Endopeptidase CLP

Barrett et *al.*, 1998.

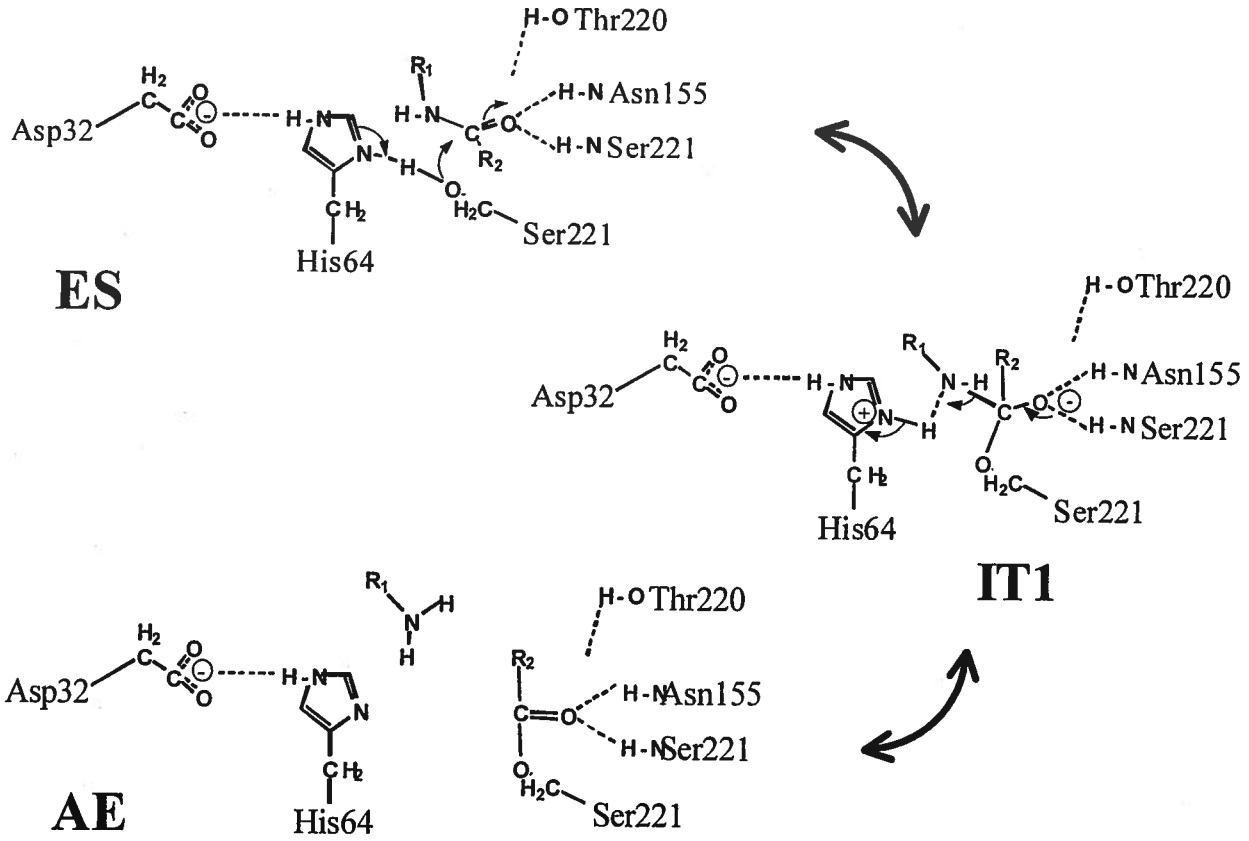


Figure 4 : Intermédiaires de la voie catalytique d'hydrolyse d'un substrat peptidique par les protéases à sérines présentant la triade Asp/His/Ser. Première étape de la réaction : l'acylation. Numérotation de la subtilisine BPN. ES = enzyme substrat, IT = intermédiaire tétraédrique, AE = acyl-enzyme. Adapté de Fink, A.L., 1989.

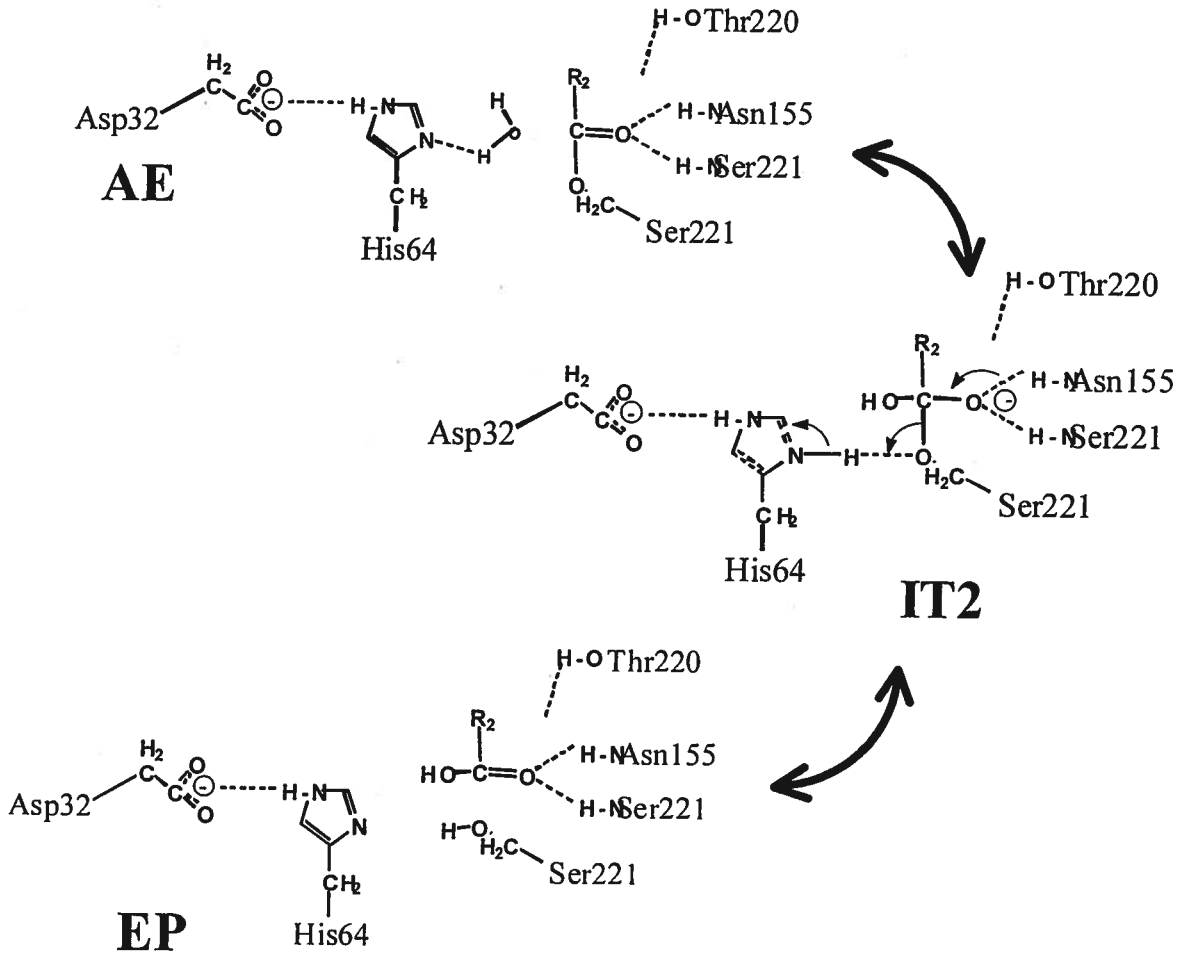


Figure 5 : Intermédiaires de la voie catalytique d'hydrolyse d'un substrat peptidique par les protéases à sérines présentant la triade Asp/His/Ser. Deuxième étape de la réaction : la déacylation. Numérotation de la subtilisine BPN. IT = intermédiaire tétraédrique, AE = acyl-enzyme, EP = enzyme produit. Adapté de Fink, A.L., 1989.

La famille des subtilisines est retrouvée chez les micro-organismes. Parmi les subtilases (la super famille S8), il s'agit d'une des familles les mieux conservées. Elle peut être divisée en trois sous groupes : les subtilisines vraies (plus de 64% d'homologie avec la subtilisine Carlsberg), les protéases alcalines (plus de 55% d'homologie) et les protéases intracellulaires (plus de 37% d'homologie) (Siezen et Leunissen, 1997). Elles sont parmi les enzymes les mieux connues et les plus étudiées (Heslot, 1996; Barrett et *al.*, 1998). Un grand nombre de structures tridimensionnelles de ces enzymes sont connues, entre autres des complexes enzyme - inhibiteur protéique (Sienzen et Leunissen, 1997; Sienzen et al., 1991). De multiples études ont été réalisées sur la spécificité, l'activité et la stabilité de ce groupe d'enzymes (Liuqin et Yongmei, 1997; Wells et Estell, 1988; Czapinska et Otlewski, 1999). Ces enzymes sont largement utilisées au niveau industriel; elles résistent bien à l'effet des détergents, elles sont produites en grande quantité par des souches modifiées génétiquement et elles se prêtent bien à des essais d'amélioration par mutagenèse dirigée (Pelmont, 1995). Une étude a même porté sur l'évolution accélérée *in vitro* et la sélection des recombinants d'un mélange de 26 gènes de ces protéases (Ness et *al.*, 1999).

L'enzyme est synthétisée sous forme d'un précurseur doté à la fois d'un peptide signal « prépeptide» (30 acides aminés) et d'un « propeptide » supplémentaire de 77 acides aminés. La prépro-subtilisine est inactive (zymogène). L'activation se réalise par l'autohydrolyse de la séquence « pro » qui joue un rôle primordial dans le repliement actif de l'enzyme (Pelmont, 1995). Seulement la subtilisine mature est détectée dans le milieu extracellulaire. Ces protéinases à sérine qui présentent une triade catalytique Asp 32, His 64, Ser 221 (numérotation de la subtilisine BPN) n'ont pas de ponts disulfures et lient les ions calcium avec une forte affinité ($k_a = 10^7\ M^{-1}$) (Glick and Pasternak, 1998). Trois enzymes de ce groupe furent particulièrement étudiées : l'enzyme Carlsberg, la subtilisine BPN et la subtilisine E. Ces enzymes présentent une forte homologie de séquence et de structure (tableau 12, figure 6).

Tableau 12 : Résultats des alignements de séquences non-conservateurs de trois subtilisines connues : la subtilisine Carlsberg de *Bacillus subtilis* (PDB : 1CSE), la subtilisine BPN de *Bacillus amyloliquefaciens* (PDB : 2SNI) et la subtilisine E de *Bacillus subtilis* (PDB : 1SCJ). Réalisé à partir du logiciel Align Plus.

Subtilisine	Carlsberg	BPN	E
Carlsberg	100,0	69,8	69,1
BPN	70,1	100,0	85,5
E	69,3	85,5	100,0

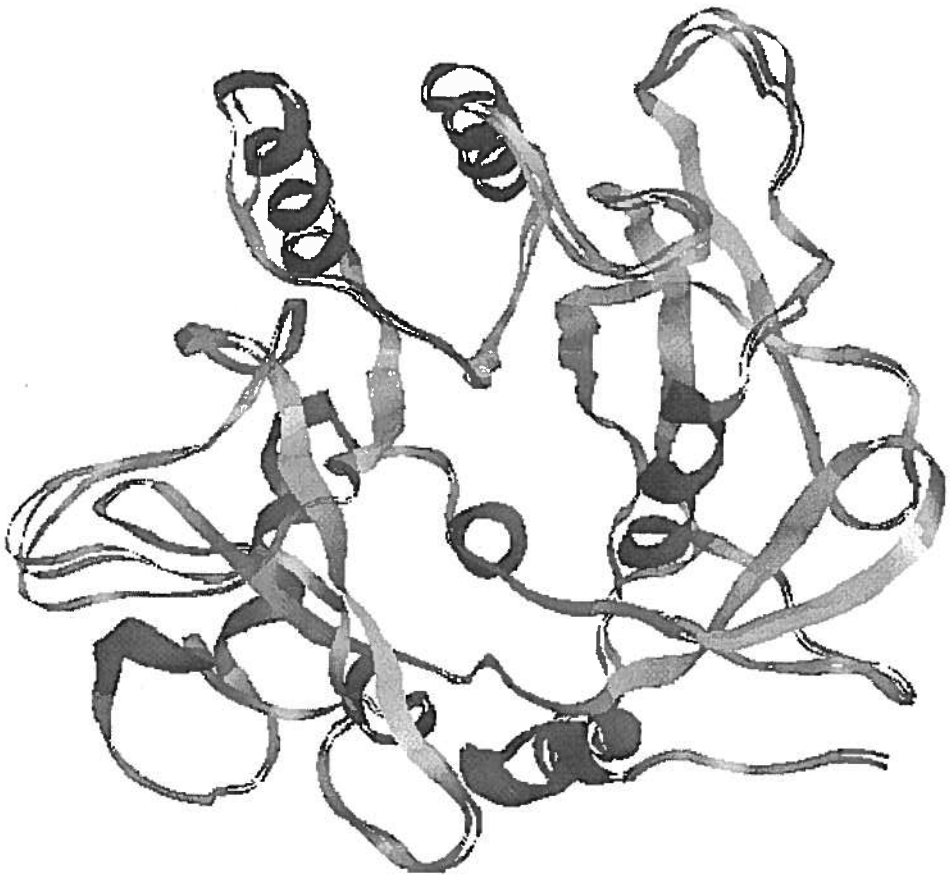


Figure 6 : Superposition de la subtilisine Carlsberg de *Bacillus subtilis* (PDB : 1CSE ; 1,2 Å de résolution), de la subtilisine BPN de *Bacillus amyloliquefaciens* (PDB : 2SNI; 2,10 Å de résolution) et de la subtilisine E de *Bacillus subtilis* (PDB : 1SCJ; 2,0 Å de résolution). RMSD : 0, 495 ("Root Mean Square Deviation"). Réalisé à partir du logiciel MOE.

Ce propeptide contient les informations manquantes à la protéine mature pour réaliser son repliement complet. Le concept de repliement assisté par le propeptide fut démontré originalement pour les protéases à sérine (Ikemura et *al.*, 1987). Les exemples les mieux connus sont ceux de la subtilisine BPN et de la subtilisine E de *Bacillus subtilis*, de la protéase α-lytique de *Lysobacter enzymogenes*, et la carboxypeptidase Y vacuolaire de *Saccharomyces cerevisiae* (Eder et Fersht, 1995). D'autres exemples de protéases peuvent s'ajouter à ceux-ci : la furine, la thermolysine, la procaricain, la prohormone convertase Kex, la cruzain et la cathepsine L qui sont des protéases à cystéine, la protéinase A une protéase à acide aspartique et même des métallo-protéases comme la métallo–protéase neutre de *Streptomyces cacaoi* (Chang et *al.*, 1994; Eder et Fersht, 1995; Shinde et Inouye, 2000).

Le repliement de ces enzymes en absence de leur propeptide, même en conditions optimales, ne permet pas l'obtention de protéases actives. Ce principe fut démontré *in vitro* et *in vivo* (Ikemura et al., 1987; Zhu et *al.*, 1989). Le propeptide n'a toutefois pas besoin d'être lié de façon covalente à la protéine pour remplir sa pleine fonction (Silen et Agard, 1989; Strausberg et *al.*, 1993a). Suite à son repliement complet, le précurseur autohydrolyse le propeptide et le libère pour donner l'enzyme mature (Volkov et Jordan, 1996; Li et Inouye, 1996). Ce processus de repliement catalysé a donné lieu au concept de chaperon intramoléculaire (CIM) (Kobayashi et Inouye, 1992; Shinde et Inouye, 1993; Shinde et Inouye, 1996).

2.3.1 Séquence et structure du propeptide

Le propeptide présente une proportion de résidus chargés plus importante que celle de l'enzyme mature (36 et 12 % respectivement). Il présente 17 résidus chargés positivement (22 % du propeptide) pour 11 résidus chargés négativement (14 % du propeptide). La séquence N-terminale (–77 à –51) est chargée positivement et la séquence C terminale (-16 à –1) est chargée négativement (le résidu N-terminal de la séquence mature étant +1 et le N-terminal du propeptide –77 (Shinde et Inouye, 1996).

L'activité de la prorégion fut étudiée en utilisant la mutagenèse aléatoire par PCR ("polymerase chain reaction") afin d'isoler des mutants incapables de permettre le repliement actif de l'enzyme (Lernet et *al.*, 1990). L'isolement d'un grand nombre de mutants négatifs a permis d'identifier 25 mutations par substitution qui causent la perte du repliement fonctionnel de l'enzyme (Kobayashi et Inouye, 1992). Environ la moitié de ces mutations se retrouvent à l'intérieur de trois secteurs hydrophobes du propeptide identifiés H1, H2 et H3 (figure 7). Le secteur H1 avait déjà été identifié comme étant un secteur de forte homologie pour les propeptides de différentes espèces de bacilles (Ikemura et *al.*, 1987). Pour le secteur H2, il est à remarquer que la substitution de n'importe quelle alanine pour une thréonine (A-31T étant toujours accompagnée de I-67T) résulte en un propeptide inactif. Étant donné que le site de liaison du substrat des subtilisines est principalement hydrophobe (Barrett et *al.*, 1998), la forte proportion de résidus chargés du propeptide (36%, Shinde et Inouye, 1996) serait orientée vers le solvant (Inouye, 1991).

Shinde et Inouye (1993), de même que Eder et ses collaborateurs (1993a et b) ont posé l'hypothèse que les chaperons intramoléculaires agissaient en abaissant la barrière énergétique entre cet état et l'état natif final (Figure 8).

2.4 Description détaillée de la réaction d'hydrolyse

Le domaine catalytique est composé et influencé par les trois résidus de la triade catalytique (Asp 32 / His64 / Ser221), par le groupement NH du squelette de la sérine active (Ser 221) et par le groupement azoté latéral de l'Asn 155 (numérotation de la subtilisine BPN), et par les interactions des groupements latéraux du substrat peptidique avec différents résidus de l'enzyme (Siezen et Leunissen, 1997). Par convention, selon Schechter et Berger (1967), les résidus du substrat sont nommés P et numérotés à partir du site catalytique; P1 à Px en direction N terminal et P1' à Px' en direction du C terminal. L'hydrolyse étant réalisée entre P1 et P1'.

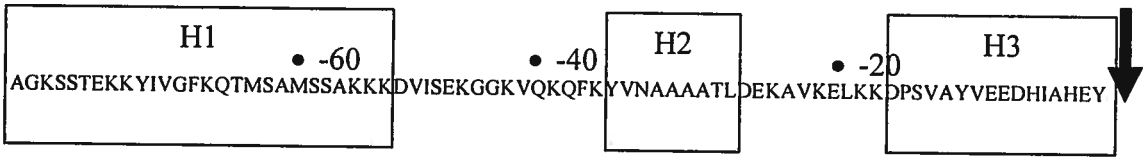


Figure 7 : Séquence du propeptide de la subtilisine E.

La flèche indique le site de coupe du propeptide. Chaque 20^{e} résidu à partir de –1 est indiqué par un point. Les boîtes H1 à H3 présentent les sections hydrophobes (adapté de Kabayashi et Inouye, 1992).

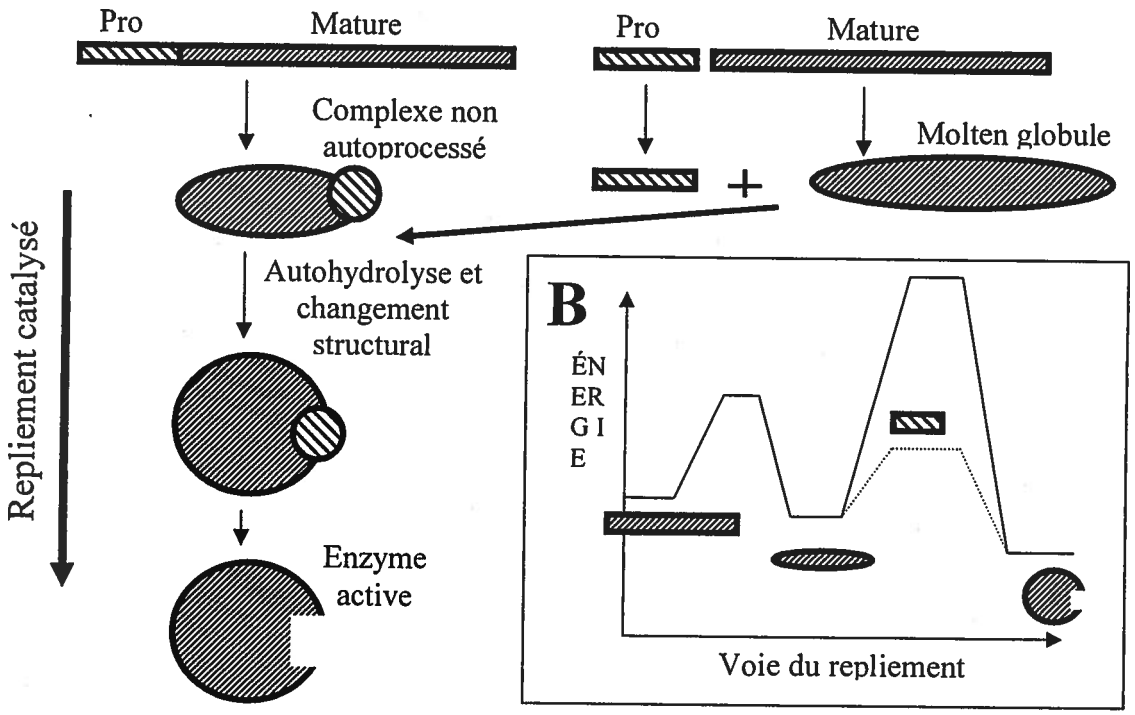


Figure 8 : Voie catalytique du repliement des subtilisines. La partie B présente un schéma des barrières cinétiques en jeux (Adapté de Shinde et Inouye, 2000).

Les sites correspondant de l'enzyme entrant en interaction avec ces résidus sont appelés sites S1 à Sx (figure 9). Les résidus P1 à P4 du substrat forment un feuillet β antiparallèle avec l'enzyme pour les clans SA et SB. Bien que ces interactions aient un rôle primordial, il faut aussi considérer les propriétés électrostatiques, dynamiques et structurales de l'enzyme (Fink, 1989; Lu et *al.*, 1997).

2.4.1 La formation de l'acyl-enzyme

A. L'enzyme native et le complexe enzyme - substrat.

Le complexe enzyme - substrat est l'association de l'enzyme et du substrat sans liaison covalente. Sa vitesse de formation est évaluée par la détermination de la constante d'association (K_1). La cinétique de la réaction peut être décrite comme l'équation à la figure 10. La constante K_2 représente le taux de formation de l'acyl-enzyme; le taux de la réaction inverse pouvant être considéré comme négligeable. La constante K_3 représente le taux de déacylation (Perona et Craik, 1995). La constante K_m, est caractéristique d'une enzyme donnée pour un substrat donné, dans des conditions données de pH et de température.

Le K_{cat} exprime la vitesse de catalyse et est donc représenté par l'étape limitante de la réaction. Pour les subtilases, la déacylation est environ 33 fois plus rapide que l'acylation qui serait l'étape limitante. Le K_m serait donc une bonne estimation de la constante d'association K_1 et le K_{cat} est représenté à la figure 12 par K_2 (Wells et Estell, 1988). Le ratio K_{cat} / K_m donne une mesure de la spécificité de l'enzyme pour son substrat (Perona et Craik, 1995).

L'interprétation correcte des constantes K_{cat} et K_m diffère selon si l'étape limitante de la catalyse est l'acylation ou la déacylation et elle varie selon l'enzyme ou selon le substrat utilisé pour une même enzyme.

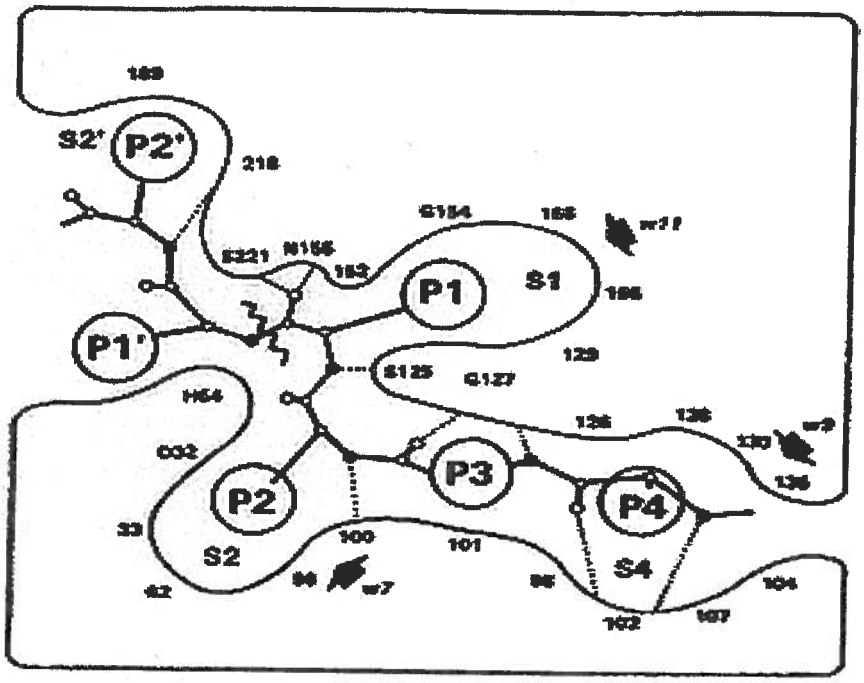


Figure 9 : Représentation schématique de la relation du substrat (ou de l'inhibiteur) avec une enzyme protéolytique du type subtilisine. La ligne ondulée correspond au site de coupe de l'enzyme. La numérotation est celle de la subtilisine BPN. Les lignes pointillées représentent des liens hydrogènes. Siezen et Leunissen, 1997.

$$\text{E-OH} + \text{R-C(=O)-X} \overset{K_1}{\longleftrightarrow} \text{E-OH•R-C(=O)-X} \overset{K_2}{\longrightarrow} \text{E-O-C(=O)-R} + \text{H-X} \overset{K_3,\ H_2O}{\longrightarrow} \text{R-C(=O)-OH} + \text{E-OH}$$

Étape limite acylation

$K_{cat} \approx K_2 \qquad K_m \approx K_1$

Étape limite déacylation

$$K_{cat} \approx K_3 \qquad K_m \approx K_1 \left[\frac{K_3}{K_2 + K_3} \right]$$

Figure 10 : Cinétique de la réaction d'hydrolyse peptidique d'une enzyme agissant comme une transférase et relation des constantes avec les paramètres de Michaelis - Menten. Adapté de Perona et Craik, 1995.

Quatre facteurs ont démontré une influence significative sur la cinétique de réaction suite à la formation du complexe enzyme - substrat : l'affinité de l'enzyme pour le substrat, la mobilité de l'enzyme, les changements de conformation de l'enzyme par rapport au substrat, et le pH, qui influencent les trois premiers facteurs. Des exemples connus de ces quatre facteurs seront décrits pour la subtilisine BPN.

Premièrement, il fut observé qu'en augmentant l'affinité de l'enzyme pour son substrat (le K_m) chez les subtilases, on modifie le K_{cat} de façon positive (Carter et Wells, 1988). Le triple mutant D32A, H64A et S221A, sans la triade catalytique, conserve une activité enzymatique (K_{cat}) de 10^3 fois plus élevée que la solution sans enzyme et ce, en ne causant que des effets mineurs sur la constante de Michaelis (K_m). Cette activité serait en partie due à la stabilisation de l'état de transition par le groupement azoté latéral de l'Asn 155 et des autres interactions de l'assemblage enzyme - substrat (ex : figure 9; liens hydrogènes : Wells et Estell, 1988). Brode et ses collaborateurs (1996), ont étudié l'activité catalytique de la subtilisine BPN par rapport à l'importance de l'adsorption de son substrat à sa surface. En solution, l'activité augmente si l'affinité pour le substrat augmente. Pour un substrat fixe cependant, l'activité diminue si l'affinité augmente due à une baisse de la mobilité de l'enzyme. La mobilité de l'enzyme devient l'étape limitante de la réaction. Ainsi, par mutagenèse dirigée, la diminution d'affinité de l'enzyme pour la surface a augmenté sa mobilité, ce qui a augmenté l'efficacité réactionnelle de l'enzyme. Les différentes interactions à l'origine de l'absorption sont les interactions hydrophobes, les interactions électrostatiques et les changements de conformation.

Certaines enzymes, comme les enzymes lipolytiques, s'activent suite à un changement de conformation induit par interaction à l'interface de l'enzyme (Brode et *al.*, 1996). Pour les subtilases, c'est aussi le cas, bien qu'aucun changement de conformation majeur ne soit observable (Schmitke et al., 1998)). L'enzyme native ne présente pas la liaison hydrogène entre les résidus sérine et histidine de la triade catalytique nécessaire à la réaction d'hydrolyse. La distance et l'orientation de ces résidus ne le permettent pas dans l'enzyme seule. La formation du complexe enzyme - substrat produit un léger mouvement du site catalytique et permet cette liaison hydrogène (Perona et Craik, 1995).

Plusieurs protéines présentent un maximum d'adsorption à la surface de l'enzyme (fixation) à leur point isoélectrique qui serait dû à une maximisation des changements de conformation pour une minimisation des répulsions électrostatiques (Brode et *al.*, 1996). Le pH a un effet non négligeable à la fois sur la liaison du substrat (K_m), et sur l'efficacité catalytique (K_{cat}) (Carter et Wells, 1988). L'activité des subtilisines augmente entre les pH 6 à 8 ($pK_A \approx 7,2$) durant lequel l'histidine 64 est déprotonée. La constante de Michaelis varie dans le même sens que le pH pour des valeurs légèrement basique. Il y a cependant une baisse d'activité de la subtilisine BPN entre les pH de 9,5 à 11, qui serait due à l'ionisation d'un résidu tyrosine entrant en contact avec le substrat. Ce résidu pourrait être le Tyr104 dans la pochette P4 ou le Tyr217 dans la pochette P1 (ou les deux). La mutation du Tyr104 par une phénylalanine a augmenté l'efficacité de l'enzyme à pH basique (Wells et Estell, 1988).

B. L'état de transition et l'intermédiaire tétraédrique.

Suite à la formation du complexe enzyme - substrat, l'oxygène de la fonction hydroxyle de la sérine 221 transférera son proton au noyau imidazole de l'histidine 64 pour effectuer son attaque sur le groupement carbonyle du lien clivé. Cette étape passe par le premier état de transition de la réaction formant un intermédiaire tétraédrique (figure 4, IT_1). Des liens hydrogènes (par le groupement NH du squelette de la sérine active (Ser 221) et par le groupement azoté latéral de l'Asn 155) viennent polariser le lien carbonyle qui subit l'attaque de façon à stabiliser cet intermédiaire et l'oxyanion qui en résulte (figure 4). Cette pochette d'interactions, pour l'oxyanion, fut décrite pour un grand nombre de protéases et est communément appelée « oxyanion hole » (Fink, 1989). Cette pochette de stabilisation fut retrouvée dans tous les sites actifs de protéases à sérine connues (Whiting et Peticolas, 1994).

L'Asp32 vient influencer le noyau imidazol de l'histidine de façon à permettre l'échange du proton et procure une compensation qui stabilise l'intermédiaire en empêchant le développement d'une charge positive sur l'imidazol. Les liens hydrogènes qui stabilisent l'oxyanion ont aussi un rôle similaire en compensant le développement d'une charge négative (figure 4) (Perona et Craik, 1995). L'orientation de l'acide aspartique de la triade par rapport à l'histidine ne serait pas aussi bien conservée que celle de la sérine par rapport à l'histidine.

Une expérience menée sur la trypsine a même démontré que le groupement carboxylé de l'Asp102 peut être déplacé à la position 214, un déplacement de 40°, tout en conservant une forte activité catalytique. Il est donc approprié de parler de l'action de deux dyades catalytiques (Ser-His et Asp-His) plutôt que d'une triade. Finalement, la possibilité que l'Asp reçoive l'hydrogène de l'histidine semble ne pas être supportée par les évidences expérimentales et théoriques (Perona et Craik, 1995).

L'interaction de l'Asn155 avec l'oxyanion fut démontrée par les travaux de Wells et ses collaborateurs (1986). Le remplacement de l'Asn par Thr, His ou Gln résulte en une diminution du K_{cat} de deux à trois fois sa valeur normale, avec seulement un changement mineur pour le K_m. Un indice du rôle de l'Asn155 est apporté par le fait qu'une diminution de stabilité de 2.2 à 4.7 Kcal / mol fut observée pour l'état de transition suite à ces modifications. Un troisième résidu interagirait (en plus de la Ser221 et de l'Asn155), la Thr220, bien que son Oγ soit à 4 Å de l'oxyanion. Cette stabilisation à distance agirait en influençant le potentiel électrostatique du site actif. Des perturbations significatives du pK_A de l'His64 surviennent suite à des mutations de résidus chargés de la surface de l'enzyme, ces résidus étant à 12 à 20 Å du site actif. Des mutations similaires de résidus chargés ont aussi affecté la stabilité de l'état de transition d'un inhibiteur. Des interactions électrostatiques à distance peuvent donc influencer légèrement, mais de façon significative, la stabilité de l'état de transition (Perona et Craik, 1995).

O'Connell et ses collaborateurs (1997), ont étudié le rôle de l'Asn155 dans la stabilisation de l'oxyanion, l'intermédiaire tétraédrique. Ils ont étudié comment ces interactions hydrogènes pourraient contribuer à l'efficacité enzymatique de la subtilisine BPN, par l'abaissement du pK_A de l'oxyanion formé. Pour la trypsine et la chymotrypsine, une telle étude par mutagenèse dirigée est impossible étant donné que les deux ponts hydrogènes stabilisateurs sont fournis par des groupements NH du squelette de l'enzyme. Ils ont démontré que l'Asn155 a un rôle significatif mais mineur pour l'abaissement du pK_A qui serait plutôt dû à l'interaction de l'oxyanion avec le cation imidazol de l'histidine du site actif (pour un complexe inhibiteur de chlorométane). Le rôle principal des liens hydrogènes avec l'oxyanion serait la solvatation localisée de celui-ci. Dans le même laboratoire, O'Sullivan et

ses collaborateurs (1999), ont étudié comment la valeur pK_A de l'oxyanion de l'intermédiaire tétraédrique est affectée par la liaison des différents résidus S1 à S4 et ce, pour la subtilisine BPN et la Chymotrypsine. Les substrats utilisés pour l'étude sont des dérivés de chlorométane présentant différents résidus aux positions P1 à P4. Il fut démontré, que lorsque le résidu P1 est la phénylalanine, le pK_A de l'oxyanion est indépendant des résidus P2 à P4. Cependant, lorsque le résidu P1 est le tryptophane ou la leucine, le pK_A de l'oxyanion augmente et il peut être influencé par les résidus en position P2 à P4.

Ces résultats ne sont cependant pas en accord avec les données cinétiques (K_m et K_{cat}) présentées par d'autres études pour des substrats peptidiques équivalents. Les modifications du pK_A présentées ne seraient pas causées par la liaison du résidu P1 dans la pochette S1. Une conformation différente de l'intermédiaire tétraédrique formé par le chlorométane expliquerait cette divergence des résultats (O'Sullivan et *al.,* 1999). Ceci remet en cause les résultats présentés auparavant sur l'effet des liens hydrogènes sur le pK_A de l'oxyanion (O'Connell et *al.,* 1997).

C. L'acylenzyme.

Comme mentionné auparavant, pour les subtilases, l'acylation est l'étape limitante de la réaction d'hydrolyse. Plusieurs facteurs affectant l'acylation affecteront aussi la déacylation qui procède par une voie similaire (Fink, 1989). La stabilité de l'acylenzyme peut entre autre être un facteur diminuant le taux de déacylation. Si la stabilité de l'acylenzyme est augmentée, l'énergie d'activation nécessaire à la déacylation sera plus élevée et le taux de déacylation sera plus bas (Tonge et Carey, 1990). Ces considérations sont abordées dans la section qui suit.

2.4.2 La déacylation

La deuxième étape, la déacylation, est l'hydrolyse de l'acylenzyme par l'attaque d'une molécule d'eau qui libère le peptide et régénère le groupement hydroxyle de la sérine de la triade (Barrett et *al.,* 1998). La force majeure responsable de la catalyse serait la distorsion non – planaire du lien à cliver, par l'enzyme, qui diminuerait l'énergie d'activation de l'hydrolyse (Fink, 1989). La déacylation sera influencée principalement par trois facteurs : la

protonation de l'histidine du site réactif, la stabilité du second état de transition et l'affinité de l'enzyme pour son substrat.

A. Le second état de transition.

L'histidine du site actif agit comme une base transférant un groupement hydroxyde au lien carbonyle de l'acylenzyme (par interaction avec une molécule d'eau : figure 5). Les travaux de Whiting et Peticolas (1994) ont démontré que l'activité de cette base est fortement influencée par le pH, le pK_A de l'histidine étant aux environs de 7. À des pH inférieurs à 7, le taux de déacylation de la subtilisine BPN diminue très rapidement à cause de la protonation de l'histidine du site actif. Sa vitesse de déacylation est 100 fois plus rapide à pH 8,6 qu'à pH 5.

Les travaux de Whiting et Peticolas (1994) ont aussi démontré que la stabilisation du second état de transition est une étape importante pour la déacylation. Par mutagenèse dirigée, ils ont démontré que la N155G subtilisine BPN présente un taux de déacylation 84 fois plus lent que l'enzyme native pour le *p*-(dimethylamino)benzoylimidazolide. Les interactions hydrogènes de « l'oxyanion hole » serviraient aussi à la stabilisation du second état de transition. De plus, la force des liens hydrogènes de « l'oxyanion hole » serait le déterminant majeur du taux de déacylation. Ces interactions augmenteraient en force de pH 5 à pH 8,3 (évaluation par différence de spectroscopie Raman).

Ces résultats sont en accord avec ceux de Tonge et Carey (1990), qui ont étudié la corrélation entre la longueur du lien carbonyle de l'acylenzyme pour une protéase à sérine et sa réactivité. Il fut démontré que la longueur du lien carbonyle est corrélée à l'énergie libre d'activation de la réaction de déacylation et est dépendante de la force du lien hydrogène formé par la présence de différents résidus à la position 155. Plus le lien hydrogène formé est fort, plus le lien carbonyle est court et plus le taux de déacylation est élevé. Cette augmentation de réactivité serait due à une légère distorsion de l'état d'équilibre de l'acylenzyme en faveur de l'intermédiaire de l'état de transition. La diminution de longueur de la liaison carbonyle peut favoriser la formation d'une charge négative sur l'oxygène. Cette

charge négative diminuerait la densité électronique du carbone carbonyle et ainsi favoriserait l'attaque nucléophile (figure 5). Cependant, d'autres facteurs viennent agir sur le taux de déacylation. Par exemple, pour un même substrat, avec un lien carbonyle ayant la même longueur, la subtilisine Carlsberg présente une valeur de K3 de 0,31 (S^{-1}) comparativement à 0,13 pour la subtilisine BPN (Tonge et Carey, 1990).

B. Le relâchement de la séquence N-terminale.

L'affinité de l'enzyme pour son substrat modulera la vitesse à laquelle la séquence N-terminale sera relâchée. Par exemple, une affinité trop grande, un nombre trop élevé d'interactions peut porter un substrat à demeurer indéfiniment absorbé à l'enzyme ; c'est ce que l'on appelle un inhibiteur. L'affinité de la plupart des peptidases à sérines pour leurs inhibiteurs provient principalement de la liaison du résidu P1 dans la pochette S1 (Lu et *al.*, 1997). McPhalen et James (1988), ont comparé la structure de la subtilisine Carlsberg complexée à l'églin – C (un inhibiteur protéique provenant d'*Hirudo medicinalis* ; une sangsue) et de la subtilisine BPN complexée à l'inhibiteur CI – 2 (« chymotrypsine inhibiteur 2 »; des grains d'orge : Figure 11 et 12).

Figure 11 : Emplacement des pochettes de spécificité P4 à P2' (selon Barrett et *al.*, 1998) du complexe enzyme inhibiteur de la subtilisine Carlsberg et de l'eglin – C (PDB : 1CSE). Les résidus sous forme d'une fine ligne sont ceux de l'inhibiteur.

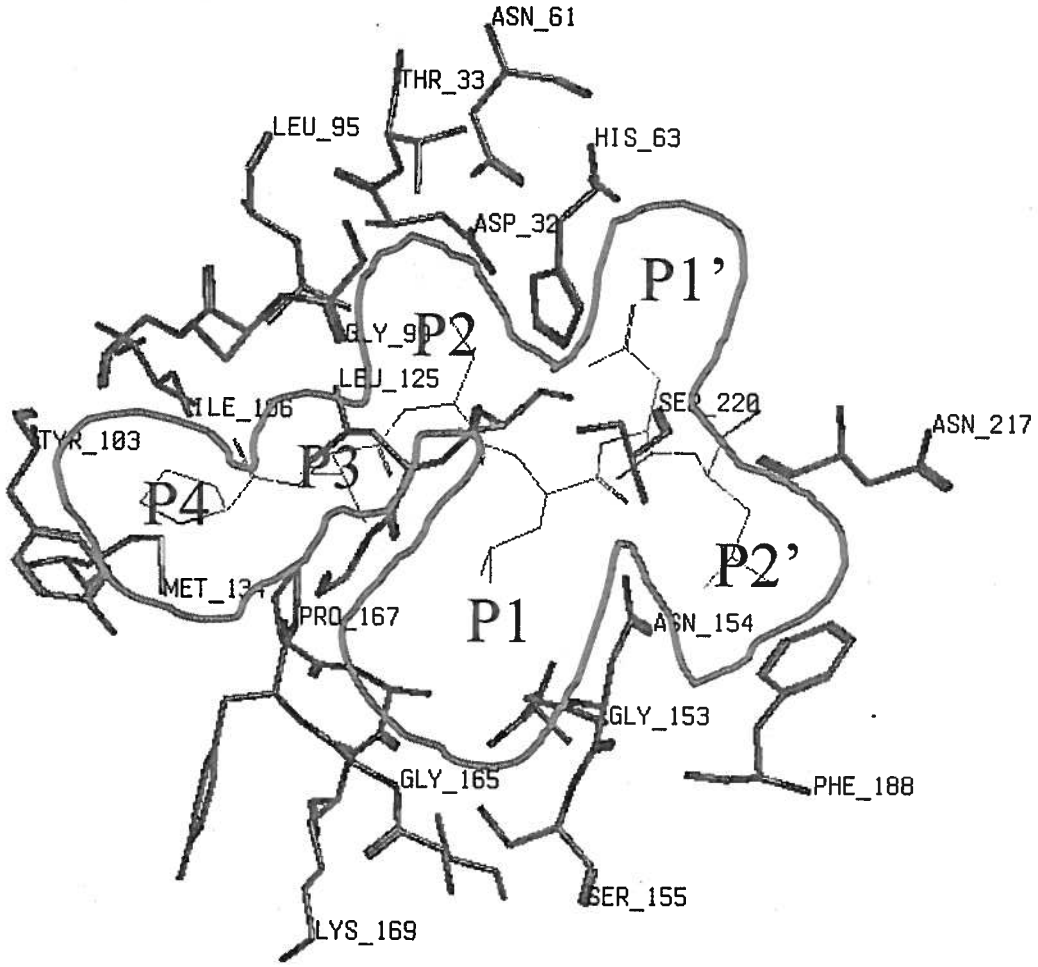
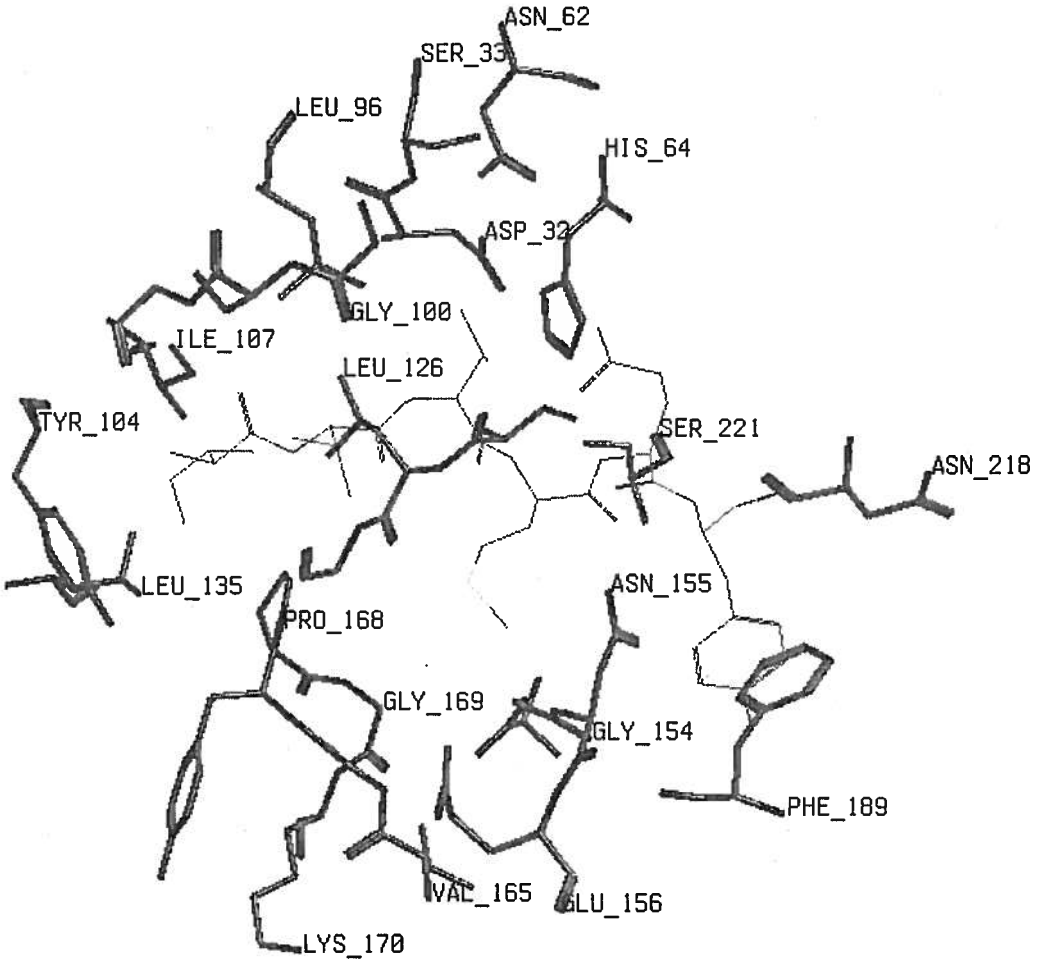


Figure 12 : Résidus entrant en interaction (selon Barrett et *al.*, 1998) entre l'enzyme et l'inhibiteur, pour le complexe enzyme inhibiteur de la subtilisine BPN complexée à CI – 2 (PDB : 2SNI). Les résidus sous forme d'une fine ligne sont ceux de l'inhibiteur.



Douze résidus de l'inhibiteur forment 134 contacts de moins de 4 Å avec 25 résidus de l'enzyme Carlsberg et 11 résidus du deuxième inhibiteur forment 134 contacts avec 22 résidus de l'enzyme BPN. De plus, les résidus P4 des inhibiteurs s'apparient parfaitement à la pochette S4 de l'enzyme. Comme mentionné précédemment, ces interactions sont similaires à celles de l'enzyme pour son propeptide. L'affinité du site de liaison de l'enzyme pour son substrat est donc le facteur primordial du relâchement de la séquence N terminale.

Chapitre 3 : Les lactobacilles.

Les lactobacilles font parti du groupe des bactéries lactiques. Il s'agit du plus important groupe de microorganismes probiotiques habituellement associé au transit intestinal. Déjà en 1908, Metchnikoff considérait que la longévité de certaines personnes était associée à leur consommation de produits laitiers fermentés (Holzapfel, et *al.*, 2001). À cette époque, des études avaient été réalisées sur la taxonomie et l'écologie microbienne de l'intestin.

Traditionnellement, les bactéries lactiques ont été classifiées sur la base de leurs propriétés phénotypiques : leur morphologie, leur mode de fermentation du glucose, leur croissance à différentes températures, le type d'acide lactique produit et leur profil de fermentation de différents sucres. Toutefois, avec l'avènement de la biologie moléculaire, il s'avère que certains regroupements générés sur la base des phénotypes et du profil de fermentation ne correspondent pas avec les branchements phylogénétiques (Schleifer, 1987; Holzapfel, et *al.*, 2001; Nigatu, 2000). Les méthodes phénotypiques furent ainsi améliorées pour inclure maintenant les analyses des composantes de la paroi cellulaire et des protéines cytoplasmiques (Klein, et *al.*, 1998; Yeung, et *al.*, 2002; Stiles et Holzapfel, 1997).

Pour les bactéries lactiques, des systèmes d'identification basés sur le profil de fermentation des sucres furent développés, mais leur utilisation demande de compléter l'information à l'aide d'outils génomiques pour assurer la validité de l'identification (Nigatu, 2000; Boyd, et *al.*, 2005; Yeung, et *al.*, 2002; Moreira, et *al.*, 2005). Ainsi, l'identification et l'étude des branchements phylogénétiques se basent maintenant sur les méthodes génomiques

incluant l'hybridation de l'ADN, l'électrophorèse en champs pulsés (PFGE), sous gradient dénaturant (DGGE) ou encore sous gradient de température (TTGE), l'amplification aléatoire par PRC (RAPD-PCR), l'analyse des plasmides, l'analyse par enzyme de restriction (REA), l'analyse de secteurs particuliers de l'ADN ribosomal (ARDAR, 16S-23S régions intergènes,), (Holzapfel, et *al.,* 2001; Klein, et *al.,* 1998; Moreira, et *al.,* 2005; Han, et *al.,* 2005; Rossetti et *al.,* 2005; Theunissen, et *al.,* 2005; Yeung, et *al.,* 2004; Kwon, et *al.,* 2004; Ogier, et *al.,* 2002; Baele, et *al.,* 2002; Ercolini et *al.,* 2001).

Une revue des différentes techniques moléculaires couramment utilisées fut produite par Satokari et ses collaborateurs (2003). L'utilisation de ces approches génomiques est aussi utile dans un contexte industriel pour caractériser et produire une empreinte génétique des microorganismes utilisés, pour effectuer des contrôles de qualité, pour surveiller l'utilisation de souches bactériennes protégées et pour déterminer la relation entre différentes souches industrielles et celle utilisées en recherche (Yeung, 2004).

Il s'avère que la comparaison des séquences des gènes de l'ARN ribosomal serait la technique la plus puissante et rigoureuse pour déterminer les relations phylogénétiques entre les microorganismes (Holzapfel, et *al.,* 2001), mais aussi une technique utilisée pour étudier des flores complexes (Byun, et *al.,* 2004, Pavlova, et *al.,* 2002, Kim, et *al.,* 2005) ou développer des méthodes diagnostiques rapides (Christensen, et *al.,* 2004). La grande abondance de séquences d'ADN de ces gènes de bactéries lactiques (plus de 100 : NCBI) permet d'utiliser la séquence du gène de l'ARN 16S pour identifier un grand nombre de microorganismes. Il s'agit d'une technique particulièrement utile pour les groupes bactériens très apparentés comme les lactobacilles du groupe des *acidophilus* (Yeung, et *al.,* 2002).

Les études génomiques effectuées permettent maintenant de classer les bactéries lactiques sous dix principaux genres : *Lactobacillus, Lactococcus, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Weissella, Carnobacterium, Tetragenococcus et Bifidobacterium.* Ce groupe est défini comme des bactéries Gram positives, catalase négative, microaérophiles ou anaérobiques, ne formant pas de spore. Les bactéries Gram positives sont divisées en deux branches principales de façon phylogénétique et les bactéries lactiques

appartiennent à l'axe des faibles contenus en G + C (moins de 50%). Le genre *Bifidobacterium* constitue la seule exception à cette règle, mais il est tout de même considéré comme appartenant aux bactéries lactiques à cause de ses similitudes physiologiques et biochimiques et dû au fait qu'il partage la même niche écologique principale : le tractus intestinal (Klein, et *al.*, 1998).

Parmi les bactéries lactiques, le genre *Lactobacillus* est le plus hétérogène englobant un large spectre de phénotypes, de propriétés physiologiques et biochimiques. Il regroupe le plus grand nombre d'espèces et il est divisé en trois sous groupes caractérisés par leur capacité de fermentation : les homofermentaires strictes, les hétérofermentaires facultatives et les hétérofermentaires strictes (Axelsson, 1998; Prescott et *al.*, 1995; Stiles et Holzapfel, 1997). Cette caractéristique provient de leur métabolisme des sucres qui ne produit que de l'acide lactique (homofermentaires strictes) sans production de CO_2 ou qui produit différents acides organiques et du CO_2 (hétérofermentaires). Ces trois groupes sont habituellement connus respectivement sous le nom du groupe des *acidophilus*, du groupe des *casei* et du groupe des *fermentum* considérant des espèces importantes de chacun de ces groupes (tableau 13) (Holzapfel, et *al.*, 2001; Stiles et Holzapfel, 1997; Klein, et *al.*, 1998).

Les bactéries du genre *Lactobacillus* sont en général tolérantes à l'acide avec des pH optimaux de croissance de 5,5 à 6,2. Certaines espèces particulièrement résistantes peuvent réduire le pH de certains aliments fermentés jusqu'à une valeur de 4 permettant ainsi une meilleure conservation de ces produits (Stiles et Holzapfel, 1997). Leurs valeurs limites de croissance se situeraient entre pH 4 et 7,2. Elles ont des exigences complexes et variables en ce qui concerne les acides aminés, les peptides, les vitamines, les sels, les acides gras et les glucides fermentescibles et ont un large spectre pour la température de croissance allant de 2 à 53 °C (Axelsson, 1998; Prescott et *al.*, 1995; Stiles et Holzapfel, 1997).

Tableau 13: Principales divisions à l'intérieur du genre *Lactobacillus* basé sur les caractéristiques phénotypiques. Le représentant de chacun des groupes est présenté en caractères gras.

Groupe 1	**Groupe 2**	**Groupe 3**
Homofermentaires strictes	Hétérofermentaires facultatives	Hétérofermentaires strictes
L. acidophilus	*L. acetotolerans*	*L. brevis*
L. amylophilus	*L. agilis*	*L. buchneri*
L. amylovorus	*L. alimentarius*	*L. collinoides*
L. aviarius	*L. bifermentans*	***L. fermentum***
subsp. araffinosus	***L. casei***	*L. fructivorans*
subsp. aviarius	*L. coryniformis*	*L. fructosus*
L. crispatus	*subsp. coryniformis*	*L. hilgardii*
L. delbrueckii	*subsp. torquens*	*L. kefir*
subsp. bulgaricus	*L. curvatus*	*L. malefermentans*
subsp. delbrueckii	*L. graminis*	*L. oris*
subsp. lactis	*L. hamsteri*	*L. panis*
L. farciminis	*L. homohiochii*	*L. parabuchneri*
L. gallinarum	*L. intestinalis*	*L. parakefir*
L. gasseri	*L. murinus*	*L. pontis*
L. helveticus	*L. paracasei*	*L. reuteri*
L. jensenii	*subsp. paracasei*	*L. sanfrancisco*
L. johnsonii	*subsp. tolerans*	*L. suebicus*
L. kefiranofaciens	*L. paraplantarum*	*L. vaccinostercus*
subsp. kefiranofaciens	*L. pentosus*	*L. vaginalis*
subsp. kefirgranum	*L. plantarum*	
L. mali	*L. rhamnosus*	
L. ruminis	*L. sake*	
L. salivarius		
subsp. salicinus		
subsp. salivarius		
L. sharpeae		

Tiré de Stiles et Holzapfel (1997).

Stiles et Holzapfel (1997) présentent une revue intéressante du genre *Lactobacillus* avec une description des différents habitats et des espèces appartenant aux trois groupes mentionnés auparavant. Il faut bien mentionner que les lactobacilles sont rarement pathogènes et généralement reconnues comme sécuritaires pour la santé humaine. Elles font entre autres parties de la flore normale du corps humain au niveau du système digestif et du vagin. Outre leur utilisation probiotique, elles ont une grande importance en alimentation et sont utilisées pour les caractéristiques de goût, d'arôme, de texture et de conservation qu'elles confèrent aux aliments. Elles sont notamment retrouvées dans les laits fermentés, les kéfirs, les légumes fermentés ainsi que plusieurs produits carnés (Stiles et Holzapfel, 1997, Axelsson, 1998; Prescott et *al.,* 1995; de Roissart et Luquet, 1994).

Problématique

Le lactosérum est le principal sous-produit de la fabrication du fromage. Ce liquide riche en sucre (50 g/L de lactose) contient des protéines ayant une forte valeur nutritionnelle et santé (Yalcin, 2006; Marshall, 2004). De plus, aucune technologie ne permet actuellement de valoriser le lactosérum de façon rentable à des volumes inférieurs à 250 000 litres transformés par jour. À cette fin, une nouvelle technologie fut développée pour valoriser le lactosérum par fermentation : la technologie Valactis™.

Cette technologie permet, par la modification des conditions de cultures, des variables de récupération et des souches bactériennes utilisées, la production d'une nouvelle gamme d'ingrédients bioactifs : les MPM (Matrices Protéiques Malléables). Les MPM se caractérisent par leur grande valeur fonctionnelle technologique (ex : propriétés émulsifiantes) et biologique (ex : simulation immunitaire). Ces propriétés fonctionnelles et leur origine naturelle en font des ingrédients de choix pour le marché de l'alimentation fonctionnelle et des nutraceutiques.

Ainsi, la technologie Valactis™ présente beaucoup d'intérêt à la fois pour son potentiel technologique de traitement du lactosérum, mais aussi pour les qualités fonctionnelles des ingrédients bioactifs obtenus. Les composantes des MPM (protéines et peptides de lactosérum, bactéries lactiques probiotiques, calcium et exopolysaccharide (EPS)) présentent des effets bénéfiques pour la santé largement documentés dans la littérature de façon individuelle. La production des MPM permet de regrouper ces ingrédients biologiques à l'intérieur d'une nouvelle gamme de produits présentant des propriétés fonctionnelles (technologiques et biologiques) exclusives qui facilitent leur intégration dans un très large éventail d'applications. Il s'agit du concept MPM qui a donné ieu à une multitude de travaux sur l'optimisation de la production, la caractérisation des produits obtenus et les possibilités d'applications de ces produits. Ce concept sera décrit du point de vu de la technologie et des produits, de la caractérisation des microorganismes utilisés et d'une possibilité de production d'enzymes protéolytique pouvant être ajoutées au procédé.

Hypothèse

Hypothèse principale.

Il est possible, à partir d'une nouvelle technologie de fermentation du lactosérum, de produire une nouvelle gamme d'ingrédients laitiers (les MPM) qui seront fonctionnels d'un point de vue biologique et technologique.

Hypothèses secondaires.

- Les produits obtenus par la technologie Valactis™ présentent des fonctionnalités biologiques et technologiques utilisables dans différents domaines.
- Les bactéries utilisées par la technologie Valactis™ appartiennent à l'espèce *L. kéfiranofaciens* et présentent des caractéristiques uniques.
- Il est possible d'utiliser les streptomycètes afin de produire des enzymes protéolytiques qui pourraient être ajoutées à la technologie Valactis™.

Objectifs

1. Caractériser les propriétés technologiques des MPM.
2. Identifier et caractériser les souches bactériennes isolées des grains de kéfir.
3. Modifier une enzyme par mutagénèse dirigée afin d'améliorer son activité protéolytique sur l'agglomération des protéines du lactosérum.
4. Sélectionner, isoler et cloner des gènes codant pour des enzymes pouvant permettent une hydrolyse appropriée des protéines du lactosérum.
5. Sélectionner les clones positifs, purifier et caractériser sommairement une enzyme.

Moyens pour atteindre les objectifs

1. Les propriétés technologiques des MPM seront évaluées en systèmes modèles (yogourt et sauce à salade : Turgeon et *al.* 1996) en mesurant la viscosité et la consistance (Adams and Birdsall, 1946), la synérèse (Keohgh et *al.*,1995), les capacités émulsifiantes (Kulmyrzaev et al., 2000) et le drainage (visuel).

2. L'identification des bactéries se fera par la caractérisation du profil de fermentation des sucres (API 50CH; bioMérieux REF 50 410 et 50 300) et elle sera confirmée par le séquençage de l'ARN 16S (Yeung, et *al.*, 2002).

3. La caractérisation des souches se fera par la description des particularités qui distinguent les bactéries lactiques (Stiles et Holzapfel, 1997) en comparaison avec les souches de référence de l'ATCC de l'espèce *L. kefiranofaciens* (ATCC 43761 et ATCC 51647).

4. La séquence protéique des subtilisines sera analysée en fonction de leurs spécificités afin de proposer des sites de mutation (Barrett et *al.*, 1998).

5. La sélection des gènes sera effectuée à partir du génome de *Streptomyces .coelicolor* (www.sanger.ac.uk/Projects/S_coelicolor/scheme.shtml) et les gènes sélectionnés seront isolés du génome de *Streptomyces lividans* (Hopwood *et al.*, 1985) et clonés à l'intérieur de *S. lividans* 10-164 (Hurtubise *et al.*, 1995).

6. La sélection des clones positifs se fera sur milieu Stewart (Prescott et al., 1995) contenant 2,5% de lait écrémé, la purification par HPLC et la caractérisation sommaire de l'enzyme sera effectuée par spectrophotométrie en utilisant les substrats succinyl-L-Ala-L-Ala-L-Pro-L-X-p-nitroanilide (Pelmont, 1995).

Description de la structure et du contenu de la thèse

Tel que stipulé dans le règlement adopté le 14 juillet 2004 par le comité de programme du doctorat en biologie de l'INRS-Institut Armand-Frappier, la thèse comporte une revue de littérature exhaustive, les articles et brevets produits, une discussion/conclusion globale et une page faisant état des contributions à l'avancement des connaissances. La contribution spécifique de l'étudiant aux travaux présentés est expliquée à chacun des chapitres sur la page de présentation du chapitre. Les articles, manuscrit et brevet sont présenté dans la forme de leur dépôt à la demande de la présidente du jury.

L'axe principal du projet est la technologie Valactis™ de production des MPM. La figure 13 présente le contenu des chapitres, des brevets, de l'article publié et des manuscrits à soumettre ou en préparation. Une section « autres contributions » contient deux autres brevets, un autre article publié ainsi que deux manuscrit soumis.

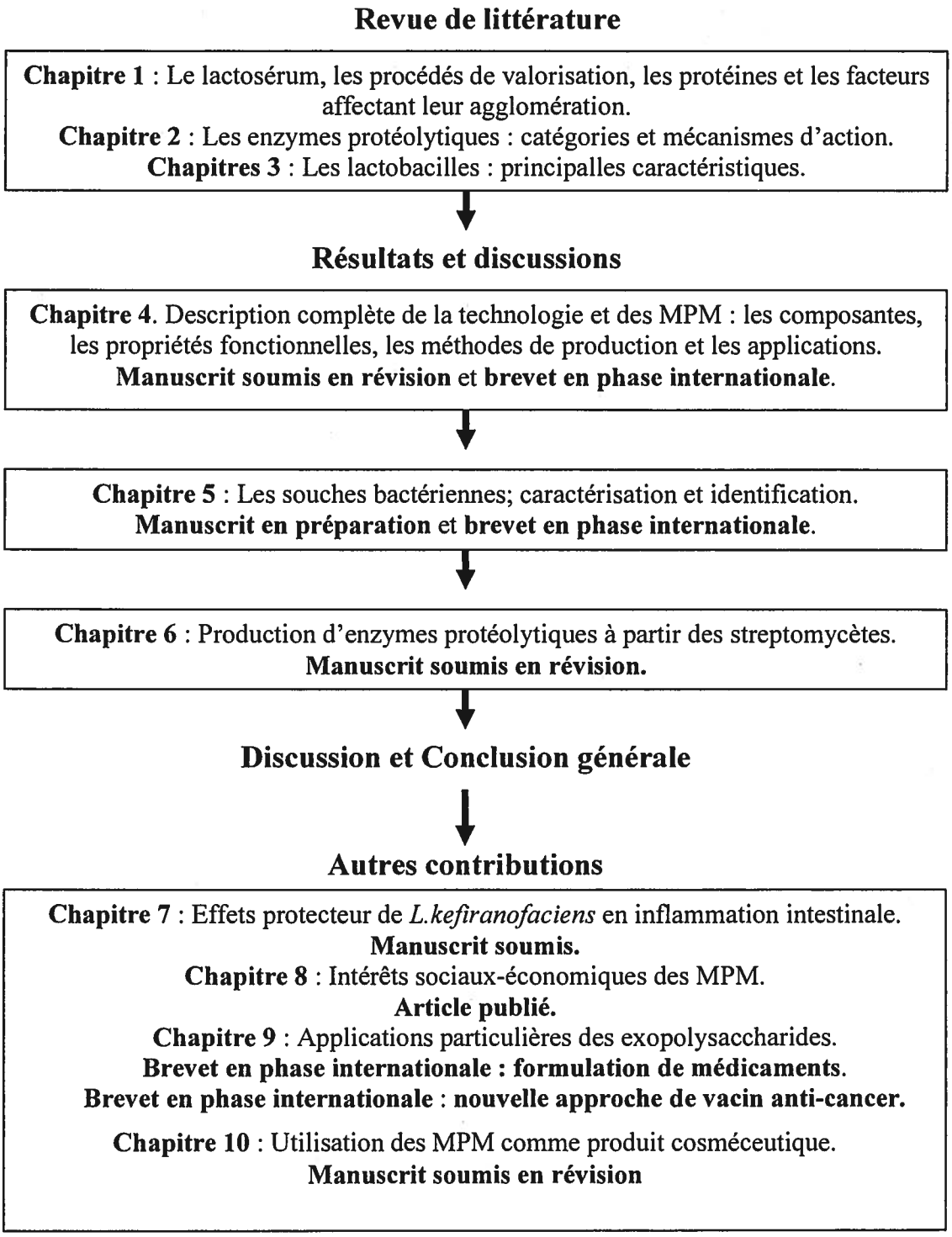


Figure 13 : Structure de la thèse en lien avec les différentes publications et brevets déposés ou publiés.

Résultats

Chapitre 4 : Description complète de la technologie; les composantes, les propriétés fonctionnelles, les méthodes de production et les applications des MPM.

Contenu du chapitre : manuscrit en préparation et brevet en dépôt international.

Manuscrit en préparation :

Simard, E., Smith, P., Mondou, M.H., Ferguson, M., Pilote, D., Goyette, P., Dupont, C., Lajoie, N., Lapointe, J.F., Lemieux, P. 2005. Nutritional and Functional Properties of a Novel Fermented Whey-Based Product. 40 pages.

Brevet :

Simard, E., Pilote, D., Dupont, Lajoie, N., Paquet, M., C., Lemieux, P., Goyette, P. 2001. International 2002. Malleable protein matrice and uses theroef. PCT/CA2002/01988. 92 pages.

Contributions : Définition des différents projets de 1995 à 1999 et contribution majeure à l'élaboration, à l'encadrement et à la réalisation des projets en collaboration avec Claude Dupont depuis 1999. Obtention de subventions de recherche comme demandeur principal. Contribution majeure à l'élaboration du projet CRSNG-Stratégique d'une durée de 5 ans et obtention des fonds en collaboration avec Claude Dupont, Pierre Lemieux, François Shareck et Daniel Oth. Réalisation d'une vaste revue des brevets existants, rédaction complète du brevet et révision du brevet pour son dépôt international.

J'ai effectué la revue de littérature sur les technologies existantes et toutes les différentes facettes du brevet. J'ai eu une contribution majeure à la revue de littérature de l'article, à la rédaction, à la compilation des données et à la coordination. J'ai aussi participé de façon majeure à l'encadrement des différentes activités de fermentation et j'ai directement encadré et dirigé les activités de caractérisation des fonctionnalités technologiques des MPM et de formulations alimentaires.

4.1 Manuscrit soumis en révision

Simard, E., Smith, P., Mondou, M.H., Ferguson, M., Pilote, D., Goyette, P., Dupont, C., Lajoie, N., Lapointe, J.F., Lemieux, P. 2005. Nutritional and Functional Properties of a Novel Fermented Whey-Based Product. 40 pages.

Manuscrit soumis à « The American Journal of Clinical Nutrition (MS 21460)» et refusé avec pour raison qu'il s'agissait d'un article de science alimentaire et non de nutrition. Des modifications ont été apportées pour ajouter des aspects de nutrition et préparer sa prochaine soumission.

J'ai eu une contribution majeure à la revue de littérature de l'article, à la rédaction, à la compilation des données et à la coordination. J'ai aussi participé de façon majeure à l'encadrement des différentes activités de fermentation et j'ai directement encadré et dirigé les activités de caractérisation des fonctionnalités technologiques des MPM et de formulations alimentaires.

INTERPRETIVE SUMMARY

A NOVEL FERMENTED WHEY-BASED PRODUCT. BY SIMARD ET AL.

NOVEL FERMENTED WHEY-BASED PRODUCT

Nutritional and Functional Properties of a Novel Fermented Whey-Based Product

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ABSTRACT

A whey protein-based product, described as a yogurt-like malleable protein matrix (**MPM**), is presented in this paper. This novel product is obtained by fermenting sweet whey with proprietary lactic acid bacteria from the *Lactobacillus* genus, followed by a protein-specific recuperation procedure. The **MPM** consists mainly (wt/wt), of water (80%), protein (8%), minerals (6%, of which calcium comprises 1,5%), carbohydrate (5%), fat (1%) and bacteria ($6X10^{11}$/100g). These components are found within the **MPM** as essential amino acids, vitamins of the B group and valuable proteins and peptides. The latter include proteins and peptides with thiol (-SH) groups, immunoglobulins, lactoferrin and an entity with transforming-growth factor like activity. The **MPM** has unique nutritional value and, it is proposed that its use as a fat substitute could provide significant health benefits for individuals with obesity-associated hyperlipidemia, inflammation and hypertension. In this study, we replaced the fat (20-40%) in 2 food formulations (yogurt and salad dressing) using the **MPM,** without affecting the texture or stability of the formulated products. The **MPM** described is, therefore, a unique nutritional ingredient that could be used in a variety of functional foods to proffer potential health benefits.

Key words: **whey protein, LAB fermentation, ingredient, functional food,**

Abbreviation key: MPM = malleable protein matrix. **BOD** = biological oxygen demand. **LAB** = lactic acid bacteria. **RT-PCR** = reverse transcriptase-polymerase chain reaction. **WSE** = water soluble extract. **AA** = amino acids. **EPS** = exopolysaccharides.

INTRODUCTION

Cheese-whey is the residual liquid obtained during cheese manufacturing. Depending on the type of cheese, it can be up to 90% of the original volume of the milk used in the manufacturing process, and contain approximately 50% of the initial constituents (Bylund, 1995). Whey was, for a long time, regarded by cheese makers as a problematic by-product because of the high volumes generated which, due the lactose content, have high biological oxygen demand (BOD) (Siso, 1996). Recently, however, a great deal of effort has been put into demonstrating the biological activities and benefits to human health of whole, individual and fractionated whey proteins and peptides (Bounous, 2000; Brock, 2002; Cross and Gill, 2000; Fitzgerald and Meisel, 2003; Floris et al., 2003; Gill and Cross, 2000; Ha and Zemel, 2003 and Smithers, et al., 1996). Recognition of the versatile functional properties (de Wit, 1989; de Wit, 1998; Morr and Ha, 1993) and high nutritional quality of whey proteins (de Wit, 1998; Ha and Zemel, 2003; Hambreus, 1992 and Smithers et al., 1996) together with the development of fractionation and conversion technologies has meant that many value-added products and ingredients from whey have begun to appear on the market (Horton, 1998; Huffman and Harper, 1999; Timmer and van der Horst, 1998). The development of new functional foods and nutraceuticals based on these whey proteins, or fractions of them, has been identified as one of the main driving forces in the whey (and dairy) industry of the future (Affertsholt, 2004; Horton, 1998; Maurice, 2001).

Probiotics have been defined as «living micro-organisms, which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition» (Guarner and Schaafsma, 1998). This definition is, however, evolving to include inactived

microorganisms or parts of their cell structures (Ouwehand et al., 2002). Probiotic strains are generally members of the lactic acid bacteria group (LAB) encountered in fermented dairy products (Stiles and Holzapfel, 1997). Amongst that group, *lactobacilli* are the most frequently used probiotics (Ouwehand, et al., 2002). Many clinically proven health benefits of probiotics have been reported in the literature (Duggan et al., 2002; Ouwehand, et al., 2002; Sartor, 2005; Sullivan and Nord, 2005; Tamboli et al., 2003). As a result of this recent research and publicity, consumer demand for cultured and/or probiotic-containing dairy products is strongly increasing (Berry, 2005 and Chandan, 1999).

Cultured products from whey are highly suited to satisfying the new and increasing consumer demand for functional foods with health benefits. A novel protein-based whey product has recently been developed (Simard et al., 2006). This product is obtained by fermenting sweet whey with a proprietary lactic acid bacterium from the Lactobacillus genus followed by protein-specific recuperation procedure. The resulting product, described as a malleable protein matrix (MPM), exhibits a yogurt-like texture. In this paper we have identified the biochemical composition and some of the functional properties of the MPM. The use of the MPM as a fat substitute in different foods is described and the potential nutritional value and health benefits of the MPM are discussed.

MATERIAL AND METHODS

Preparation of the MPM

The MPM are produce by Technologie Biolactis inc. at industrial scale by twelve hours fermentation of *Lactobacillus Kefiranofaciens* R2C2 strain, follow-up by the addition of calcium chloride 0,3% (wt/vol) and the adjustment of the pH to 7,5 with NaOH. The agglomerate proteins were recovered with a VNPX710 clarifying unit of Alpha Laval (Alfa Laval, Sweden) and the consistency at 14°C, evaluated by a USDA consistometer spreading plate with concentric chart (Adams and Birdsall, 1946). The consistency was adjusted to a yoghurt-like for a value of eight on the chart by clarifying recuperation ajustement.

The pH of the coagulated whey protein was measured with an Ag-AgCl flat surface electrode for solid food (Orion, Beverly, MA, USA).

Analysis of the MPM

Compositional analysis of the MPM. Compositional analysis was performed by Bodycote Canada (Laval, QC, Canada). Protein, fat, moisture, vitamins and ash were determined using the methods of the Association of Official Analytical Chemists (AOAC, 1990). Lactose content was determined using a Lactose/D-galactose enzymatic test kit (Boehringer Mannheim Biochemical Inc, Indianapolis, IN, USA). All minerals and oligo-elements (Ca, Cu, Fe, K, Na, Mg, Mn, Zn, Se) were determined by an atomic absorption spectrophotometric method (AOAC, 1990) while phosphorus was evaluated by a UV spectrophotometric method (Allen, 1940). The lactic acid bacterial (LAB) count was also

evaluated under anaerobic condition using the RCW media (Kojima and al, 1993). Amino acid (AA) analysis was performed using acid hydrolysis followed by the Pico-Tag method (Water Co, Milford, MA). All analyses were performed in triplicate.

Protein profile of the MPM. The protein profile of the **MPM** was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (Laemmli, 1970). The molecular weight markers (Full-range Rainbow™), ranging from 10 to 250kDa, were purchased from Amersham Biosciences (Piscataway, NJ, USA). Purified Lactoferrin (LF; 80 kDa) was purchased from the Sigma Chemical Co (St-Louis, MO, USA). The higher and lower molecular weight proteins were resolved on 10% and 15% polyacrylamide gels respectively. The gels were stained with Coomassie brilliant blue R-250.

The protein concentration of samples was determined with a Bio-Rad assay protein kit (Bio-Rad Laboratories, Hercules, CA, USA). BSA was used as the standard protein. In order to confirm the identity of the proteins, tryptic degradation of the protein bands on the SDS-PAGE gels was performed using sequencing grade modified trypsin (Promega, Madison, WI.) as described by (Shevchenko et al., 1996). The tryptic peptides were purified using a ZipTip C_{18} pipette tip (Millipore Corporation, Bedford, MA, USA) and analyzed with a Micromass Quattro II triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA), equipped with a Nanospray and a Z-spray interface. Samples were introduced into the mass spectrometer using type D Nanospray probe tips in positive mode. The capillary voltage was 1.0 keV and the cone was at 35 V.

Thiol (-SH) content. The concentration of thiol (SH) groups in the MPM (total) and in the water-soluble extract (WSE) of the MPM was measured using a Bioxytech® GSH-420TM kit (Oxis International, Portland, OR, USA) with HMS-90 (Immunotech, Vaudreuil-Dorion, QC, Canada) used as the standard thiol-containing protein. The WSE was prepared by centrifuging the MPM (or HMS 90, 100 mg/ml) at 4000×g for 8 minutes at room temperature. The aqueous phase was separated and the protein fraction precipitated by the addition of trichloroacetic acid (TCA, 5%, wt/vol, final concentration). The mixture was centrifuged once more (4000×g for 8 minutes at room temperature) and the protein-free, aqueous phase collected. Samples (100 µl of total MPM or HMS 90 at 100 mg/ml; 200 µl of the WSE of the MPM or HMS 90) were buffered by the addition of potassium phosphate (200µl), diethylenetriaminepentaacetic acid (DTPA) and Lubrol at pH 7.8. Tris (2-carboxyethyl) phosphine (TCEP, 200µl) was added to reduce any oxidized thiol groups. The chromogenic reaction was initiated by the addition of 4-chloro-1-methyl-7-trifluoromethylquinolinium methylsulfate (200µl) in HCl followed by the addition of NaOH (200µl) to obtain a pH greater than 13. The reaction mixture was kept in the dark for 30 minutes and the absorbance measured at 420 nm with a standard UV/Vis spectrophotometer (Varian).

Gene Modulatory effects of MPM

Microarray experiment. Adult human epithelial keratinocytes (HEKa), grown in Medium 154 supplemented with human keratinocyte growth supplement (HKGS), were obtained from Cascade Biologics, (Portland, OR, USA). HEKa cells were exposed to various dilution of MPM (wt/vol; 1:1000, 1:10,000, 1:100,000) in phosphate buffer saline

for 16 h. Control, Lipopolysaccharide, Exopolysaccharides, and calcium were also compared. Total RNA was isolated using a RNeasy® purification Kit (Qiagen, Germantown, MD, USA). The RNA samples from control and exposed HEKa cells were labelled with cyanine dyes Cy3 and Cy5 respectively for competitive hybridization and applied to the Galactis™ human microarray screening system (Technologie Biolactis Inc., Laval, QC, Canada). Gene activation observed with the Galactis™ screening system was confirmed using the reverse transcriptase-polymerase chain reaction (RT-PCR) in accordance with the standard protocol (O'Connell, 2002). The following genes were analyzed; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), defensin 2 (Def2), tumor necrosis factor α (TNFα), interleukin-6 (IL-6) and transforming growth factor β (TGFB-i).

The use of MPM as a fat substitute

Low-fat yogurt model. Skimmed milk powder was reconstituted to produce skimmed milk (12% wt/vol). The milk was heated at 85°C for 30min then cooled to 40-43°C before the addition of commercial yoghurt culture (1% wt/vol) containing *Streptococcus thermophillus*, *Lactobacillus acidophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* (Lyo-San Lachute, QC, Canada). The mixture was incubated at 42°C ± 0.5°C for 4 hours or until the pH reached 4.5. The yoghurt was then chilled quickly to 10°C before the addition of MPM (0, 5, 25, 50 and 75% (wt/wt) with a viscosity of approximately 3000cP. The viscosity of the MPM was determined at 5°C with a Brookfield viscometer (Model LV-DVII+, Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) fitted with a vane spindle #V-72 according to the method of Adams and Birdsall (1946).

The yoghurts were stored at 4°C for 24 hours before further analysis. The pH values of the yoghurts were determined at 4°C with a pH meter (Orion 310, Orion, Boston, MA, USA). Total solids were determined by drying duplicate samples at 110°C for 2 hours, followed by a cooling step in a dessicator. Various rheological properties of the yoghurts were then measured: The consistency at 4°C was determined with an Adams consistometer (Cole-parmer, USA) according to the method of Adams and Birdsall (1946). Briefly, this method measures the rate of flow of a semi fluid food along two axes. The apparent viscosity (AV), at 5°C, of a homogenous yoghurt sample in a 500ml beaker was recorded with a Brookfield viscometer (Model LV-DVII+, Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) fitted with vane spindle # V-71. The spindle was allowed to travel for 5 min and the data recorded are the averages of highest and lowest readings obtained. In a method adapted from that of Keogh et al. (1995), syneresis was evaluated as follows: a sample of yoghurt (30g) was weighed into a plastic tube, and left overnight at 4°C. The supernatant was removed with absorbent paper and the remaining gel weighed. The percentage of syneresis was calculated according to equation [1]:

$$Syn = 100\frac{(m_1 - m_2)}{m_1} \qquad [1]$$

where *Syn* is the syneresis (%) and m_1 and m_2 are the weights (g) of the sample before and after the allowed period of syneresis respectively. All analyses were carried out in duplicate. Drainage and conservation data were also monitored over time during 4 weeks at 4 °C with visual observation of drainage, syneresis and mycelium contamination.

Salad dressing model. The formulation of the salad dressing was adapted from that of Turgeon et al. (1996). The non-MPM part of the formulation was composed of corn oil (66.7%, wt/wt) (Best Foods, Etobicoke, ON, Canada), vinegar (29.3%, wt/wt, comprised of 5% acetic acid) and sugar (4%). Three different concentrations of the MPM were incorporated with this oil/sugar/vinegar mix: 0% (control), 15%, 25% and 40% (wt/wt). Firstly, the sugar and vinegar were mixed together using a household blender until all the sugar had dissolved. Next, the MPM was added and stirred thoroughly for 30s. Finally, the oil was added and the formulation stirred for 1 min. Each formulation was prepared in triplicate. Various rheological properties of the salad dressing formulations were evaluated. The creaming index (C_r) of each salad dressing formulation was determined according to the method of Kulmyrzaev et al (2000): Samples (25ml) were transferred into 50 ml conical tubes and the C_r evaluated according to equation [2] :

$$C_r = 100 \times \frac{H_O}{H_E} \qquad [2]$$

where H_O is the total volume (ml) of the separated oil phase measured after a storage period of 24h at 30°C and H_E is the initial volume (ml) of the salad dressing sample evaluated. The long-term emulsion stability of the salad dressing formulations at 4°C was also evaluated at various time intervals by the same procedure.

Finally, the viscosity of the different salad dressing formulations was evaluated using the Adams consistometer (Cole-parmer, USA) at 4°C (Adams and Birdsall, 1946).

Results

Analysis of the MPM

Description of the MPM. Fresh **MPM**, prepared according to the method described, has a yogurtl-like texture, is beige in color, a neutral taste and smell, and a neutral pH (7-7.5). It has a firm, cohesive and spreadable consistency as pictured in Figure 1.

Composition of the MPM. The compositional analysis results are shown in Table 1. The **MPM** is composed of water (80%), protein (8%), 6% minerals (6%, including calcium (1,5%)), carbohydrate (5%, including lactose (2.7%)) and fat (<1%). On average, the manufacturing process leading to the production of fresh **MPM** resulted in a 10-fold enrichment of the proteins and an 8-fold enrichment of the minerals contained in the initial non-fermented sweet whey. Copper, Iron (six-fold increase) and Manganese, together with some vitamins of B group, were also enriched in the **MPM**. Vitamin C was not detected in the **MPM**. There was an increase in fat content (2.4-fold) in the **MPM** but, although the lactose level was almost half that of the initial non-fermented sweet whey, the total carbohydrate level remained approximately the same in the **MPM**. Finally, $6X10^{11}$ CFU of LAB per 100 g were detected within the frech **MPM**.

Protein profile of the MPM. The SDS-PAGE protein profiles of the **MPM** and the initial non-fermented sweet whey are shown in Figure 2. From the figure we can see that the fermentation procedure resulted in an increase in the ratio of high to low molecular weight proteins within the MPM compared to the non-fermented sweet whey. Two lower-molecular weight bands (≤ 25kDa) can be seen in the **MPM** profile in Fig. 2b corresponding to the 2 major whey proteins α-lactalbumin (LA, Mr 14 kDa (Brew and

Grobler, 1992)) and β-lactoglobulin (β-LG, Mr 18kDa; (Hambling et al., 1992)). Higher-molecular weight bands (>75kDa; lanes 7-10 on fig. 2-a,b) associated with LF and various immunoglobulins (Ig) were also found within the **MPM**. The identity of the protein associated with LF was further confirmed by tryptic degradation and mass spectrometry analysis (data not shown).

Content in thiol(-SH) groups

The results regarding the thiol(-SH) content of the total MPM and its WSE are presented in figure 3. The total **MPM** and its WSE exhibit higher level of thiol(-SH) groups as compared to HMS 90, a commercial whey-based protein product reported to contain high levels of thiol(-SH) groups (Micke et al., 2002). The MPM level of the AA containing thiol(-SH) Cys is 240 mg/100g (humid basis) (Table 3).

Gene Modulatory effects of MPM

Microarray experiment. RNA samples were collected from HEKa cells exposed to the **MPM.** The resulting gene activation within those samples identified using the Galactis™ screening system and confirmed by RT-PCR are shown in Figure 4. Several genes associated with immune modulation and cell growth were activated upon exposure to the **MPM**, specifically the TGFβ-i gene. The genes for Defensin 2, TNFα, and IL-6, all associated with the immune modulation process (Abbas et al., 1996; Sharma and Bose, 2001; Ouellette, 2004) were also activated. These genes were not activated by calcium and exopolysaccharides.

The use of MPM as a fat substitute

Low-fat yogurt model. The pH value, solid content and rheological properties of low-fat yoghurt formulations, upon incorporation with the **MPM**, are shown in Table 3. The pH of the low-fat yoghurts increased with increasing content of the **MPM**, from pH 4.56 with no **MPM** added up to pH 5.83 with 75% **MPM** added (wt/wt). The solid content of the low-fat yoghurts similarly increased with increasing amounts of **MPM** added. Regarding the rheological properties, addition of 25% to 50% of the **MPM** is required to increase the spreading and viscosity values. The syneresis values are increased in the yogurts made with 5% and 25% **MPM** as compared to the control batch but she is reducing at higher concentration (50% and 75% **MPM**).

Salad dressing model. The physical characteristics and stability of a salad dressing formulation, upon incorporation with the **MPM**, are shown in Table 4. The salad dressing containing 10% **MPM** was pourable, as indicated by its high consistency spreading value. Compared to the control (0% added **MPM**), its C_r (60%) was lower and its long-term stability was improved. In contrast, the addition of 25% or 40% **MPM** resulted in mayonnaise-type emulsions with consistency spreading values much lower than those found with the control and 10% added **MPM**. The long-term stability of these 2 formulations was greatly increased up to180 days with 25% added **MPM** and more than 365 days with 40% added **MPM**. At 365 days the salad dressing formulation containing 40% MPM, but containing no added preservatives, showed no signs of creaming, drainage or spoilage.

DISCUSSION

Compositional data, nutritional value and potential health benefits of the MPM

The MPM described herein is a novel protein-based whey product representing a new class of whey-protein ingredients. The **MPM** is different from protein-based polymers obtained by thermomechanical processes or protein microprecipitation of whey-protein concentrates (Cheftel and Dumay, 1993; Mai et al., 1990; Queguinier et al., 1992). To date, fermented protein-based whey products are not available commercially. Although a complex product, it can be seen as a yogurt-like whey protein network where Ca^{2+} bridges between proteins probably play a major role.

Although the lactose content decreased during the fermentation process, the total carbohydrate content within the **MPM** remained unaltered. This is probably due to the synthesis of exopolysaccharides (EPS) by *L. Kefiranofaciens* R2C2 during the fermentation process. *L. Kefiranofaciens subsp.* strains are well-known EPS producers (Fujisawa et al., 1988; Vancanneyt et al., 2004). A high concentration of LAB was found in the final **MPM** product compared to other commercially available fermented dairy products (Table 5). Many health benefits are actually associated with different EPS (Welman and Maddox, 2003).

In comparison with common and specialized fermented dairy products, the **MPM** contains more calcium, phosphorus, vitamin B and proteins but with a similar calorific content (Table 5). The increased Na^{+} (3.3-fold increase) and Ca^{2+} (32-fold increase) content within the **MPM** are to be expected as sodium hydroxide (NaOH) and chloride calcium ($CaCl_2$) were added during the manufacturing process to maintain the pH, in the

case of the NaOH and to increase the final **MPM** yield, in the case of the $CaCl_2$. The increased phosphorus content (15.9-fold increase) may be due to the formation of phosphate salts upon the by the fact that some phosphate salts (especially Ca- phosphates) formed following the addition of Ca^{2+} have limited solubilities (CRC Handbook of chemistry and physics, 1980). Calcium and phosphorus are important minerals for bone formation and health (Cashman, 2002; Fishbein, 2004; Ilich and Kerstetter, 2000). The fact that part of the calcium is most likely to be found bounded to whey proteins might also improve its nutritional bioavailability (Fishbein, 2004). Vitamins of the B group are reported to contribute to the energy metabolism as well as tissue formation and blood lipid control in humans (Woolf and Manore, 2006).

From the SDS-PAGE profiles of the **MPM** and the whey, we can see that the protein recovery procedure increased the ratio of high to low molecular weight proteins. High molecular weight whey proteins such as LF and IG have demonstrated many biological activities to be used in functional food with health benefits (Brock, 2002; Cross and Gill, 2000; Floris, et al., 2003; Smithers, et al., 1996). Moreover, a reduction in the low molecular weight proteins β-LG and LA could be beneficial as these proteins have both been shown to be immunogenic and responsible for many food allergies during infancy (Kaminogawa and Totsuka, 2003; Wal, 2002). This could be due in part to degradation of the major whey proteins LA and β-LG during the fermentation process by the action of endogenous proteases of the *L. kefiranofaciens* strain used. This could decrease the ability of these 2 proteins to agglomerate during the calcium-induced recuperation procedure and in turn reduce their relative concentration in the **MPM**. However, although the proteolytic

action on caseins of many *L.* species is well-known (Kunji et al., 1996), no actual data is reported regarding the proteolytic system of *L. kefiranofaciens* subsp. This is partially confirmed by thiol(-SH) groups in the WSE of the MPM, suggesting the presence of thiol(-SH) containing AA (Cys) or peptides in the WSE. The increase of thiol(-SH) groups in the WSE during fermentation should however be confirmed.

High thiol (-SH) content in the diet has been shown to increase the level of the antioxidant Glutathione (GSH) (Bounous et al., 1989; Bounous and Gold, 1991; Parodi, 1998), thought to be of major importance to the immune system and, in particular, the immunological response to cancer (Bounous and Molson, 2003). From amino acid analysis of the **MPM,** shown in Table 3**,** we can see that of all the essential amino acids only Phe and Thr had chemical scores below 100% indicating that the **MPM** retains the nutritional value of whey proteins (Hambreus, 1992). High content in the branch-chain AA Leu is also found.

For the genomics evaluation, the gene is activated by the transforming growth factor β (TGFβ), the most common growth factor found in latent form in sweet whey (Rogers et al., 1996). The latent form of TGFβ can be activated by lowering the pH, raising the temperature or by protease activity (Rogers et al., 1996). Such conditions for the activation of TGFβ are all met during the manufacturing process of the MPM. This suggests that the polypeptide fraction (initial whey proteins and/or new peptides generated during the fermentation) recovered in the MPM is still able to exert biologically activity. These results seem to be related to the polypeptide fraction since other

components found in the MPM such as calcium and exopolysaccharides did not triggered the activation of the same genes.

Functional properties

In this study, the **MPM** was successfully used as a substitute for fat in a low-fat yogurt and a salad dressing. In the low-fat yogurt model, up to 75% **MPM** could be added as a texturizing and thickening agent without adversely affecting the final rheological properties of the formulated yogurts. At 75% **MPM**, it resulted in yogurts containing approximately 0.9% of fat with comparable spreading consistency, viscosity and syneresis characteristics. The increase in pH of the yoghurt with increasing content of the **MPM,** from pH 4.56 (0% **MPM,** wt/wt) to pH 5.83 (75% **MPM**, wt/wt) is to be expected as the pH of the **MPM** varies from 7.0 to 7.5.

In the salad dressing model, the **MPM** behaved as an emulsifying and thickening agent resulting in a mayonnaise-type emulsion at 25% and 40% added **MPM** with long-term stability characteristics (see Table 4). The addition of 40%**MPM** resulted in a mayonnaise-type salad dressing containing approximately 40% of fat. Surface properties of whey proteins allow them to behave as emulsifying agents in a variety of food applications (de Wit, 1998; Morr and Ha, 1993). This property appears to be preserved in the **MPM**, despite its high calcium content which is reported to be detrimental to the emulsification properties of proteins (Morr and Ha, 1993).

CONCLUSION

The **MPM** is a novel, protein-based whey product representing a new class of whey protein ingredients that can be obtained by fermenting sweet whey with a proprietary LAB strain from the *Lactobacillus* genus and a subsequent protein-specific recuperation procedure.

The **MPM** exhibits a unique composition compared to common and specialized fermented dairy products, particularly in terms of essential AA, thiol(-SH) and LAB content. Moreover, the **MPM** displayed good functionality and texture properties in low-fat yogurt and salad dressing models.

The **MPM** is thus a complex product with good technological functionality, nutritional value and numerous potential health benefits. The **MPM** could thus be use in the formulation of innovative functional products with health benefits. Research efforts are actually devoted to explore its synergistic immunomodulatory anti-inflammatory properties (Beaulieu et al., 2007) and its blood lipid lowering effect as well as the probiotic ability of the LAB strain. Further characterization of individual components of the **MPM** such as the polypeptide fraction and the EPS produced by the LAB strain has also been initiated.

TABLES

FIGURES:

Figure 1: Visual representation of the malleable protein matrix (**MPM**).

Figure 2: Comparison of the protein profiles of the malleable protein matrix (**MPM**) and initial (non-fermented) sweet whey by SDS-PAGE with a) 10% and b) 15% acrylamide. Molecular weigh markers (lane 1), 5μg/ul? LF (lane 2), initial (non-fermented) sweet whey at 1:1, 1:2, 1:10 and 1:100 dilution ratios (lanes 3-6) and humide **MPM** at 1:10, 1:20, 1:50 and 1:100 dilution ratios (lanes 7-10).

Figure 3: Content in thiol (-SH) groups in the total and water soluble (WSE) extract of the malleable protein matrix (**MPM**).

Figure 4: Activation of the genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), defensin 2 (Def2), tumor necrosis factor α (TNFα), interleukin-6 (IL-6) and growth factor β (TGFB-i).as revealed Reverse transcriptase-polymerase chain reaction (RT-PCR) data from adult human epithelial keratinocytes (HEKa) exposed to the malleable protein matrix (**MPM**). Control (lane 1); 10 μg/ml lipopolysaccharide (lane 2), 10 μg/ml exopolysaccharide (lane 3), 1.5mM calcium ($CaCl_2$) (lane 4) and humide of the **MPM** at 1:1000, 1:10000 and 1:100000 dilution ratios (lanes 5-7).

Figure 1: Visual representation of the malleable protein matrix (**MPM**).

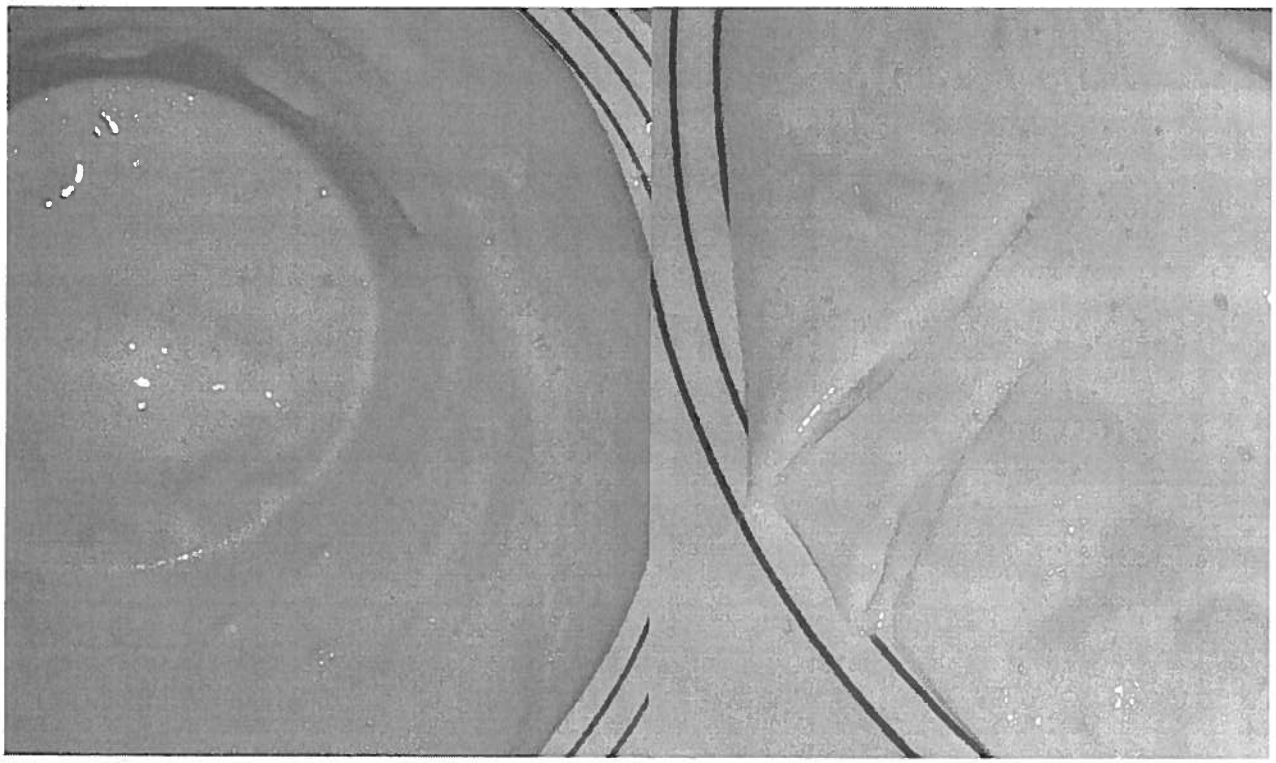

Figure 2 Comparison of the protein profiles of the malleable protein matrix (**MPM**) and initial (non-fermented) sweet whey by SDS-PAGE with a) 10% and b) 15% acrylamide. Molecular weigh markers (lane 1), 5μg/ul, LF (lane 2), initial (non-fermented) sweet whey at 1:1, 1:2, 1:10 and 1:100 dilution ratios (lanes 3-6) and humide **MPM** at 1:10, 1:20, 1:50 and 1:100 dilution ratios (lanes 7-10).

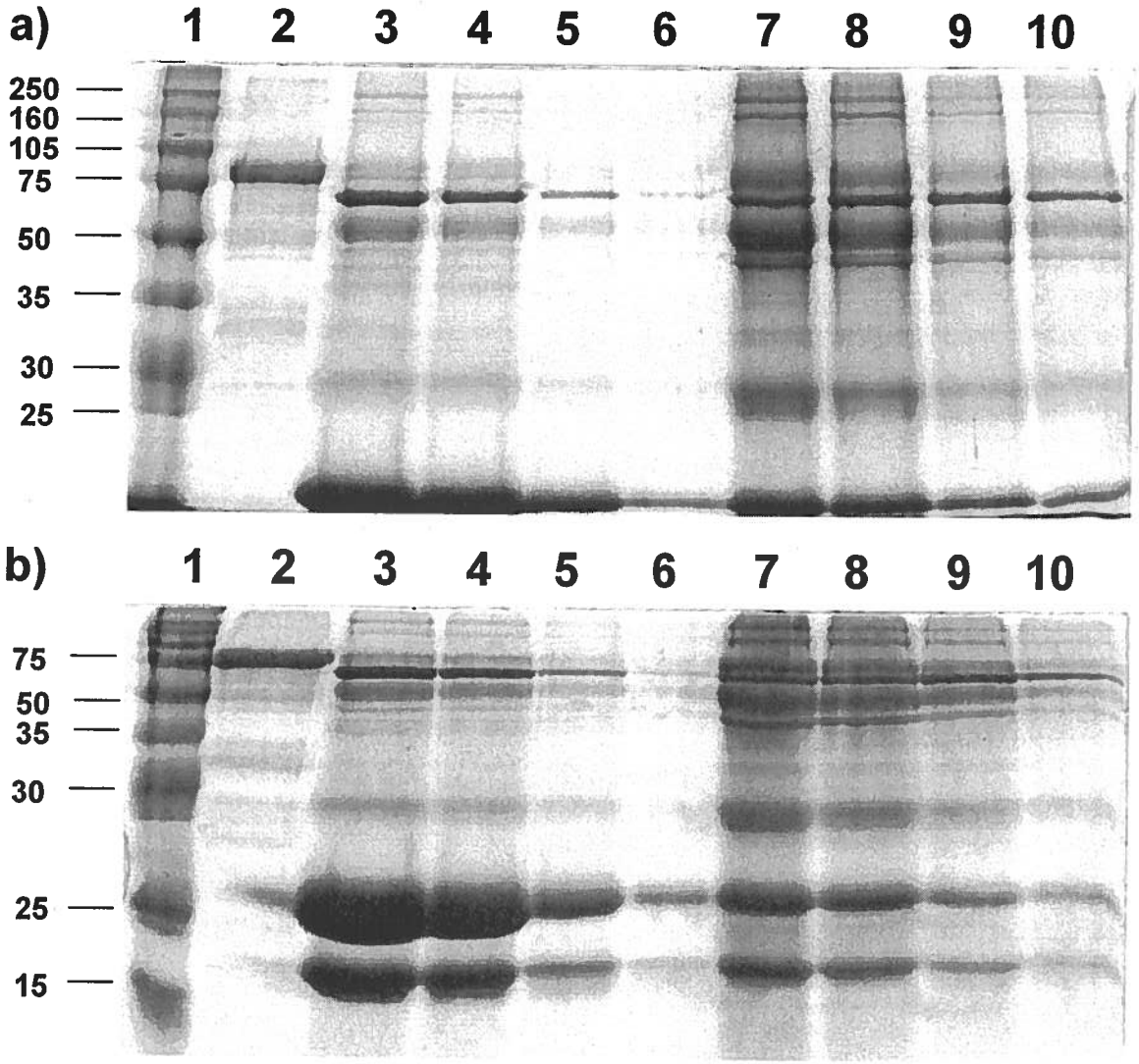

Figure 3: Content in thiol(-SH) groups in the total and water soluble (WSE) extract of the malleable protein matrix (**MPM**).

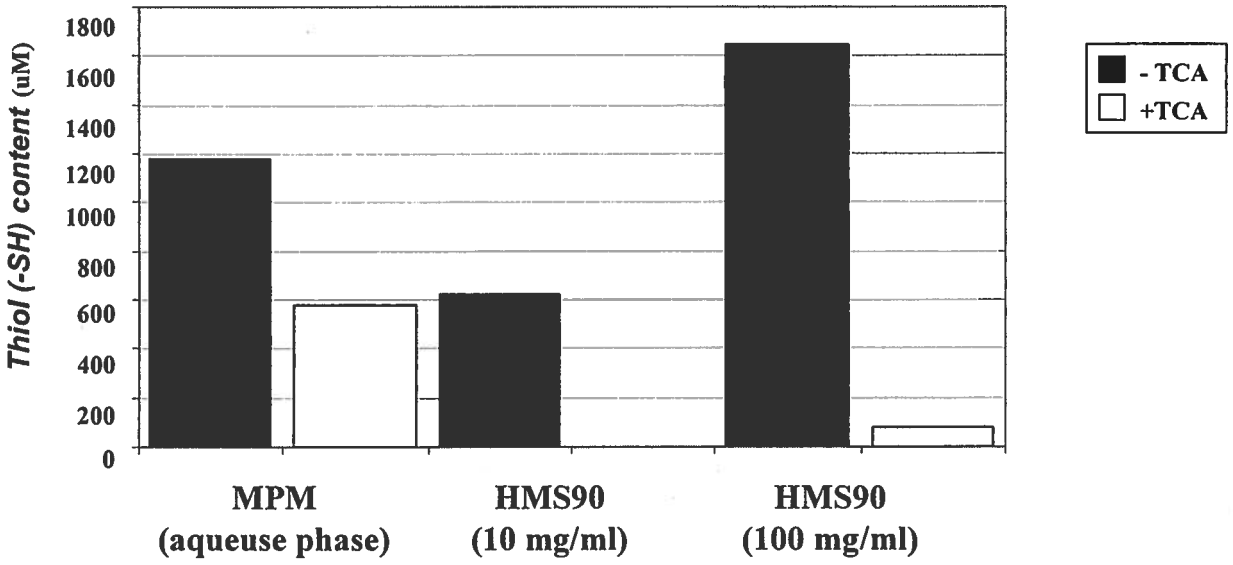

Figure 4 : Activation of the genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), defensin 2 (Def2), tumor necrosis factor α (TNFα), interleukin-6 (IL-6) and growth factor β (TGFB-i).as revealed Reverse transcriptase-polymerase chain reaction (RT-PCR) data from adult human epithelial keratinocytes (HEKa) exposed to the malleable protein matrix (**MPM**). Control (lane 1); 10 μg/ml lipopolysaccharide (lane 2), 10 μg/ml exopolysaccharide (lane 3), 1.5mM calcium ($CaCl_2$) (lane 4) and humide of the **MPM** at 1:1000, 1:10000 and 1:100000 dilution ratios (lanes 5-7).

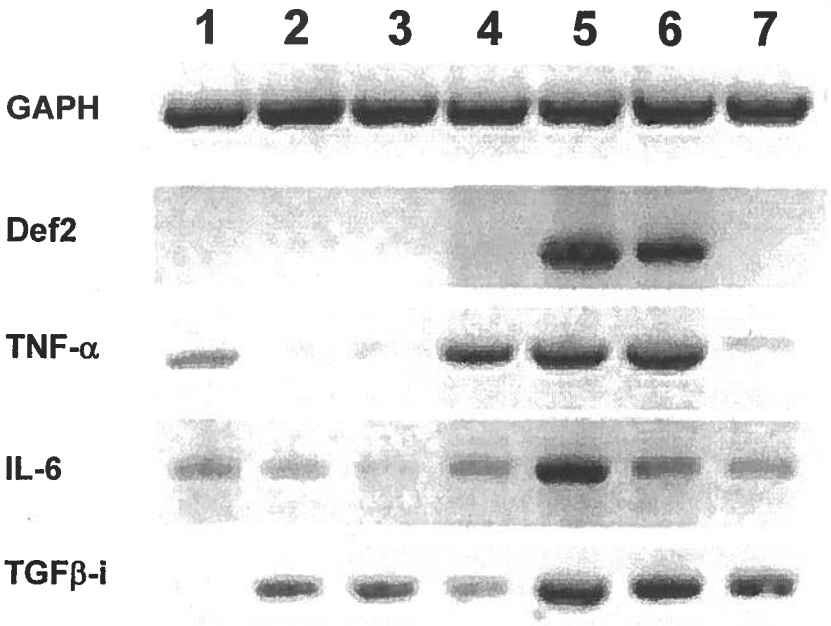

Table 1: Average and specific composition (wt/wt; humid basis) of the malleable protein matrix (**MPM**) as compared to the initial (non-fermented) liquid sweet whey.

	MPM	sweet whey[1]
Average composition (g/100g)	*n=5*	
Humidity	80.0	93.7.
Protein	8.3	0.8
Fat	1.2.	0.5.
Ash (minerals)	5.9	0.5.
Carbohydrates	4.7	5.3.
Lactose	2.7.	4.8.
Galactose	0.2.	Not detected
Minerals (mg/100g) *n=5*		
Potassium	142.9	161.0
Sodium	175.2	54.0.
Calcium	1507.4.	47.0
Phosphorus	730.3.	45.9
Selenium	< 0.1.	0
Magnesium	5.4	2.2.
Oligo-elements (mg/100g)	*n=3*	
Copper	0.07	0
iron	0.24	0.04
Manganese	0.05	0
Zinc	0.13	0.12
Vitamins (/100g)	n=3	
Riboflavine (B2)	0.32 mg	0.16 mg
Niacin (B3)	1.00 mg	0.17 mg
Pyridoxine (B6)	0.04 mg	0.04 mg
Cobalamine (B12)	Not detected	0.28 ug
Ascorbic acid (C)	Not detected	0.9 mg
Folic acid	5 µg	5 µg[2]
Bacterial count (CFU/100g)	*n=?*	
LAB	$6X10^{11}$	0 ?

[1] taken from (Kosikowski, 1982) and (Nutritiondata.com)

[2] Le lait et les produits laitiers dans la nutrition humaine (WOA).

Table 2: Amino acids (AA) profile of the malleable protein matrix (**MPM**) as compared to the initial (non-fermented) sweet whey. The chemical score of each indipensable and strictly indispensable is also presented.

nutritional requirement	AA	MPM		Sweet whey[1]
		content (mg/100g)	chemical[2] score (%)	content (mg/100g)
dispensable	Asp/Asn	720		83
	Glu/Gln	1030		148
	Ala	330		39
	Arg	220		25
	Gly	290		18
	Ser	380		41
	Pro	340		52
indispensable	His	240	100	16
	Ile	350	100	47
	Leu	720	100	78
	Val	340	100	46
	Trp	?		?
	Met	160	100	16
	Cys[3]	240		17
	Phe	260	95.6	27
	Tyr	240		24
strictly indispensable	Thr	560	100	54
	Lys	610	100	68

[1] taken from (Nutritiondata.com)

[2] For the calculation of the chemical score, the content of each indispensable and strictly indispensable AA (mg/g protein) was divided by the the reference value for indispensable AA as reported in (Bos et al., 2000). Met + Cys and Phe + Tyr are calculated together.

[3] Not indispensable, but used in the calculation of the chemical score of Met.

Table 3 : Characteristics of the low-fat yogurts formulated with various content of the malleable protein matrix (MPM).

	MPM added (wt/wt %)				
	0	5	25	50	75
pH	4.56	4.61	5.04	5.42	5.83
Solid content (wt/wt %)	12.6	12.8	14.1	15.1	15.7
spreading consistency (cm)	5.5	5.0	6.7	6.5	4.5
viscosity (cP)	1200	610	1400	8700	*1500?*
Syneresis (%)	0	58	*69*	*15*	*7*

Table 4 : Characteristics of the salad dressings formulated with various content of malleable protein matrix (MPM).

	MPM added (wt/wt %)			
	0	15	25	40
emulsion type	unstable	creamy	mayonnaise-type	mayonnaise-type
spreading consistency (cm)	++[1]	++	8	5
C_r (%)	70	60	0	0
long-term stability[2] (d)	1	4	180	>365

[1] spreading is > than the limit of the apparatus

[2] day(s) before C_r

Table 5: Energy and content, of selected nutrients (per 100 g) of the malleable protein matrix (**MPM**), as compared with common fermented dairy products.

	MPM	Kefir[1] (plain)	Bio K+ [2] (original)	Yogurt[3] (Activia, sweetened plain)	Recommended daily value[6]
Energy (kcal)	60[4]	51	45	97	nA
Protein (g)	8.2	2.9	5.1	4.4	nA
Fat (g)	1.2	1.7	0	3.5	65
Carbohydrate (g)	4.8	13.7	6.1	11.5	300
Calcium (mg)	1500	94	168	155	1100
Phosphorus (mg)	730	nA[5]	nA	126	1100
Vitamin B2 (mg)	0.3	nA	nA	0,17	1,6
Vitamin B3 (mg)	1	nA	nA	nA	23
LAB/probiotic (CFU/100g)	$6x10^{11}$	nA	$5x10^{10}$	nA	nA

[1] Produits Liberté Inc. Brossard, PQ, Canada (www.liberte.qc.ca)

[2] Bio-K+ international Inc. Laval, PQ, Canada (www.biokplus.com)

[3] Danone Canada. Boucherville, PQ, Canada (www.activia.ca)

[4] Energy was calculated

[5] nA = not-available

[6] Canadian food labelling (ACIA)

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REFERENCES

Adams, M. C. and E. L. Birdsall. 1946. New consistometer measures corn consistency. J. Food Industries. 18(6):78.

Affertsholt, T. 2004. Global market study on whey products and its high value derivatives. How dairy companies are turning a commodity into a valuable specialties products. New Ingredients derived from Components in Milk and Whey, Aarhus, Denmark.

Abbas AK, Murphy KM, Sher A. 1996. Links Functional diversity of helper T lymphocytes. Nature. 383(6603):787-93.

Allen, R. J. L. 1940. The estimation of phosphorus. Biochem. J. 34:858-865.

AOAC. 1990. Method number 989.05 : Ash of milk. Gravimetric method. Final Action. Method number 948.15 : Fat in seafood. Acid hydrolysis method. Final Action. Method number 928.08 : Nitrogen in meat. Kjeldhal method. Final action 1974. In : Official methods of analysis, 15th ed. Association of Official Analytical Chemist, Washington, DC.

Beaulieu, J., Dubuc, R., Beaudet, N., Dupont, C., and Lemieux, P. 2006 Immunomodulatory potential of a malleable matrix composed of fermented whey proteins and lactic acid bacteria. *Journal of Medicinal food.* (Accepted to be published in 2007 Spring issue.

Berry, D. 2005. Cultured dairy foods: demographically desirable. *in* Dairy Foods.

Bos, C., C. Gaudichon, and D. Tome. 2000. Nutritional and physiological criteria in the assessment of milk protein quality for humans. J Am Coll Nutr. 19(2 Suppl):191S-205S.

Bounous, G. 2000. Whey protein concentrate (WPC) and glutathione modulation in cancer treatment. Anticancer Res. 20(6C):4785-4792.

Bounous, G., G. Batist, and P. Gold. 1989. Immunoenhancing property of dietary whey protein in mice: role of glutathione. Clin Invest Med. 12(3):154-161.

Bounous, G. and P. Gold. 1991. The biological activity of undenatured dietary whey proteins: role of glutathione. Clin Invest Med. 14(4):296-309.

Bounous, G. and J. H. Molson. 2003. The antioxidant system. Anticancer Res. 23(2B):1411-1415.

Brew, K. and J. A. Grobler. 1992. a-lactalbumin. Pages 191-229 *in* Advanced dairy chemistry-1 : Proteins. P. E. Fox, ed. Elsevier Applied Science, N-Y, USA.

Brock, J. H. 2002. The physiology of lactoferrin. Biochem Cell Biol. 80(1):1-6.

Bylund, G. 1995. Dairy processing handbook. Tetra Pak Processing Systems AB, S-221 86 Lund, Sweden.

Cashman, K. D. 2002. Calcium intake, calcium bioavailability and bone health. Br. J. Nutr. 87(Suppl 2):S169-177.

Chandan, R. C. 1999. Enhancing Market Value of Milk by Adding Cultures. J. Dairy Sci. 82(10):2245-2256.

Cheftel, J. C. and E. Dumay. 1993. Microcoagulation of proteins for development of «creaminess». Food Rev. Int. 9(4):473-502.

CRC Handbook of chemistry and physics. 1980. 61st edition ed. CRC Press, Boca Raton, FL, USA.

Cross, M. L. and H. S. Gill. 2000. Immunomodulatory properties of milk. Br J Nutr. 84 Suppl 1:S81-89.

de Wit, J. N. 1989. Functionnal properties of whey proteins. Pages 285-321 *in* Developments in dairy chemistry. P. F. Fox, ed. Elsevier applied science, N-Y.

De Wit, J. N. 1998. Marschall Rhone-Poulenc Award Lecture. Nutritional and functional characteristics of whey proteins in food products. J Dairy Sci. 81(3):597-608.

Duggan, C., J. Gannon, and W. A. Walker. 2002. Protective nutrients and functional foods for the gastrointestinal tract. Am J Clin Nutr. 75(5):789-808.

Fishbein, L. 2004. Multiple sources of dietary calcium--some aspects of its essentiality. Regulatory Toxicology and Pharmacology. 39(2):67-80.

Fitzgerald, R. J. and H. Meisel. 2003. Milk protein hydrolysates and bioactive peptides. Pages 675-698 *in* Advanced dairy chemistry-1 Proteins partB. 3rd ed. P. F. Fox and P. L. H. McSweeney, eds. Kluwer academic/Plenum, New York.

Floris, R., I. Recio, B. Berkhout, and S. Visser. 2003. Antibacterial and antiviral effects of milk proteins and derivatives thereof. Curr Pharm Des. 9(16):1257-1275.

Fujisawa, T., S. Adachi, T. Toba, K. Arihara, and T. Mitsuoka. 1988. Lactobacillus Kefiranofaciens sp. nov. isolated from kefoir grains. Int J Syst Bacteriol. 38(1):12-14.

Gill, H. S. and M. L. Cross. 2000. Anticancer properties of bovine milk. Br J Nutr. 84 Suppl 1:S161-166.

Guarner, F. and G. J. Schaafsma. 1998. Probiotics. Int J Food Microbiol. 39(3):237-238.

Ha, E. and M. B. Zemel. 2003. Functional properties of whey, whey components, and essential amino acids: mechanisms underlying health benefits for active people (review). The Journal of Nutritional Biochemistry. 14(5):251-258.

Hambling, S. G., A. S. McAlpine, and L. Sawyer. 1992. b-lactoglobulin. Pages 141-189 *in* Advanced dairy chemistry-1 : Proteins. P. F. Fox, ed. Elsevier Applied Science, N-Y, USA.

Hambreus, L. 1992. Nutrional aspects of milk proteins. Pages 457-490 *in* Advanced dairy chemistry. Vol. 1. P. E. Fox, ed. Elsevier Applied Science, N-Y,USA.

Horton, B. 1998. The whey processing industry into the 21st century. Whey. Proceedings of the 2nd International Whey Conference, Chicago.

Huffman, L. M. and W. J. Harper. 1999. Maximizing the value of milk through separation technologies. J Dairy Sci. 82(10):2238-2244.

Ilich, J. Z. and J. E. Kerstetter. 2000. Nutrition in Bone Health Revisited: A Story Beyond Calcium. J Am Coll Nutr. 19(6):715-737.

Kaminogawa, S. and M. Totsuka. 2003. Allergenecity of milk proteins. Pages 647-674 *in* Advanced dairy chemistry-1 Proteins partB. 3rd ed. P. F. Fox and P. L. McSweeney, eds. Kluwer academic/Plenum, New York.

Keohgh, M. K., K. I. Lainé, and J. F. O'Connor. 1995. Rheology of sodium caseinate-carrageenan mixtures. J. Texture Studies. 26:635-652.

Kosikowski, F. V. 1982. Cheese and fermented milk products. 2nd edition ed. Brooktondale, N-Y.

Kulmyrzaev, A., M. P. C. Sivestre, and D. J. McClements. 2000. Rheology and stability of whey protein stabilized emulsions with high CaCl2 concentrations. Food Research International. 33(1):21-25.

Kunji, E. R., I. Mierau, A. Hagting, B. Poolman, and W. N. Konings. 1996. The proteolytic systems of lactic acid bacteria. Antonie Van Leeuwenhoek. 70(2-4):187-221.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227(5259):680-685.

Mai, J., D. Breibart, and C. D. Fisher, Mai, J., D. Breibart, and C. D. FisherMai, J., D. Breibart, and C. D. Fishers. 1990. Proteinaceous material. Unilever, assignee. Eur. Pat. 0,400,714.

Maurice, T. 2001. You used to do what with Whey ? : From disposal problem to value-added opportunity. Ingredients Mag. August, 2001.

Micke, P., K. M. Beeh, and R. Buhl. 2002. Effects of long-term supplementation with whey proteins on plasma glutathione levels of HIV-infected patients. Eur J Nutr. 41(1):12-18.

Morr, C. V. and E. Y. W. Ha. 1993. Whey protein concentrates and isolates : processing and functional properties. Critical reviews in food science and nutrition. 33(6):431-476.

Nutritiondata. 2006. http://www.nutritiondata.com.

O'Connell, J. 2002. RT-PCR protocols. Vol. 193. Humana Press, Totowa, NJ.

Ouellette AJ. 2004. Links Defensin-mediated innate immunity in the small intestine. Best Pract Res Clin Gastroenterol. 18(2):405-19.

Ouwehand, A. C., S. Salminen, A. C. Ouwehand, E. Isolauri, and S. Salminen. 2002. Probiotics: an overview of beneficial effects. Antonie van Leeuwenhoek. 82(1-4):279-289.

Parodi, P. W. 1998. A role for milk proteins in cancer prevention. Aust. J. Dairy Technol. 53:37-47.

Rogers, M.L., Goddard, C., Regester, G.O., Ballard, F.J., Belford, D.A. 1996. Transforming growth factor beta in bovine milk: concentration, stability and molecular mass forms. J Endocrinol. 151(1):77-86.

Queguinier, C., E. Dumay, C. Salou-Cavalier, and J. C. Cheftel. 1992. Microcoagulation of a whey protein isolate by extrusion cooking at acid pH. J. Food Sci. 57(3):610-616.

Sartor, R. B. 2005. Probiotic therapy of intestinal inflammation and infections. Curr Opin Gastroenterol. 21(1):44-50.

Sharma, S. Bose, M. 2001. Links Role of cytokines in immune response to pulmonary tuberculosis. Asian Pac J Allergy Immunol. 19(3):213-9.

Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem. 68(5):850-858.

Simard, E., D. Pilote, C. Dupont, N. Lajoie and M. Paquet. 2006. United States Patent Application A1 20060057131. Malleable Protein Matrices and use thereof.

Siso, M. I. G. 1996. The biotechnological utilization of cheese whey: A review. Bioresource Technology. 57(1):1-11.

Smithers, G. W., F. J. Ballard, A. D. Copeland, K. J. De Silva, D. A. Dionysius, G. L. Francis, C. Goddard, P. A. Grieve, G. H. McIntosh, I. R. Mitchell, R. J. Pearce, and G. O. Regester. 1996. New Opportunities from the Isolation and Utilization of Whey Proteins. J. Dairy Sci. 79(8):1454-1459.

Stiles, M. E. and W. H. Holzapfel. 1997. Lactic acid bacteria of foods and their current taxonomy. International Journal of Food Microbiology. 36(1):1-29.

Sullivan, A. and C. E. Nord. 2005. Probiotics and gastrointestinal diseases. J Intern Med. 257(1):78-92.

Tamboli, C. P., C. Caucheteux, A. Cortot, J. F. Colombel, and P. Desreumaux. 2003. Probiotics in inflammatory bowel disease: a critical review. Best Pract Res Clin Gastroenterol. 17(5):805-820.

Timmer, J. M. K. and H. C. van der Horst. 1998. Whey processing and separation technology : state-of-the-art and new developments. Whey Proceedinds of the 2nd International Whey Conference, Chicago:40-65.

Turgeon, S. L., C. Sanchez, S. F. Gauthier, and P. Paquin. 1996. Stability and rheological properties of salad dressing containing peptidic fractions of whey proteins. International Dairy Journal. 6(6):645-658.

Vancanneyt, M., J. Mengaud, I. Cleenwerck, K. Vanhonacker, B. Hoste, P. Dawyndt, M. C. Degivry, D. Ringuet, D. Janssens, and J. Swings. 2004. Reclassification of *Lactobacillus kefirgranum* Takizawa et al. 1994 as Lactobacillus kefiranofaciens subsp. kefirgranum subsp. nov. and emended description of L. kefiranofaciens Fujisawa et al. 1988. Int J Syst Evol Microbiol. 54(2):551-556.

Wal, J. M. 2002. Cow's milk proteins/allergens. Ann Allergy Asthma Immunol. 89(6 Suppl 1):3-10.

Welman, A. D. and I. S. Maddox. 2003. Exopolysaccharides from lactic acid bacteria: perspectives and challenges. Trends Biotechnol. 21(6):269-274.

Woolf, K. and M.M. Manore. 2006. B-vitamins and exercise: does exercise alter requirements? Int J Sport Nutr Exerc Metab. 16(5):453-84.

4.2 Brevet en phase internationale

Simard, E., Pilote, D., Dupont, Lajoie, N., Paquet, M., C., Lemieux, P., Goyette, P. 2001. International 2002. Malleable protein matrice and uses theroef. PCT/CA2002/01988. 92 pages.

Ce brevet fut déposé le 20 décembre 2001 en dépôt provisoire au États-Unis. Le dépôt international fut effectué le 20 décembre 2002 pour le Mexique, les États-Unis, l'Europe, le Canada et le Japon. Une brève description de l'invention est présentée et le brevet complet est joint par la suite.

J'ai réalisé une vaste revue des brevets existants, la rédaction complète du brevet et la révision du brevet pour son dépôt international. J'ai aussi effectué la revue de littérature sur les technologies existantes et toutes les différentes facettes du brevet.

4.2.1 Description sommaire de l'invention

Le procédé de production des MPM utilisé à l'échelle industrielle (technologie Valactis™), est basé sur la fermentation du lactosérum avec une souche pure de *Lactobacillus kefiranofaciens* isolée d'un consortium obtenu à partir de grains de kéfir (souche R2C2). En cours de croissance la bactérie produit des protéases indigènes et des exopolysaccharides (EPS). Les protéases s'attaquent aux protéines du lactosérum et produisent des peptides variant en longueur et en hydrophobicité. Suite à la fermentation, un ajout de $CaCl_2$ est effectué (1% p/v) et le pH de la solution est ajusté à 7,5 pour favoriser l'agglomération des protéines. Le lactosérum contenant les bactéries, les peptides, les sous-produits de la fermentation et les agglomérats est centrifugé pour récupérer les MPM (5% du volume); ce qui permet, entre autre, d'augmenter la concentration des bactéries probiotiques d'un facteur 20. Les agglomérats ainsi regroupés forment un réseau protéique fortement hydraté (80% d'eau) similaire à un yogourt brassé en apparence et en texture.

Cette hydrolyse partielle des protéines améliore l'odeur et le goût âcre naturel des protéines du lactosérum tout en générant des fonctionnalités technologiques et biologiques importantes. Il s'agit du seul produit laitier fermenté à base de protéines de lactosérum. En plus des avantages bénéfiques pour la santé de ces protéines et du calcium, il contient une forte concentration de bactéries lactiques probiotiques. Ces différentes fonctionnalités permettent des applications alimentaires de nutrition, des applications comme ingrédient bénéfique pour la santé dans le domaine des nutraceutiques et des aliments fonctionnels, des applications cosmétiques/cosméceutiques et des applications pharmaceutiques.

Le brevet en phase nationale (Canada, États-Unis, Europe, Mexique, Japon) couvre l'état des connaissances et des brevets apparentés, les différentes méthodes de production possibles, les souches bactériennes qui furent déposées ainsi que la composition et les utilisations envisageables des MPM pour les différents domaines d'application. Il n'existe aucune technologie ni produit similaires actuellement.

4.2.2 Comparaison aux technologies et produits existants

Les technologies actuelles s'orientent vers la purification/concentration de composés à forte valeur ajoutée pour augmenter le nombre de produits issus du lactosérum. Par filtration et échange ionique, les constituants sont concentrés dans des fractions spécifiques qui seront utilisées pour la production de lait maternisé, de suppléments nutritifs ou d'aliments fonctionnels. La production de fractions concentrées de lactoferrine en est un bon exemple. Les technologies utilisées sont dispendieuses d'implantation et d'utilisation, ce qui fait que la grande majorité du volume de lactosérum est simplement séché ou concentré et ensuite séché (Nielsen et Affertsholt, 2003). Les produits actuellement obtenus sont donc soient des concentrés de protéines de lactosérum et des poudres de lactosérum qui présentent des fonctionnalités technologiques restreintes (faible coût de production et faible valeur marchande) ou des produits spécialisés (isolats de protéines ou hydrolysats) présentant des propriétés technologiques et biologiques supérieurs (coût de production plus élevé avec une plus forte valeur marchande).

La technologie Valactis™ permet de concentrer à faible coût (procédé de fermentation) des principes actifs (hydrolysats, bactéries, calcium, EPS) à l'intérieur d'un même produit tout en alliant des fonctionnalités technologiques uniques (propriétés d'hydratation, émulsifiantes, moussantes, épaississantes, etc.). Jusqu'à maintenant, il s'agit du seul procédé de valorisation du lactosérum par fermentation pour la production d'ingrédients pour consommation humaine. Cette nouvelle voie technologique innovatrice est basée sur un équilibre fin entre les avantages apportés par le processus de fermentation et l'atteinte d'un rendement de récupération des protéines économiquement rentable. Par exemple, une hydrolyse enzymatique insuffisante produira un produit âcre aux fonctionnalités technologiques médiocres (ex : faible capacité d'incorporation des huiles). De façon inverse, une hydrolyse trop importante diminue les rendements en récupération de protéines par la production de peptides ne participant pas à l'agglomération. La MPM n'est donc pas un produit unique, mais une nouvelle gamme d'ingrédients à forte valeur ajoutée qui se différencie par un coût de revient peu élevé pour un grand nombre de fonctionnalités technologiques et biologiques. La figure 14 résume le procédé industriel actuellement en application.

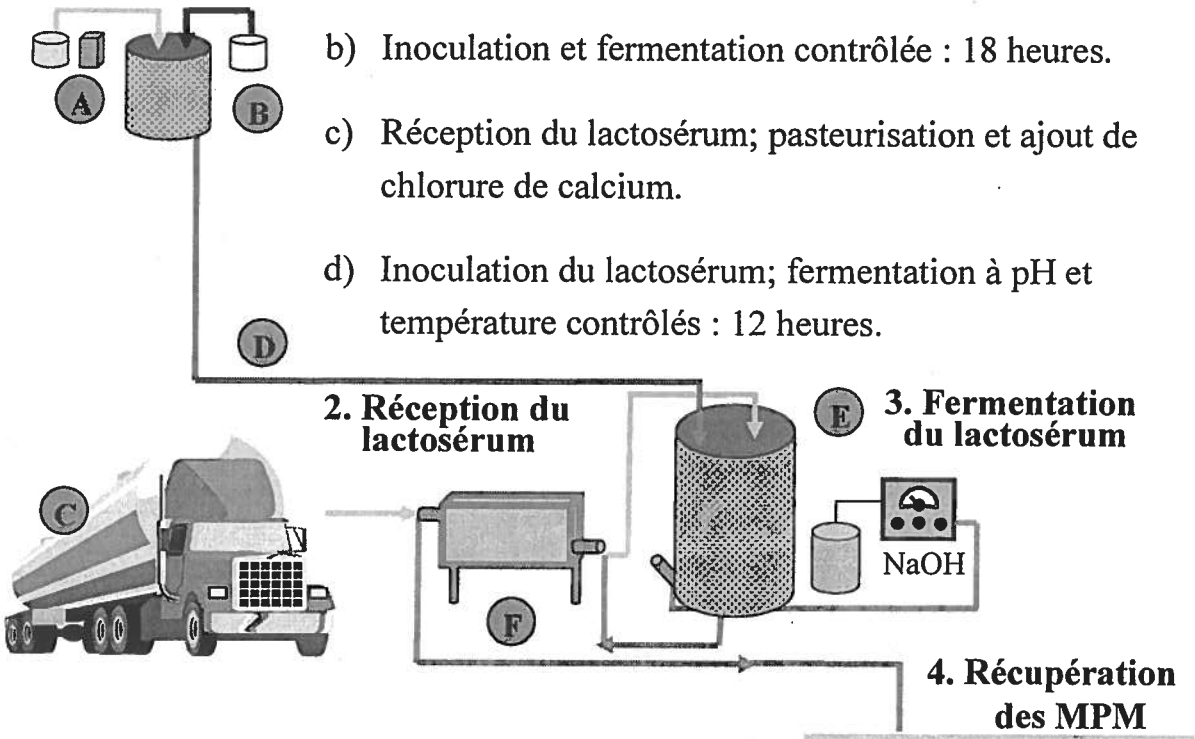
1. Pré-culture

a) Préparation et pasteurisation du milieu de pré-culture.

b) Inoculation et fermentation contrôlée : 18 heures.

c) Réception du lactosérum; pasteurisation et ajout de chlorure de calcium.

d) Inoculation du lactosérum; fermentation à pH et température contrôlés : 12 heures.

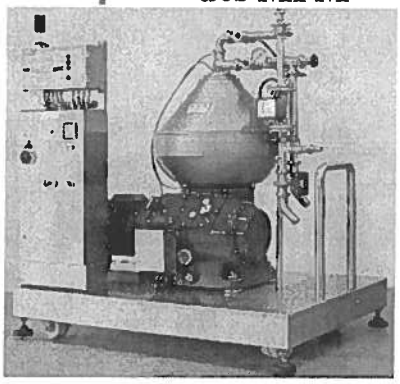


e) Ajustements physico-chimiques : pH de 7,5.

f) Pasteurisation et récupération des MPM à l'aide d'un clarificateur industriel pour ajustée la densité selon l'application visée.

g) La solution résiduelle est utilisée pour la production d'une biomasse pour l'alimentation animale.

h) Les MPM sont entreposés à 4^0C durant 7 jours pour compléter les analyses de microbiologie avant l'expédition aux clients.



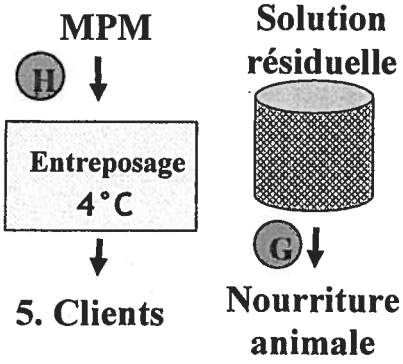


Figure 14 : Procédés utilisé pour la production industrielle des MPM.

4.2.3 Brevet de la technologie Valactis™.

MALLEABLE PROTEIN MATRICE AND USES THEREOF

ABSTRACT

The present invention relates to a malleable protein matrice (MPM), which is the reaction product of the agglomeration of proteins after a fermentation process and is exhibiting biological activities and is suitable for the incorporation (or encapsulation) of various hydrophilic or lipophylic substances. The present invention also relates to the process for the preparation of the malleable protein matrice and its usages.

MALLEABLE PROTEIN MATRICE AND USES THEREOF

BACKGROUND OF THE INVENTION

(a) Field of the Invention

This invention relates to a biodegradable and natural malleable protein matrice, method of preparation thereof, and compositions thereof, such as food, cosmetic, nutraceutical, probiotic, functional food and pharmaceutical compositions.

(b) Description of Prior Art

The high demand for low fat product lead the food industry to develop substitute food. The high demand for such product is based on studies recommending a decreased in daily fat consumption. It is important for the substitute food to have interesting sensorial characteristics as the original food (taste, smell, texture, etc.). Another field being in intensive increase is the nutraceutical and functional foods. The functional food is food with beneficial effect on health. The world consumption of these new foods is of about 70MM$ on an annual basis. The popularity of these products is so high that worldwide sales are expected to be of 500MM$ in 2010.

In the cosmetic and pharmaceutical industry, there is a long felt need for raw material for formulations, protection and controlled liberation of active ingredients. Several products are already existing, but most of them are very expensive. The industry is always in need for new technologies and products that will produce better results at a lower cost.

Ultrafiltration, reverse osmosis and drying processes are among the methods currently used for the valorization of whey proteins from the so-called serum lactis, the by-product of cheese making. These methods are efficient but extremely expensive and do not generate a readily useable product in a variety of industrial sectors. The fact that the cost of the installations for the above-mentioned methods is high is a problem for the cheese industry in general. Only large cheese manufacturers with strong financial positions and generating large volumes of serum lactis can reach profitability with the above-mentioned methods despite the high costs. Since serum lactis cannot be discarded freely in the environment it constitutes a pollutant *per se*, the small

manufacturers have therefore to spend money to discard the serum lactis which is mainly used for animal feed.

Simpler and less costly processes were developed to retrieved whey proteins but with also with concomitant drawbacks. Methods using temperature, pH, salt, enzymes, fermentation and flocculent are among the main parameters used to help the retrieval of whey proteins but generally lead to isolates exhibiting poor commercial quality and value. Patent CA 2,247,824 by Lewandoski and co-inventors describes a process for the production of microbial biomass from the effluent of dairy products. The resulting biomass from that process is used for animal feeding only. However, this product is not having functional properties such as emulsifiant properties that are needed for applications in human food.

Many processes and methods are offered to replace fat in food products. Agglomerates of whey proteins are used to replace fat like as described in U.S. Patent 5,358,730. The process involves a thermal treatment of whey proteins at a pH above their isoelectric point with the addition of salt. The process leads to the formation of curds (solid gels that can be shopped off in little pieces) that can be used in fat replacement. Whey proteins are extensively used in the food industry for their functional properties. However, this product is a solide and non-malleable product that is difficult to use in most of the food, cosmetic, pharmaceutical and nutraceuticals applications.

Proteins are also excellent film formers, conditioning agents, and moisturizers for hair and skin. However, natural proteins generally have limited use in cosmetics and toiletries because they are somewhat unstable and tend to precipitate or denature when exposed to high temperatures or salt solutions. In addition they are often hydrolyzed by chemical reagents or acids and bases. Even if these difficulties are overcome, the formulation of cosmetic products containing proteins is further fraught with difficulty since each protein has an isoelectric point i.e. a pH at which the protein is neutral. If it is desired to form compositions having a pH which is below the isoelectric point of the protein, the protein may possibly form an insoluble precipitate.

Furthermore, a large number of food products like mayonnaise, dressings, margarine, spreads or low-fat or zero-fat substitutes, can be

stabilized by polysaccharides as emulsion stabilizers or thickening agents. Also in the medical, pharmaceutical and cosmetic fields, polysaccharides they are used as emulsion stabilizers. Well known polysaccharides are obtained from a variety of plant seeds, e.g. guar gum from Cyamopsis tetragonaloba (guar) or locust bean gum (LBG) from locust bean. Other well-known sources are seaweed, giving carrageenan, alginates or agar.

The use of polysaccharides and proteins in cosmetic compositions is well known in the art. Polysaccharides are known to be good humectants, film formers, and function as skin moisturizers. Certain polysaccharides also have gelling ability and are useful in formation of higher viscosity liquid or solid, compositions. However, polysaccharides may tend to provide a heavy, sticky feel on the skin and, when used in quantities sufficient to cause gelling, may provide products which are not aesthetically pleasing.

Food science

The process described in the U.S. Patent 4,699,793 is used to produce seasoning. Because of the heat treatment performed before the fermentation, the resulting product has an undesirable taste and a poor homogeneity, which are the most important parameters in food science.

It is known that the presence of certain bacteria is associated with numerous beneficial effects on health (Gomes *et al.* (1999) *T. Food Sc. & Tech.* **10**:139-157). The microorganisms are present in many foods and are frequently used as probiotics to improve some biological functions in the host. Clinical trials have demonstrated that selected probiotic strains can influence the composition of the intestinal microflora and modulate the host immune system. Pre-, pro- and synbiotics offer both protection against and cure a variety of endemic and acute diseases.

More particularly, the lactic acid bacteria (LAB) are known for their several beneficial effects on health. Perdigon et al. (Curr Issues Intest Microbiol., 2001, Mar 2(1):27-42), have proceed with an important review of the lactic bacteria on health, particularly on immune system. The activation of the systemic and secretory immune response by LAB requires many complex interactions among the different constituents of the intestinal ecosystem (microflora, epithelial cells and immune cells). Through different mechanisms

they send signals to activate immune cells. Thus the knowledge of the normal intestinal microflora, the contribution of LAB and their role in the numerous functions in the digestive tract as well as the functioning of the mucosal immune system form the basis for the study and selection of a probiotic strain with immunostimulatory properties. In the selection of LAB for their immunostimulatory capacity it helps to know not only the effect which they have on the mucosal immune system, but the specific use to which these oral vaccine vectors are being put.

Pharmaceutical

Delivery of therapeutic agents to a mammalian host can frequently be as important as the activity of the drug in providing effective treatment. For the most part, drugs are delivered orally, frequently initially at a dosage below the therapeutic dosage and by repetitive administration of the drug, the dosage is raised to a therapeutic level or a level exceeding the therapeutic level. In many cases, the fact of having a dosage above therapeutic level provides for adverse effects, since most drugs are not only effective for the intended purpose, but frequently have adverse side effects. Various proposals have been made to avoid these problems, such as slow-release capsules, depots, pumps, and the like. These various approaches have numerous short comings for general applications where one wishes to maintain the presence of a therapeutic agent at a therapeutic dosage for an extended period. Invasive procedures are frequently undesirable, requiring surgery for introduction of the delivery device, followed by subsequent removal. Where the delivery device is placed on the skin, the agent must be capable of transport across the skin at the desired rate. Slow release particles have a limited time span and when introduced into the blood stream will be rapidly phagocytosed.

Oral administration in the form of a conventional tablet, pill or capsule constitutes the generally preferred route for administration of pharmaceuticals since this route is generally convenient and acceptable to patients. Unfortunately such compositions may be associated with certain disadvantages, particularly in the treatment of pediatric or geriatric patients,

who may dislike or have difficulty in swallowing such compositions, or where administration of a conventional tablet, pill or capsule is not duable.

The field of biodegradable polymers has developed rapidly since the synthesis and biodegradability of polylactic acid was first reported by Kulkami et al., 1966 "Polylactic acid for surgical implants" Arch. Surg., 93:839, Several other polymers are known to biodegrade, including polyanhydriques and polyorthoesters, which take advantage of labile backbone linkages, as reported by Heller et al., 1990, Biodegradable Polymers as Drug Delivery Systems; Chasin, M. and Langer, R., Eds., Dekker, New York, 121-161. Since it is desirable to have polymers that degrade into naturally occurring materials, polyaminoacids have been synthesized for *in vivo* use. This was the basis for using polyesters of alpha-hydroxy acids (viz., lactic acid, glycolic acid), which remain the most widely used biodegradable materials for applications ranging from closure devices (sutures and staples) to drug delivery systems (U.S. Pat. No. 4,741,337 to Smith et al.; Spilizewski et al., 1985 "The effect of hydrocortisone loaded poly(dl-lactide) films on the inflammatory response, " J. Control. Rcl. 2:197-203). Despite the development of novel biodegradable polymers, there is still a need for inexpensive and efficient delivery systems.

Exopolysaccharides act as biological response modifier as reported by Ruiz-Bravo A. (Clinical and Diagnostic Laboratory Immunology 2001, Jul; 8(4)-706-10). U.S. patents 5,888,552; 5,456,924; 5,451,412; 5,290,571; 5,230,902 describe compositions and methods to improve immune responses at large either for cancer or HIV-patients. U.S. patent 5,888,552 describes anti-cancer therapeutic compositions containing whey proteins while U.S. 5,456,924 describes a method of treatment of HIV-seropositive individual with dietary whey proteins.

It would be highly desirable to be provided with a biodegradable and non-toxic malleable protein matrice and a process to produce such that would turn or convert an industrial waste into a product with a commercial value and a biological activity.

SUMMARY OF THE INVENTION

One object of the present invention is to provide a biodegradable and non-toxic malleable protein matrice (MPM).

Preferably, the invention relates to matrice of whey proteins and exopolysaccharides. In addition, the matrice of the present invention is advantageously used to replace fat or for the incorporation or encapsulation of various hydrophilic or lipophylic substances and particularly substances used in the food, cosmetic, nutraceuticals and pharmaceutical sectors.

Another object of the present invention, there is to provide a novel method for the retrieval of whey proteins from the serum lactis which leads to a new kind of whey protein-based product. This new product is referred hereto as a malleable protein matrice (MPM), which is the reaction product of the agglomeration of whey proteins present in the serum lactis after a fermentation process. It has the texture of a malleable cream exhibiting biological activities and unique properties for the incorporation (or encapsulation) of various hydrophilic or lipophylic substances.

It is also an object of the invention to prepare various types of MPMs with different properties, characteristics and multiple applications and to prepare them directly from an industrial waste (whey or serum lactis).

In accordance with the present invention, there is provided a malleable protein matrix comprising:

- a precipitate of a protein of interest in solution;

- at least one microorganism capable of fermenting the solution containing the protein; and

-a matrix carrier allowing fermentation of the protein and the microorganism.

The matrix in accordance with a preferred embodiment of the present invention, wherein the fermentation is promoted by co-culture of at least two microorganisms simultaneously or successively.

The matrix in accordance with a preferred embodiment of the present invention, further comprising a fermentation by-products of the fermentation of the solution containing the protein by the microorganism.

The matrix in accordance with a preferred embodiment of the present invention, further comprising a peptide.

The matrix in accordance with a preferred embodiment of the present invention, wherein the peptide comprises at least two amino acid residues.

The matrix in accordance with a preferred embodiment of the present invention, wherein the peptide comprises more than one hundred amino acid residues.

The matrix in accordance with a preferred embodiment of the present invention, further comprising components obtained during agglomeration of the protein.

The matrix in accordance with a preferred embodiment of the present invention, further comprising components present in aqueous phase.

The matrix in accordance with a preferred embodiment of the present invention, wherein the protein is selected from the group consisting of natural protein, plant protein, animal derived protein and synthetic protein.

The matrix in accordance with a preferred embodiment of the present invention, wherein the protein is selected from the group consisting of albumen, amylase, amyloglucosidase, arginine/lysine polypeptide, casein, catalase, collagen, crystalline, cytochrome C, deoxyribonuclease, elastin, fibronectin, gelatin, gliadin, glucose oxidase, glycoproteins, hexyldecyl ester of hydrolyzed collagen, human placental protein, human placental enzymes, iodized corn protein, keratin, lactalbumine, lactoferrin, lactoglobulin, lactoperoxidase, lipase, milk protein, hyristoyl glycin/histidine/lysin polypeptide, nisin, oxido reductase, pancreatin, papaïne, pepsin, placental protein, protease, saccharomyces polypeptides, serum albumin, serum protein, silk, sodium stearoyl lactalbumin, soluble proteoglycan, soybean palmitate, soy, egg, peanut, cottonseed, sunflower, pea, whey, fish, seafood, subtilisin,

superoxide dismutase, sutilains, sweet almond protein, urease, wheat germ protein, wheat protein, whey protein, zein and hydrolyzed vegetable protein.

The matrix in accordance with a preferred embodiment of the present invention, wherein the protein is whey protein.

The matrix in accordance with a preferred embodiment of the present invention, wherein the fermentation by-products is polysaccharide.

The matrix in accordance with a preferred embodiment of the present invention, wherein the polysaccharide is selected from the group of exopolysaccharide and anionic polysaccharide.

The matrix in accordance with a preferred embodiment of the present invention, wherein the polysaccharide contains at least four saccharide moieties.

The matrix in accordance with a preferred embodiment of the present invention, wherein the saccharide moieties are selected from the group consisting of D and L forms of glucose, fructose, xylose, arabinose, fucose, galactose, pyruvic acid, succinic acid, acetic acid, 3,6-anhydrogalactose sulfate, galactose-4-sulfate, galactose-2-sulfate, galactose-2, 6-disulfate, mannose, glucuronic acid, mannuronic acid, guluronic acid, galactouronic acid, and rhamnose.

The matrix in accordance with a preferred embodiment of the present invention, wherein the polysaccharide have molecular weight ranging from about 500 to about 15,000,000 daltons.

The matrix in accordance with a preferred embodiment of the present invention, wherein the molecular weight is ranging from about 5,000 to 6,000,000 daltons.

The matrix in accordance with a preferred embodiment of the present invention, wherein the molecular weight is ranging from about 25,000 to 1,000,000 daltons.

The matrix in accordance with a preferred embodiment of the present invention, wherein the polysaccharide is selected from the group consisting of heteropolysaccharides, homopolysaccharides, galactans,

galactomannans, glucomannans, polyuronic acids, dextran sulfate, heparin, pectin, sodium alginate and mixtures thereof.

The matrix in accordance with a preferred embodiment of the present invention, wherein galactan is selected from the group consisting of agar, agarose, kappa-carageenan, iota carageenan and lambda carageenan.

The matrix in accordance with a preferred embodiment of the present invention, wherein galactomannan is selected from the group consisting of locust bean gum and guar.

The matrix in accordance with a preferred embodiment of the present invention, wherein glucan is selected from the group consisting of cellulose and derivatives thereof, starch and derivatives, dextrans, pullulan, beta 1,3-glucans, chitin, xanthan and tamatind.

The matrix in accordance with a preferred embodiment of the present invention, wherein glycomannan is konjac.

The matrix in accordance with a preferred embodiment of the present invention, wherein polyuronic acid is selected from the group consisting of algin, alginate and pectin.

The matrix in accordance with a preferred embodiment of the present invention wherein heteropolysaccharide is selected from the group consisting of gellan, welan, gum arabic, karaya gum, okra gum, aloe gum, gum tragacanth, gum ghatti quicessed gum , psyllium and starch arabinogalactan.

The matrix in accordance with a preferred embodiment of the present invention, wherein the microorganism is selected from the group consisting of *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium animalis*, *Bifidobacterium asteroides*, *Bifidobacterium bifidum*, *Bifidobacterium boum*, *Bifidobacterium breve*, *Bifidobacterium catenulatum*, *Bifidobacterium choerinum*, *Bifidobacterium coryneforme*, *Bifidobacterium cuniculi*, *Bifidobacterium dentium*, *Bifidobacterium gallicum*, *Bifidobacterium gallinarum*, *Bifidobacterium indicum*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium longum* DJO10A, *Bifidobacterium longum* NCC2705, *Bifidobacterium magnum*, *Bifidobacterium merycicum*, *Bifidobacterium*

minimum, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium pseudolongum*, *Bifidobacterium pseudolongum subsp. globosum*, *Bifidobacterium pullorum*, *Bifidobacterium ruminantium*, *Bifidobacterium saeculare*, *Bifidobacterium scardovii*, *Bifidobacterium subtile*, *Bifidobacterium suis*, *Bifidobacterium thermacidophilum*, *Bifidobacterium thermacidophilum subsp. suis*, *Bifidobacterium thermophilum*, *Bifidobacterium urinalis*, *Lactobacillus acetotolerans*, *Lactobacillus acidipiscis*, *Lactobacillus acidophilus*, *Lactobacillus agilis*, *Lactobacillus algidus*, *Lactobacillus alimentarius*, *Lactobacillus amylolyticus*, *Lactobacillus amylophilus*, *Lactobacillus amylovorus*, *Lactobacillus animalis*, *Lactobacillus arizonensis*, *Lactobacillus aviarius*, *Lactobacillus bifermentans*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus cellobiosus*, *Lactobacillus coleohominis*, *Lactobacillus collinoides*, *Lactobacillus coryniformis*, *Lactobacillus coryniformis subsp. coryniformis*, *Lactobacillus coryniformis subsp. torquens*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus cypricasei*, *Lactobacillus delbrueckii*, *Lactobacillus delbrueckii subsp. bulgaricus*, *Lactobacillus delbrueckii subsp. delbrueckii*, *Lactobacillus delbrueckii subsp. lactis*, *Lactobacillus durianis*, *Lactobacillus equi*, *Lactobacillus farciminis*, *Lactobacillus ferintoshensis*, *Lactobacillus fermentum*, *Lactobacillus fornicalis*, *Lactobacillus fructivorans*, *Lactobacillus frumenti*, *Lactobacillus fuchuensis*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus graminis*, *Lactobacillus hamsteri*, *Lactobacillus helveticus*, *Lactobacillus helveticus subsp. jugurti*, *Lactobacillus heterohiochii*, *Lactobacillus hilgardii*, *Lactobacillus homohiochii*, *Lactobacillus intestinalis*, *Lactobacillus japonicus*, *Lactobacillus jensenii*, *Lactobacillus johnsonii*, *Lactobacillus kefir*, *Lactobacillus kefiri*, *Lactobacillus kefiranofaciens*, *Lactobacillus kefirgranum*, *Lactobacillus kimchii*, *Lactobacillus kunkeei*, *Lactobacillus leichmannii*, *Lactobacillus letivazi*, *Lactobacillus lindneri*, *Lactobacillus malefermentans*, *Lactobacillus mali*, *Lactobacillus maltaromicus*, *Lactobacillus manihotivorans*, *Lactobacillus mindensis*, *Lactobacillus mucosae*, *Lactobacillus murinus*, *Lactobacillus nagelii*, *Lactobacillus oris*, *Lactobacillus panis*, *Lactobacillus pantheris*, *Lactobacillus parabuchneri*, *Lactobacillus paracasei*, *Lactobacillus paracasei subsp. paracasei*, *Lactobacillus paracasei subsp. tolerans*, *Lactobacillus parakefiri*, *Lactobacillus*

paralimentarius, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus perolens*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus psittaci*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus ruminis*, *Lactobacillus sakei*, *Lactobacillus sakei* L45, *Lactobacillus salivarius*, *Lactobacillus salivarius subsp. salicinius*, *Lactobacillus salivarius subsp. salivarius*, *Lactobacillus sanfranciscensis*, *Lactobacillus sharpeae*, *Lactobacillus sp.* NGRI 0001, *Lactobacillus suebicus*, *Lactobacillus thermotolerans*, *Lactobacillus vaccinostercus*, *Lactobacillus vaginalis*, *Lactobacillus vermiforme*, *Lactobacillus versmoldensis*, *Lactobacillus zeae*, *Lactococcus garvieae*, *Lactococcus lactis*, *Lactococcus lactis subsp. cremoris*, *Lactococcus lactis subsp. hordniae*, *Lactococcus lactis subsp. lactis*, *Lactococcus lactis subsp. lactis bv. diacetylactis*, *Lactococcus piscium*, *Lactococcus plantarum*, *Lactococcus raffinolactis*, *Leuconostoc argentinum*, *Leuconostoc carnosum*, *Leuconostoc citreum*, *Leuconostoc fallax*, *Leuconostoc ficulneum*, *Leuconostoc fructosum*, *Leuconostoc gasicomitatum*, *Leuconostoc gelidum*, *Leuconostoc inhae*, *Leuconostoc kimchii*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Leuconostoc mesenteroides subsp. cremoris*, *Leuconostoc mesenteroides subsp. dextranicum*, *Leuconostoc mesenteroides subsp. mesenteroides*, *Leuconostoc mesenteroides subsp. mesenteroides* ATCC 8293, *Leuconostoc pseudomesenteroides*, *Propionibacterium acidipropionici*, *Propionibacterium acnes*, *Propionibacterium australiense*, *Propionibacterium avidum*, *Propionibacterium cyclohexanicum*, *Propionibacterium freudenreichii*, *Propionibacterium freudenreichii subsp. freudenreichii*, *Propionibacterium freudenreichii subsp. shermanii*, *Propionibacterium granulosum*, *Propionibacterium jensenii*, *Propionibacterium lymphophilum*, *Propionibacterium microaerophilum*, *Propionibacterium propionicum*, *Propionibacterium thoenii*, *Saccharomyces delbrueckii*, *Saccharomyces cerevisiae*, *Saccharomyces unisporus*, *Saccharomyces globosus*, *Saccharomyces carlsbergensis*, *Kluyveromyces fragilis*, *Kluyveromyces bulgaricus*, *Kluyveromyces lactis*, *Torula holmii*, *Candida tenuis*, R2C2, INIX, ES1 and K2.

The matrix in accordance with a preferred embodiment of the present invention, wherein the microorganism is selected from the group

consisting of *L. rhamnosus, L. acidphilus, L. casei, L. lactis, L. plantarum, L. Kefirgranum,* R2C2, INIX, ES1 and K2.

The matrix in accordance with a preferred embodiment of the present invention, wherein the microorganism is R2C2.

The matrix in accordance with a preferred embodiment of the present invention, wherein the microorganism is INIX.

The matrix in accordance with a preferred embodiment of the present invention, wherein the microorganism is *L. Kefirgranum.*

The matrix in accordance with a preferred embodiment of the present invention, wherein the microorganism is *bacillaceae, bifidobacteriaceae, enterobacteriaceae, enterococcaceae, lactobacillaceae; propionibacteriaceae* and yeast.

The matrix in accordance with a preferred embodiment of the present invention, wherein the bacillaceae is *Bacillus subtilis.*

The matrix in accordance with a preferred embodiment of the present invention, wherein the bifidobacteriaceae is one selected from the group consisting of *Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis* and *Bifidobacterium lactis.*

The matrix in accordance with a preferred embodiment of the present invention, wherein the *enterobacteriaceae* is *Escherichia coli* Nissle 1917.

The matrix in accordance with a preferred embodiment of the present invention, wherein the *enterococcaceae* is *Enterococcus faecium.*

The matrix in accordance with a preferred embodiment of the present invention, wherein the *lactobacillaceae* is one selected from the group consisting of *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus crispatus, Lactobacillus fermentum, Lactobacillus johnsonii, Lactobacillus paracasei, Lactobacillus plantarum, Lactobaillus reuteri, Lactobacillus rhamnosus* and *Lactobacillus salivarius.*

The matrix in accordance with a preferred embodiment of the present invention, wherein the yeast is *saccharomyces cerevisiae boulardii.*

In accordance with the present invention, there is provided a microorganism R2C2 isolated from a consortium obtained from Kefir grain.

In accordance with the present invention, there is provided a microorganism K2 isolated from a consortium obtained from Kefir grain.

In accordance with the present invention, there is provided a microorganism ES1 isolated from a consortium obtained from Kefir grain.

In accordance with the present invention, there is provided a microorganism INIX isolated from ATCC 43761 strain.

In accordance with the present invention, there is provided a process for manufacturing the matrix of the present invention, the process comprising the steps of:

a) fermenting a protein solution with a microorganism in a medium;

b) precipitating protein from the proteins solution of step a); and

c) isolating precipitated proteins from supernatant.

The process in accordance with a preferred embodiment of the present invention, wherein the fermenting step is promoted by co-culturing at least two microorganisms simultaneously or successively.

The process in accordance with a preferred embodiment of the present invention, wherein the process further comprises a step between steps a) and b) for addition of a polysaccharide.

The process in accordance with a preferred embodiment of the present invention, wherein the process further comprises a step between steps b) and c) for addition of a polysaccharide.

The process in accordance with a preferred embodiment of the present invention, further comprising a step of pasteurization of the proteins solution before step a). This process can further include a sterilization step after the pasteurization step.

The process in accordance with a preferred embodiment of the present invention, wherein precipitation of fermented proteins is effected by at least one method selected from the group consisting of salt addition, pH modulation, thermal treatment, proteolytic enzymes addition and floculent addition.

The process in accordance with a preferred embodiment of the present invention, wherein the flocculent is a bacterial flocculent.

The process in accordance with a preferred embodiment of the present invention, wherein the bacterial flocculent is *L. Kefirgranum*.

The process in accordance with a preferred embodiment of the present invention, wherein separation of precipitated proteins from supernatant is effected by a method selected from the group of centrifugation and filtration.

In accordance with the present invention, there is provided a composition comprising the matrix of the present invention in association with a pharmaceutically acceptable carrier.

In accordance with the present invention, there is provided the use of the matrix of the present invention, wherein the use is for the manufacture of a product selected from the group of food product, medical product, pharmaceutical product, cosmetic product, probiotic, functional food and nutraceutical.

In accordance with the present invention, there is provided the use of the matrix of the present invention, wherein the use is for the manufacture of a food product.

The use in accordance with a preferred embodiment of the present invention, wherein the matrix is used as an emulsion stabilizer or thickening agent.

The use in accordance with a preferred embodiment of the present invention, wherein the food product is selected from the group consisting of mayonnaise, dressing, margarine, spread, butter, whipped cream, cream, yogurt, cheese and low-fat substitute.

The use in accordance with a preferred embodiment of the present invention, wherein the matrix is used as a delivery vehicle.

In accordance with the present invention, there is provided the use of the matrix of the present invention for the preparation of a probiotic.

In accordance with the present invention, there is provided the use of the matrix of the present invention, wherein the use is for cosmetic product.

The use in accordance with a preferred embodiment of the present invention, wherein the cosmetic product is selected from the group consisting of skin lotion, cream, sunscreen, blush, mascara, eyeshadow, shampoo and conditionner.

In accordance with the present invention, there is provided the use of the matrix of the present invention for increasing immune response in a subject.

In accordance with the present invention, there is provided a method of increasing immune response in a subject, comprising administering an effective amount of the matrix of the present invention to the subject.

In accordance with the present invention, there is provided the use of the matrix of the present invention for reducing triglyceride level in a subject.

In accordance with the present invention, there is provided a method for reducing triglyceride level in a subject, comprising administering an effective amount of the matrix of the present invention to the subject.

In accordance with the present invention, there is provided the use of the matrix of the present invention for reducing TNF-α level in a subject.

In accordance with the present invention, there is provided a method for reducing TNF-α level in a subject, comprising administering an effective amount of the matrix of the present invention to the subject.

In accordance with the present invention, there is provided the use of the matrix of the present invention for increasing gluthatione level in a subject.

In accordance with the present invention, there is provided a method for increasing gluthatione level in a subject, comprising administering an effective amount of the matrix of the present invention to the subject.

The MPM of the present invention also fulfill a long-felt need in different sectors, namely in food (fat replacement, thickening agent), cosmetic (delivery systems, physiological effects), nutraceuticals, functional food, probiotic and pharmaceutical (oral delivery systems, biological response modifier drug delivery systems).

All the references herein are incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates a general schema of the preparation process for MPM;

Fig. 2 illustrates a detailed schema of the matrix formation;

Fig. 3 illustrates the formulation of matrix formulation;

Fig. 4 illustrates one example of an industrial implementation of the present invention; and

Figs. 5A-E illustrate homology between gene ARN165 of strains INIX (SEQ ID NO:1), K2 (SEQ ID NO:2), R2C2 (SEQ ID NO:3), ES1 (SEQ ID NO: 4), ATCC 43761 (SEQ ID NO:5) and ATCC 51647 (SEQ ID NO:6).

DETAILED DESCRIPTION OF THE INVENTION

The invention consists in a malleable protein matrice (MPM) produced from fermented residual whey obtained from the cheese industry. The MPM is obtained by triggering agglomeration of whey proteins, which are then retrieved by various means. The process allows the production of insoluble and malleable protein matrices composed of 1) proteins and/or peptides, 2) one or several bacterial strains, 3) fermented by-products, 4) other components obtained during the agglomeration and retrieval process of the agglomerates and 5) components present in the aqueous phase. Following the agglomeration, the resulting matrix is retrieved by filtration, centrifugation or with any other methods allowing such retrieval. The protein agglomeration can be triggered by, but not limited to, a modulation of pH,

temperature, the addition of salts, the addition of proteolytic enzymes, the addition of flocculent or the combination of all or some of those methods. The invention also describes various parameters that can affect the resulting characteristics of the matrix like the bacterial component of the MPM.

This matrix and its production process present major advantages over the matrice and production processes known in the art. The production process as described below allows the obtention of a uniform formulation directly from lactoserum or other primary protein source when all the components are present prior agglomeration. The components are found either in the agglomerate and the aqueous phase of the post-agglomeration fermentation product. A formulation containing MPM is produced in mixing the MPM and other products to have introduced in the formulation in water, oil or other liquid suitable for such formulation. Another formulation is produced in lyophilizing MPM and hydrating them with a solution containing other products to be introduced in the formulation.

The polymers used can be from different origins, such as from a microorganism, from plant and they also can be synthetic. The polymer is being mixed to the proteins before, during or after the process of agglomeration. The amount of polymers trapped in the matrix may vary to form the resulting matrix. The source of proteins used in the agglomeration process can be from either pure whey obtained from a cheese factory or from a concentrate of whey proteins (WPC, CPI) resuspended in an aqueous solution. The agglomeration process is preceded by a fermentation process or of any other methods to improve the quality of the final product obtained: flavor, color, texture, conservation time, functional properties, nutritional properties, biological properties, pharmaceutical properties.

Fig. 1 illustrates the preferred embodiment of the process of the present invention consisting in a fermentation process of whey with a pure strain of lactobacillus isolated from a consortium obtained from Kefir grain (R2C2 strain accession number: 041202-3 National Microbiology Laboratory, Health Canada, 1015 Arlington Street, Winnipeg, Manitoba, Canada, R3E 3R2). The first step is a pre-culture where freeze-dried, or frozen ferment culture is used to inoculate whey or seed medium suitable for the species

used like pure strain of lactobacillus isolated from a consortium obtained from Kefir grain (R2C2 strain). The fermentation is continued to get a concentration of bacteria of 10^8 to 10^9 bacteria per ml of pre-culture. The pre-culture is then inoculated in whey or protein solution in an amount of from 1 to 12.5%. Whey may be used as is, or supplemented with different culture additives suitable for the species used. During the proliferation process, the lactobacillus produces an exopolysaccharide (EPS), which is secreted in the medium, along with some endogenous proteases. The endogenous proteases present in the medium hydrolyze the whey proteins to generate peptides with various length and hydrophobicity. The whey medium is maintained under appropriate culture conditions to promote a rapid multiplication of the microorganisms used. If needed, a constant temperature, pH, agitation, aeration and other culture conditions are supplied. For triggering agglomeration of whey proteins, several means can be used to facilitate or inducing the formation of agglomerates like pH modification, salt addition and heat treatment. Agitation is needed to provide good homogeneity of the resulting matrice which contain microorganisms, peptides, proteins, fermented by-products. Continuous centrifugation is preferred to promote a better homogeneity of the matrice but various retrieval means can also be used.

MPM can be used under a humid form or dried and can be lyophilized or dried by other means and once dried the MPMs are also compressible with a Carver press to form solid tablets. Lyophilized MPMs are compressible without the need to add any excipients to form tablets that could have multiple applications like incorporation of probiotics or drugs. The tablets hydrate slowly because of their high content of proteins and are protecting the incorporated agents while passing in the stomach environment. MPM can integrate water, oil or other solvent to improve its general properties. The compositions and/or formulations obtained are useful in food science, cosmetic, nutraceuticals, functional food, probiotic and pharmaceuticals.

Fig. 2 illustrates the matrix formation and Fig. 3 illustrates formulations produced with MPM.

The process described to produce MPMs is preferably made of non-concentrated sources of whey proteins like serum lactis. The MPMs exhibit an

improved homogeneity and a product with improved functional and organoleptic properties as well as beneficial effects on health because the fermentation process of the present invention is performed in a non-concentrated solution. There is therefore no need to homogenize the resulting matrix with high shear conditions as for the processes known in the art.

Fig. 4 illustrates an example of an industrial implementation of the process of the present invention. As shown in Fig. 4, a preculture medium is prepared with whey and yeast extract, followed by the pasteurization of this preparation. The pasteurized solution is then inoculated with ferment and fermented under control to the obtention of a bacterial culture of 10^8-10^9 bacteria per ml of preculture. One person skilled in the art would understand that the preculture medium preparation does not need to be part of the production process.

Whey is then provided in fermentor to which is added the preculture medium for fermentation. After completion of the fermentation process, the precipitation of the fermented proteins is achieved by one or more of the methods previously described and the precipitated proteins are isolated from the supernatant and stored until delivery.

The MPMs described above have multiple applications that are listed below. MPMs is an inexpensive product with a variety of competitive advantages and applications. In the food industry/functional food/nutraceuticals, the MPMs can be used as a fat replacement agent, as a protein supplement, as a functional food product having a specific feature (stimulation of the immune system, decreasing levels of triglyceride), as a bio-vehicle for ingredients, flavors, supplements, food additives, vitamins. In the cosmetic and as a cosmeceutical, the MPMs can be used as fat and/or petroleum replacement agent, as a protein supplement in body lotion and cream, as a cosmeceutic product having specific features (increase *in situ* production of collagen), as a bio-vehicle for therapeutic agents, supplements, and vitamins. From the pharmaceuticals point of view, the MPMs can be used as a bio-vehicle for therapeutic agents, to increase oral formulation or generic drugs (excipient), to improve therapeutic indices of drugs (synergy), to reduce drug side effects and to increase bioavailability.

Proteins

Although the preferred source of protein of the invention is the serum lactis, the process can also be applied to diluted protein solution. A variety of proteins are suitable to make the MPM. The term "protein" when used in accordance with this invention means a peptide chain having at least two amino acid residues, preferably at least four, and more preferably more than one hundred amino acid residues. Most preferably the protein is a high molecular weight polypeptide which is preferably water soluble, and may be natural, plant (vegetable) proteins, or animal derived proteins, as well as synthetic proteins.

Examples of natural proteins include albumen, amylase, amyloglucosidase, arginine/lysine polypeptide, casein, catalase, collagen, crystalline, cytochrome C, deoxyribonuclease, elastin, fibronectin, gelatin, gliadin, glucose oxidase, glycoproteins, hexyldecyl ester of hydrolyzed collagen, human placental protein, human placental enzymes, iodized corn protein, keratin, lactoferrin, lactoglobulin, lactoperoxidase, lipase, milk protein, hyristoyl glycine/histidine/lysin polypeptide, nisin, oxido reductase, pancreatin, papaïne, pepsin, placental protein, protease, saccharomyces polypeptides, serum albumin, serum protein, silk, sodium stearoyl lactalbumin, soluble proteoglycan, soybean palmitate, soy, egg, peanut, cottonseed, sunflower, pea, whey, fish, seafood, subtilisin, superoxide dismutase, sutilains, sweet almond protein, urease, wheat germ protein, wheat protein, whey protein, zein, hydrolyzed vegetable protein, and the like. Preferred is whey which is a mixture of whey proteins obtained from cow's milk following the cheese making.

Synthetic proteins or polypeptides are also suitable. Synthetic proteins are produced by solid phase synthesis, or via recombinant biotechnology processes. MPM can become a solution to the difficulties encountered in the formulation of proteins. MPM can be formulated in creams for instance under either acidic or alkaline conditions without affecting the texture and appearance of the cream.

Polymers

Several polymers may be used in the production of MPMs. They are either synthetic or natural. However a variety of exopolysaccharides and polysaccharides are suitable for the preparation of the MPM used in the compositions of the invention, provided that the exopolysaccharides and polysaccharide contains a sufficient number of hydrophilic groups to cause the resulting MPM. In addition, the polysaccharide must be capable of reacting with the protein to form an MPM having a protein/polysaccharide ratio enough to cause aggregation. The term "polysaccharide" when used in accordance with the invention means a polysaccharide which contains at least four saccharide moieties. The term "saccharide moiety" means a polyhydroxy aldehyde or ketone, or acid hydrolysis product thereof, which, preferably, has the general formula $C_x(H_2O)_y$. Examples of saccharide moieties include the D and L forms of glucose, fructose, xylose, arabinose, fucose, galactose, pyruvic acid, succinic acid, acetic acid, galactose, 3,6-anhydrogalactose sulfate, galactose-4-sulfate, galactose-2-sulfate, galactose-2, 6-disulfate, mannose, glucuronic acid, mannuronic acid, guluronic acid, galactouronic acid, rhamnose, and so on. Preferably the polysaccharides used to make the MPM have molecular weights ranging from about 500 to 15,000,000 daltons, preferably 5,000 to 6,000,000, more preferably 25,000 to 1,000,000 daltons.

These polysaccharides are either added exogenously or produced by a microorganism. Examples of suitable anionic polysaccharides include galactans, galactomannans, glucomannans, polyuronic acids, and the like, which exhibit the requisite number of pendant hydrophilic groups. Suitable galactans are agar, agarose, kappa carageenan, iota carageenan, lambda carageenan, and the like. Examples of suitable galactomannans are locust bean gum and guar; examples of glucans are cellulose and derivatives thereof, starch and derivatives, dextrans, pullulan, beta 1,3-glucans, chitin, xanthan, tamarind and the like; examples of glucomannans are konjac; examples of polyuronic acids are algin, alginates, pectins; examples of heteropolysaccharides are gellan, welan, gum arabic, karaya gum, okra gum, aloe gum, gum tragacanth, gum ghatti quinceseed gum, psyllium, starch arabinogalactan and so on. Also suitable are dextran sulfate, heparin, pectin, sodium alginate, and mixtures thereof.

These polysaccharides may be further modified as taught in Aoki, T. T.; Araki & M. Kitamikado; 1990, Vibrio sp. AP-2. Eur. J. Biochem, 187, 461-465, provided it contains the requisite number of hydrophilic pendant groups. Also suitable for use in the compositions of the invention are chemically modified galactans, such as those taught in an article authored by K. B. Guiseley in Industrial Polysaccharides;Genetic Engineering, Structure/Property Relations and Applications, Edited by M. Yalpani, 1987, Elsevier Science Publishers. The Guiseley article teaches methods for the chemical modification of agar to obtain optimum gelling properties. In general, any modification of the galactans which does not affect the helical conformation (i.e. which is obtained via linkage of the O6 and O4 of galactose to the O2 of 3,6-anhydrogalactose) will preserve the gelling capability and is suitable for use in the compositions of the invention provided the requisite number of hydrophilic groups are present. The hydrophilic groups provide a polysaccharide which is water soluble. Many other polymers can be added before, during or after the fermentation process. They can be used to change 1) the functional properties of the MPMs, 2) the physical chemistry properties of the MPMs, 3) the aggregation of proteins, 4) the capacity to formulate or encapsulate various components from the various sectors like food, cosmetics, nutraceuticals and pharmaceuticals and 5) the biological activity of the MPMs. Examples of polymers like polyethylene glycol, polyehtyleneimine, polyesters, mono-, di-, or tri-block copolymers or any polymers helping the formation of colloid systems could be used to improve MPMs.

Microorganisms

Although that the preferred microorganism used in the invention is R2C2 (Accession Number 041202-3, National Microbiology Laboratory, Health Canada, 1015 Arlington Street, Winnipeg, Manitoba, Canada, R3E 3R2), the process is not limited to one or several specific species or strain and can integrate a variety of other microorganisms either alone or in combination like *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium animalis*, *Bifidobacterium asteroides*, *Bifidobacterium bifidum*, *Bifidobacterium boum*, *Bifidobacterium breve*, *Bifidobacterium catenulatum*, *Bifidobacterium choerinum*, *Bifidobacterium coryneforme*, *Bifidobacterium cuniculi*,

Bifidobacterium dentium, *Bifidobacterium gallicum*, *Bifidobacterium gallinarum*, *Bifidobacterium indicum*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium longum* DJO10A, *Bifidobacterium longum* NCC2705, *Bifidobacterium magnum*, *Bifidobacterium merycicum*, *Bifidobacterium minimum*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium pseudolongum*, *Bifidobacterium pseudolongum subsp. globosum*, *Bifidobacterium pullorum*, *Bifidobacterium ruminantium*, *Bifidobacterium saeculare*, *Bifidobacterium scardovii*, *Bifidobacterium subtile*, *Bifidobacterium suis*, *Bifidobacterium thermacidophilum*, *Bifidobacterium thermacidophilum subsp. suis*, *Bifidobacterium thermophilum*, *Bifidobacterium urinalis*, *Lactobacillus acetotolerans*, *Lactobacillus acidipiscis*, *Lactobacillus acidophilus*, *Lactobacillus agilis*, *Lactobacillus algidus*, *Lactobacillus alimentarius*, *Lactobacillus amylolyticus*, *Lactobacillus amylophilus*, *Lactobacillus amylovorus*, *Lactobacillus animalis*, *Lactobacillus arizonensis*, *Lactobacillus aviarius*, *Lactobacillus bifermentans*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus cellobiosus*, *Lactobacillus coleohominis*, *Lactobacillus collinoides*, *Lactobacillus coryniformis*, *Lactobacillus coryniformis subsp. coryniformis*, *Lactobacillus coryniformis subsp. torquens*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus cypricasei*, *Lactobacillus delbrueckii*, *Lactobacillus delbrueckii subsp. bulgaricus*, *Lactobacillus delbrueckii subsp. delbrueckii*, *Lactobacillus delbrueckii subsp. lactis*, *Lactobacillus durianis*, *Lactobacillus equi*, *Lactobacillus farciminis*, *Lactobacillus ferintoshensis*, *Lactobacillus fermentum*, *Lactobacillus fornicalis*, *Lactobacillus fructivorans*, *Lactobacillus frumenti*, *Lactobacillus fuchuensis*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus graminis*, *Lactobacillus hamsteri*, *Lactobacillus helveticus*, *Lactobacillus helveticus subsp. jugurti*, *Lactobacillus heterohiochii*, *Lactobacillus hilgardii*, *Lactobacillus homohiochii*, *Lactobacillus intestinalis*, *Lactobacillus japonicus*, *Lactobacillus jensenii*, *Lactobacillus johnsonii*, *Lactobacillus kefir*, *Lactobacillus kefiri*, *Lactobacillus kefiranofaciens*, *Lactobacillus kefirgranum*, *Lactobacillus kimchii*, *Lactobacillus kunkeei*, *Lactobacillus leichmannii*, *Lactobacillus letivazi*, *Lactobacillus lindneri*, *Lactobacillus malefermentans*, *Lactobacillus mali*, *Lactobacillus maltaromicus*, *Lactobacillus manihotivorans*, *Lactobacillus mindensis*, *Lactobacillus*

mucosae, *Lactobacillus murinus*, *Lactobacillus nagelii*, *Lactobacillus oris*, *Lactobacillus panis*, *Lactobacillus pantheris*, *Lactobacillus parabuchneri*, *Lactobacillus paracasei*, *Lactobacillus paracasei subsp. paracasei*, *Lactobacillus paracasei subsp. tolerans*, *Lactobacillus parakefiri*, *Lactobacillus paralimentarius*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus perolens*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus psittaci*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus ruminis*, *Lactobacillus sakei*, *Lactobacillus sakei* L45, *Lactobacillus salivarius*, *Lactobacillus salivarius subsp. salicinius*, *Lactobacillus salivarius subsp. salivarius*, *Lactobacillus sanfranciscensis*, *Lactobacillus sharpeae*, *Lactobacillus sp.* NGRI 0001, *Lactobacillus suebicus*, *Lactobacillus thermotolerans*, *Lactobacillus vaccinostercus*, *Lactobacillus vaginalis*, *Lactobacillus vermiforme*, *Lactobacillus versmoldensis*, *Lactobacillus zeae*, *Lactococcus garvieae*, *Lactococcus lactis*, *Lactococcus lactis subsp. cremoris*, *Lactococcus lactis subsp. hordniae*, *Lactococcus lactis subsp. lactis*, *Lactococcus lactis subsp. lactis bv. diacetylactis*, *Lactococcus piscium*, *Lactococcus plantarum*, *Lactococcus raffinolactis*, *Leuconostoc argentinum*, *Leuconostoc carnosum*, *Leuconostoc citreum*, *Leuconostoc fallax*, *Leuconostoc ficulneum*, *Leuconostoc fructosum*, *Leuconostoc gasicomitatum*, *Leuconostoc gelidum*, *Leuconostoc inhae*, *Leuconostoc kimchii*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Leuconostoc mesenteroides subsp. cremoris*, *Leuconostoc mesenteroides subsp. dextranicum*, *Leuconostoc mesenteroides subsp. mesenteroides*, *Leuconostoc mesenteroides subsp. mesenteroides* ATCC 8293, *Leuconostoc pseudomesenteroides*, *Propionibacterium acidipropionici*, *Propionibacterium acnes*, *Propionibacterium australiense*, *Propionibacterium avidum*, *Propionibacterium cyclohexanicum*, *Propionibacterium freudenreichii*, *Propionibacterium freudenreichii subsp. freudenreichii*, *Propionibacterium freudenreichii subsp. shermanii*, *Propionibacterium granulosum*, *Propionibacterium jensenii*, *Propionibacterium lymphophilum*, *Propionibacterium microaerophilum*, *Propionibacterium propionicum*, *Propionibacterium thoenii*, *Saccharomyces delbrueckii*, *Saccharomyces cerevisiae*, *Saccharomyces unisporus*, *Saccharomyces globosus*, *Saccharomyces carlsbergensis*, *Kluyveromyces fragilis*, *Kluyveromyces bulgaricus*, *Kluyveromyces lactis*, *Torula holmii*,

Candida tenuis, ES1 (Accession Number 041202-2, National Microbiology Laboratory, Health Canada, 1015 Arlington Street, Winnipeg, Manitoba, Canada, R3E 3R2), INIX (Accession Number 041202-4, National Microbiology Laboratory, Health Canada, 1015 Arlington Street, Winnipeg, Manitoba, Canada, R3E 3R2) and K2 (Accession Number 041202-1, National Microbiology Laboratory, Health Canada, 1015 Arlington Street, Winnipeg, Manitoba, Canada, R3E 3R2). The microorganisms are preferably homolactic but can be heterolactic.

The invention can use various genders and species and examples will be given to demonstrate that they modify functional properties of the MPMs like their hydration and emulsification capacities, their beneficial effects of health or their respective conservation times. MPMs generated with probiotic strain like *Lactobacillus plantarum* allows a better stimulation of the intestinal flora. MPMs generated with *Lactococcus lactis* allow the production of bacteriocin, NISIN, leading to an improved conservation time of the matrices. MPMs generated with *lactobacillus kefiranofaciens* or *L. ramnosus* 9595 allow a better positive overall stimulation of the immune system by their EPS, Murofushi et al. 1986. Immunopharmacology. Vol. 12. pp 29-35. MPM can prolong conservation of products in which it was added or incorporate. Yogurt containing MPM exhibited a better shelf life than the MPM-free yogurt. In addition, MPM can help maintaining survival of microorganisms for a prolonged period of time. Furthermore, MPM in yogurt serve as a stabilizing agent and replace either gelatine, pectine or corn starch.

In the process of the present invention, the culture of one microorganism can favorise the growth of a second or more microorganism in a sequential fermentation. A first fermentation of the lactic bacteria allows the growth of more demanding bacteria like bifidobacteria and propionibacteria.

The isolation of the bacterial strains (R2C2, K2, ES1) was performed on RCW agars as described by Kojima, S. et al., 1993, Biosci. Biotech. Biochem. Vol. 57, No. 1. pp: 119-120. Kefir grains were homogenized with a blender in an isotonic and sterile solution (tryptone 8,5 g/l + NaCl 1 g/l). This solution was used for RCW agar inoculation.

Different types of colonies were isolated. The selected strains are gram positives, non-mobile, catalase negatives and homofermetative strains. The strains are optionally anaerobic, not growing at 15°C and are having a samel physiology than the species described in Fujisawa et al., International journal of Systematic Bacteriology. Vol. 38. No. 1. pp:12-14. The strains were compared to the reference strain ATCC # 43761 for sugar fermentation pattern as illustrated at Table 1. Moreover, the strains were compared to the reference strains ATCC 43761 and 51647 for 16S homology as shown in Figs. 5A-E. The strains were compared to the reference stain ATCC 43761 for sugar fermentation pattern (as illustrated at Table 1) and to the reference strain ATCC 43761 and 51647 for 16S rRNA homology (as illustrated at Table 2). The isolated strains were classified in the genus *Lactobacillus*, and the species *kefiranofaciens*.

Table 1

Fermentation profile of sugar from APA 50 CH and medium API 50 CHL

Substrates	**R2C2**	**INIX**	**K2**	**ES1**
Glycerol	-	-	-	-
Erythritol	-	-	-	-
D-Arabinose	-	-	-	-
L-Arabinose	-	-	-	-
Ribose	-	-	-	-
D-Xylose	-	-	-	-
L-Xylose	-	-	-	-
Adonitol	-	-	-	-
β-Methyl glycoside	-	-	-	-
Galactose	+++	+++	+++	+++
D-Fructose	+++	+++	+++	+++
D-Mannose	++	+++	+++	+++
L-Sorbose	-	-	-	-
Rhamnose	-	-	-	-
Dulcitol	-	-	-	-
Inositol	-	-	-	-

Substrates	R2C2	INIX	K2	ES1
Mannitol	+++	+++	-	+++
Sorbitol	-	-	-	-
α-Methyl-D-Mannoside	-	-	-	-
α-Methyl-D-Glucoside	-	-	-	-
N-acetyl glucosamine	++	++	++	++
Amygdaline	-	-	-	-
Arbutine	-	-	-	-
Esculine	++	-	+++	+++
Salicine	+	++	+++	-
Cellobiose	-	-	+++	-
Maltose	++	+++	-	+++
Lactose	+++	+++	+++	+++
Melbiose	-	-	-	-
Saccharose	+++	++	-	+++
Trehalose	+++	+++	-	+++
Inuline	-	-	-	-
Melezitose	-	-	-	-
D-Raffinose	+	++	-	+++
Starch	-	-	-	-
Glycogene	-	-	-	-
Xylitol	-	-	-	-
β-Gentibiose	-	-	+++	-
D-Turanose	-	-	-	-
D-Lyxose	-	-	-	-
D-Tagarose	-	-	-	-
D-Fucose	-	-	-	-
L-Fucose	-	-	-	-
D-Arabitol	-	-	-	-
L-Arabitol	-	-	-	-
Gluconate	-	-	-	-

Substrates	R2C2	INIX	K2	ES1
2-ceto-gluconate	-	-	-	-
5-ceto-gluconate	-	-	-	-

Factors influencing agglomeration

Salt is one factor for the retrieval of the MPM. In a preferred embodiment, $CaCl_2$ is used but could also be replaced by any salt that is known in the art to have an effect on protein agglomeration like sodium pyrophosphate. For the same purpose, parameters like pH, temperature, enzymatic hydrolysis can be varied to promote agglomeration of proteins to form a matrix.

Food science

A need exists for a polysaccharide produced by a food-grade microorganism, having properties similar to or even superior to xanthan gum. Such an Exopolysaccharide (EPS) can either be added to the food product and the resulting product has to be labeled (but then the product is a so-called "friendly labeled" additive), or it can be produced *in situ* without the necessity of any labeling, because the microorganism is food-grade. The use of such microorganisms for the MPM production is preferred so the MPM produced offer either proteins characteristics and EPS properties. The MPMs can be used as a fat replacement agent, as a protein supplement, as a functional food product having a specific function (stimulation of the immune system, decreasing levels of triglyceride), as a bio-vehicle for ingredients, flavors, supplements, food additives, vitamins, etc.

Cosmetic

The MPM combined in one matrix polysaccharides and proteins can be used in a wide variety of compositions, including foundation make-ups, skin lotions and creams, sunscreens, blushes, mascara, eye shadows, in addition to hair care products such as shampoos, conditioners, and the like. Suggested ranges of MPM are 0,01-95%, preferably 0,05-50%, more preferably 0,1-30% by weight of the total composition. The composition into which the MPM is

incorporated can contain at least one surfactant, which may be an anionic, amphoteric, nonionic, cationic, or zwitterionic surfactant.

The MPM are suitable for use in foundation makeup or color cosmetics such as eye shadow, blush, concealer, or eyeliner compositions in the liquid, cream, solid, or stick form. Suitable compositions may be water-in-oil or oil-in-water emulsions, but are preferably oil-in-water emulsions. Such compositions generally comprise: 0,01-95% MPM, 0,5-95% water, 0,5-25% particulate matter, 0,01-20% surfactant, and 0,1-95% oil. In addition, these compositions may further contain ingredients selected from the group of humectants, preservatives, nonvolatile or volatile oils, gellants, and mixtures thereof.

Nutraceuticals

The MPM of the present invention possesses the synergical sum of the physiological effects of Exopolysaccharides, whey proteins, bacteria, fermentation products that are parts of the MPM, and thus may be used in nutraceuticals.

MPMs have also multiple advantages in the field of probiotics. First, MPMs constitute a mean to produce probiotics at a low cost. During the MPMs retrieval, all bacteria in suspension are retrieved in the MPMs, which represents around 5% of the fermented volume and thus a concentration factor of 20. A fermented solution, containing $1x10^9$ bacteria generates a concentration of $2x10^{10}$ bacteria in the MPMs. Production of probiotics will at the same time lead to the retrieval of components having health benefits like proteins, peptides and fermentation by-products such as exopolysaccharide, vitamins, bacterial proteins, etc. MPMs constitute also a multifunctional vehicle for probiotics. MPMs allow the incorporation of hydrophobic or hydrophilic substances that an be used to protect, synergize and feed the probiotics. For instance, vitamin C, which is hydrophilic, helps maintaining viability of concentrated probiotics. Presence of certain exopolysaccharides (as for example oligogalactosaccharides) has a prebiotic effect (synergy) on the stimulation of the intestinal flora or the presence of vitamin E (hydrophobic), has protecting effect on the viability of the microorganism (antioxidant) and a nutritive effect (vitamin). In addition, because of its

composition in proteins, MPMs are potentially capable of protecting the viability of probiotics. Finally, MPM can serve as a vehicle in different forms, humid, lyophilized or in compressed tablets. All forms constitute advantages in the field of probiotics. Humid MPMs are easy to formulate as shown in food formulations, cosmetics thus suggesting the same for the formulation of probiotics. Lyophilized MPMs, offer an important protection potential because of its content in proteins and the possibility of the incorporation of hydrophilic and hydrophobic protecting substances. Lyophilized MPMs are compressible without the need to add any excipients to form tablets which can be used for incorporation of probiotics or drugs.

Pharmaceuticals

Several drugs may be formulated with the MPM and they may be delivered orally and topically.

A plurality of pharmaceutically related products and drugs or bioactive materials can be formulated with the MPM like small molecules of various classes (hydrophilic and hydrophobic), proteins, RNA, oligonucleotides, DNA, viruses, bacterias. Examples or types of bioactive materials that are used in the MPM and methods of the present invention include any pharmaceutical agents, including, but not limited to anti-inflammatory drugs, analgesics, anti-arthritic drugs, antispasmodics, antidepressants, antipsychotics, tranquilizers, antianxiety drugs, narcotic, antagonists, antiparkinsonism agents, cholinergic agonists, chemotherapeutic drugs, immunosuppressive agents, antiviral agents, antibiotic agents, appetite suppressants, antiemetics, anticholinergics, antihistaminics, antimigraine agents, coronary, cerebral or peripheral vasodilators, hormonal agents, contraceptives, antithrombotic agents, diuretics, antihypertensive agents, cardiovascular drugs, opioids, and the like.

Suitable bioactive materials also include therapeutic and prophylactic agents. These include, but are not limited to any therapeutically effective biological modifier. Such modifiers include, but are not limited to lipids, organics, proteins and peptides (synthetic and natural), peptide mimetics, hormones (peptides, steroid and corticosteroid), D and L amino acid polymers, oligosaccharides, polysaccharides, nucleotides, oligonucleotides

and nucleic acids, including DNA and RNA, protein nucleic acid hybrids, small molecules and physiologically active analogs thereof. Further, the modifiers may be derived from natural sources or made by recombinant or synthetic means and include analogs, agonists and homologs. As used herein "protein" refers also to peptides and polypeptides. Such proteins include, but are not limited to enzymes, biopharmaceuticals, growth hormones, growth factors, insulin, monoclonal antibodies, interferons, interleukins and cytokins. Organics include, but are not limited to pharmaceutically active chemicals with amino, imino and guanidino groups. Suitable steroid hormones include, but are not limited to estrogen, progesterone, testosterone and physiologically active analogs thereof. Numerous steroid hormone analogs are known in the art and include, but are not limited to estradiol, SH-135 and tamoxifen. As used herein, "nucleic acids includes DNA, RNA and physiologically active analogs thereof. The nucleotides may encode single genes or may be any vector known in the art of recombinant DNA including, but not limited to, plasmids, retroviruses and adeno-associated viruses.

The MPMs described in the present invention have an advantage over conventional tablets pill in the above-described patients as it is a non-solid, creamy biodegradable vehicle that can be easily swallowed. Certain polysaccharides found in the MPMs, like kefiran products by *L. Kefiranofaciens*, are known to pass into blood circulation. These polysaccharides, peptides and bacteria found in the different MPM increase the absorption of some medicaments.

The following non-limiting examples further illustrate the invention and must not be contemplated as to limit the scope of the present invention.

EXAMPLE 1

Preparation of MPM

The preparation of a typical MPM is described in the following example.

Whey obtained from cheddar production is sterilized by filtration (0.22 μm). The sterilized whey is contained in a fermentation chamber at the time of inoculation with the R2C2 strain. A pre-culture is prepared to get a

concentration of bacteria of 10^8 to 10^9 per ml of preculture medium. The inoculation is done with a volume of preculture medium (10^8 R2C2/ml) corresponding to 1% and 15% but preferably 10% of the final volume of whey. The fermentation process is done at 37°C and at pH controlled at 5. The pH is controlled by the addition of NaOH. Agitation is maintained to a minimum to allow a uniform distribution but without causing an excessive aeration. The fermentation process is carried out at over a period of 16 to 36 hours depending of the characteristics needed. Following the fermentation process, between 0,1% and 1,5%, but preferably 1% of $CaCl_2$ (w/v) is added and the pH adjusted between 6,5 and 8, but preferably 7,5. The resulting MPMs is malleable, looks like a pudding of white creamy color with no noticeable taste or smell. Retrieval of the matrix is performed by centrifugation. Numerous centrifugal forces are appropriate depending on the functional characteristics desired. However, many tests indicated that a centrifugal force of 3500 RCF (Relative Centrifugal Force) is preferred for the production of a matrix with appropriate functional properties and that a centrifugal force between 3500 and 7476 RCF is also suitable.

An alternative process is using ES1, INIX, K2, R2C2, *Lactobacillus helveticus* ATCC 10386, *Lactobacillus kefirgranum* ATCC 51647, *Lactobacillus ramnosus* ATCC 7469, *Lactobacillus zeae* ATCC 15820 or *Lactococcus lactis* ATCC 11454 and controlling culture conditions of pH and temperature at the controlling values shown in Table 2. An alternative process is adjusting initial pH at the controlling value of microorganism used as shown in Table 2.

Table 2

Parameters for different strains

Strains	Temperature (°C)	pH
ES1	37	5
INIX	37	5
K2	37	5
R2C2	37	5
ATCC10386	42	5,5
ATCC 51647	30	5,5
ATCC 7469	42	6
ATCC 15820	42	6
ATCC 11454	24	6

In another alternative process, the lactoserum is pasteurized before fermentation and the fermentated solution is pasteurized again before separation of the MPMs from the co-products. This alternative however is not used when active bacteria are needed in the final product.

In a further alternative process, a double inoculation is performed with R2C2 and *Lactococcus lactis* to get a co-culture. The two species can also be cultivated together for future innoculation.

In another alternative process, the strain used is *Propionibacterium acidipropionici* ATCC 4875, which produce propionic acid. Fermentation temperature is 30°C, pH is 7, and fermentation is performed for a period of 96 hours. Yeast extracts can be supplemented in proportions of 0.5 to 1% (w/v) to stimulate propionic acid production. When anaerobic bacteria are used, the process may use addition of gas like CO_2 or nitrogen to remove oxygen and/or increase CO_2 partial pressure.

EXAMPLE 2

Composition of spoonable salad dressing

The matrix is incorporated in the proportions as illustrated in Table 3, for producing a spoonable salad dressing. In this manner, a tasty, creamy and firm dressing similar to mayonnaise is obtained.

Thus, the formulation of the dressing is characterized by the fact that the dressing, when refrigerated at 4°C, keep all is properties. The matrix can replace egg yolks as emulsifiers and stabilizers in oil-water emulsions and the matrix can emulsified as much as its own volume of oil.

The salad dressing is prepared by adding sugar to MPM with agitation to prevent clumping, adding vinegar and corn syrup and stir with an Osterizer blender at maximum speed for 30 seconds, adding corn oil rapidly to the blender jar and maintaining mixing for 1 minute and store salad dressing at 4°C for at least 24 hours.

Table 3

Composition of spoonable salad dressing

	%
MPM	37.6
Salad oil (corn)	37.3
Corn syrup	3.7
Sugar	1.5

EXAMPLE 3

Composition of chocolate milk

A chocolate milk is produced by mixing milk and MPM with an ultraturrax homogenizer, adding dry ingredient, adding liquid ingredients until dry ingredients are completely in solution and refrigerating at 4°C for at least 24 hours. The ingredients are listed in Table 4.

Table 4

Composition of chocolate milk

	%
Milk	53.5
MPM	40.8
Sugar	4.7
Chocolate flavor	0.2
Cocoa	0.1
Dairy Enhancer	To suit 100%

EXAMPLE 4

Composition of light butter

A light butter is produced by softening butter at ambient temperature, mixing butter and MPM, homogenize mixture by using ultra-turrax homogenizer until smooth-mixture is reached and refrigerate at 4°C for at least 24 hours. The ingredients are listed in Table 5.

Table 5

Composition of light butter

	%
Butter	50-85
MPM	15-50

EXAMPLE 5

Use of MPM as thickening agent in yogurt

Yogurt is a dairy product obtained through the fermentation of milk by specific bacterial strains converting part of the lactose into lactic acid such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus.* The milk coagulates when a sufficient quantity of lactic acid is produced.

Since the virtues of yogurt are associated among other things with the bacteria's action in the intestine, their presence in a sufficient number is important. With respect to this, yogurt should by law, in several countries, contain at least 10 million bacteria per gram at the time it is marketed.

The composition standards stipulate that yogurt must contain not less than 9.5% non-fat milk solids and not less than 3.0% protein. It may also contain some ingredients that come from milk (either whole or skim milk powder, or concentrated evaporated milk), fruits, fruit juices or extracts, jams, cereals or any other flavouring, sweeteners, a quantity not exceeding 2.0% of texturizing agents (stabilizers, gelling, thickening or emulsifying agents), citric acid, food colouring and, in the case of yogurt with added fruit, fruit juices or extracts or jams, a preservative not exceeding 50 ppm.

Potential use of the MPM, as thickening agent in yogurt, may be possible to enhance texture and viscosity in replacement of carrageenan, pectin, gelatin and corn starch among others. The MPM can be of interest as a dairy ingredient in a dairy product since it is a low calorie ingredient.

EXAMPLE 6

MPM as a vehicle to preserve microorganisms alive

MPM can maintains bacteria in life for a long period of time. Three samples of MPM R2C2 (produced with three different centrifuge force: 1592, 3500 and 7476 RCF) were conserved at 4°C during 11 months. After that period of time, a small quantity of MPM was inoculated on RCW agar. The gels were incubated without oxygen at 37°C for 5 days. After incubation, the R2C2 strain was isolated from the samples. It was found that MPM can maintains bacteria in life for a prolonged period of time and therefore can be used as probiotic vehicle.

EXAMPLE 7

Production of MPM containing a probiotic strain

Table 6

Production parameters

Parameters	Lactobacillus rhamnosus 7469
* bacterial count	5,1 x 10^8 b/ml
Lactic acid; t0	0,439 g/l
Lactic acid, End of fermentation	1,67 g/l
Lactic acid in Residual Solution	0,215 g/l
MPM	15,82 g/kg
Total glucide, t0	59,25 g/l
Glucide at the end	51,0 g/l
Glucide in MPM	48,0 g/l
Lactose at T0	45,52 g/l
Lactose at the end	39,68 g/l
Lactose in Residual solution	42,40 g/l
Lactose in MPM	20,70 g/kg
Yield of MPM	28,69 g/l
% humidity	84,67%
% organic matter	9,01%
% minerals	4,95%

* from the culture before retrieval of the MPMs. The count in the MPMs is 20 times higher.

EXAMPLE 8

Makeup production

The MPM as prepared from the method described at the Example 1 was used to prepare two formulations of makeup sticks as follows:

Table 7

Makeup formulations

Formula	Components	w/w %
A	Sodium stearate	7.56
	Water	43.77
	Phenoxyethanol	0.50
	Propyl paraben	0.10
	Methyl paraben	0.30
	Butylene glycol	13.04
	Calcium chloride	0.80
	MPM	0.80
	PEG-20 methyl glucose sesquiisostearate	3.49
B	Hydrogenated castor oil	2.00
	Isostearyl alcohol	5.74
	Titanium dioxide	0.74
	Iron oxide yellow	1.05
	Iron oxide red	0.33
	Iron oxide black	0.13
	Talc	2.23
	Dimethicone	11.63
	Titanium dioxide/trimethylolethane	6.30

EXAMPLE 9

Preparation of a skin lotion

A skin lotion was made according to the following formula:

Table 8

Skin lotion formulation

Components	w/w %
MPM	1.60
Trisodium EDTA	0.10
Butylene glycol	5.00
Sorbitan stearate/sucrose cocoate	6.00
Methyl paraben	0.25
Ethyl paraben	0.15
Xanthan gum	0.30
Octyl methoxycinnamate	7.50
Octyl salicylate	5.00
Benzophenone-3	3.00
C12-15 alkyl benzoate	8.00
Cetyl alcohol	1.00
Phenoxyethanol	1.00
Propyl paraben	0.10
Water	QS

EXAMPLE 9

Encapsulation of trypan blue and fluorescein with MPMs

Marker molecules like trypan blue and fluorescein were encapsulated in MPMs at various concentrations. These formulations were used to perform *in vitro* and *in vivo* experiments to study pharmacokinetic parameters like absorption, bioavailability, distribution, metabolism, and excretion of MPM-based oral formulations. Solubility, stability, liberation were analyzed and compared to the free markers. MPMs were found to have high

encapsulation capacity and allow an overall improvement of general pharmacokinetic parameters.

To evaluate the efficacy of a fluorescent probe (Fluorescein (Sigma, Canada)) to penetrate the bloodstream in complexation with a potential drug carrier, female Wistar rats aged 8 weeks were used. The average rat weight was 200 g and they were fed *ad libitum*. Fluorescein (5mg/Kg of body weight) was vortexed either in saline 0,9% or in MPM before gavage. 3 rats/group were fed 1ml of mixed solution via feeding needles at T0 (Ttime). Fluorescein was only present in the first gavage. Rats were gavaged twice a day at an interval of 4 hours. 300µl blood samples were collected at T1, T3,5. When possible, urine was collected before bleedings. Blood was centrifuged at 13000 rpm for 5 minutes. Plasma was collected. 20 µl of plasma were diluted in 2 ml of PBS pH 7,2. Readings were recorded in a spectrofluorometer Eclipse (Varian, Australia) at an excitation wavelength of 490nm and an emission of 514nm. Concentrations were determined against a standard curve of fluorescein. The data below were collected showing that in fluorescein is entering blood circulation slightly better than when formulated in saline and excreted better in animal gavaged with MPMs suggesting a better absorption of fluorescein.

Table 9

Fluorescein concentration in blood over time

Time (hours)	1h (ng/ml)	3h30 (ng/ml)
Saline 0,9%	336,699	99,693
MPM-R2C2	415,701	116,622
MPM-Inix	323,532	84,645

Table 10

Fluorescein concentration in urine over time

Time (hours)	1h (ug/ml)	3h30 (ug/ml)
Saline 0,9%	96,3072	125,6508
MPM-R2C2	176,49423	126,027

MPM-Inix 213,58755 125,0865

EXAMPLE 10

Conditions for industrial production of MPMs

The preculture medium is a medium composed of whey permeate (62,5 g/l) containing 10g/l yeast extract (1%). Sterilized water is used to reconstitute powder whey. Powder of whey permeat is added along with the yeast extract. The medium is pasteurised at 90°C for 30 minutes avoiding the caramel-formation phenomenon and allowing a better ferment growth.

The preculture is generated in fermentor in the following conditions: 39°C, initial pH of 5 is controlled at 4,3 during fermentation, minimal agitation (50 RPM) for 18 to 20 hours with a ratio of initial inoculation between 1 to 15%, but preferably 10% (10^8 bacteria/ml). The inoculation is performed from frozen ferments. The same conditions are used to ferment whey in order to produce the MPMs, but 1% $CaCl_2$ is added to cruce whey, just before the pH adjustment at 5 with HCl. This minimizes the risks of contamination and allows to bring the starting pH close the optimal growth of the bacterial strain used. Once the pH adjusted, whey is pasteurized in the fermentor at 70°C for 40 minutes, temperature is brought to 39 °C and whey is inoculated with 0,5 to 5%, but preferably 2,5% of preculture as defined previously. The fermentation of whey lasts 16 hours with the R2C2 strain. The pH is controlled by adding NaOH. Agitation is maintained to a minimal to allow a good distribution but without causing an excessive aeration. Reajustement of the pH at 7,5 and the retrieval of the MPMs is performed with an industrial clarifier Westfalia model NA-7. The yields obtained depend on the desired firmness and vary between 30 to 50 g/l of fermented solution. The MPM retrieval trigger the recuperation practically of all bacteria found in suspension.

EXAMPLE 11

Inoculation with frozen ferment in whey for MPM production

The MPM was prepared in the same conditions as in the Example 10 except that the fermentation was inoculated without preculture but directly in whey with a frozen ferment. The growth of the ferment is slower and

requires a longer fermentation time (24 hours). The quality and the yield of retrieved MPM in those conditions is comparable to the use of ferment by picking in a preculture as described previously.

EXAMPLE 12

Storage of R2C2 preculture for the MPM production

The preculture of R2C2 as described in the Example 10 can be refrigerated and stored for 2 days without any noticeable alteration. A longer period of refrigeration of 72 hours triggers the apparition of latent phase of the ferment during a subsequent whey fermentation.

EXAMPLE 13

Pasteurization of the fermented whey

Fermented whey can be submitted to a thermal treatment of 65°C for 30 minutes in order to ensure the absence of viable contaminants. The pasteurization of the fermented solution has however to be avoided if ones want to preserve the beneficial effects of the probiotics when used in the fermentation process.

EXAMPLE 14

The use of a clarifier to retrieve the MPMs

Following a fermentation as described in the Example 10 of MPMs, an equipment Westfalia, model NA-7, was tested to continuously retrieve by means of nozzle or to retrieve by periodical discharges. The retrieval with nozzle generates a less dense and liquid MPM. This kind of MPM did not answer the needs for food formulations. In order to increase the density, a short residence time in the clarifier is preferable. This was accomplished by using the periodical discharges. The desired density of MPMs can be adjusted to satisfy the needs for food formulators for different products (butter and cream, etc.). The NA-7 is used at maximal speed of rotation with a flow of 330 L/h of fermented solution and discharged every 7 minutes intervals. The time of aperture of the bowl is adjusted to allow a partial appropriate discharge, eg. allowing neither the MPM accumulation inside the NA-7, nor a complete discharge of the bowl. The yields obtained are 40 to 45 g/L for

MPMs having a spreading measurement of 8 to 10 cm while MPM obtained at a lower yield of 30 to 35 g/L for a spreading measurement of 3 cm. All agglomerates and microorganisms in suspension are retrieved.

EXAMPLE 15

Retrieval of MPM with a LAPX404

The LAPX404 machine from ALFA Laval is also utilized at a flow rate of 130 L/h, at a maximal rotation speed of 9500 RPM discharging every 7 minutes. The discharged volume with LAPX404 is partial but constant. In those conditions, density of the MPMs retrieved is appropriate for food formulations. The density can be adjusted by varying the discharges intervals and the flow rate. All agglomerates and microorganisms in suspension are retrieved.

EXAMPLE 16

MPM production from concentrated whey

In the same conditions as described in Example 10, concentrated whey (13% solid matter) is utilized as the fermentation start up solution. In those conditions, yields obtained for MPM production increased to 85 g per liter of fermented solution fermented with the R2C2 strain.

EXAMPLE 17

Protein recuperation by fermentation with specific microorganism

This example describes the use of *Lactobacillus kefirgranum* to help retrieving proteins in fermented solutions. *Lactobacillus kefirgranum* is inoculated from a lyophilised biomass in PL-salt medium as described: Tryptone peptone (casein) 1%, MgSO4 0,02%, MnSO4 0,005%, Sodium acetate 0,2%, Tween 80 0,05%, Yeast Extract 1%, Powder of permeat of whey 62,5g/L. Complete with distilled water, adjust pH between 5,0 and 5,5 with HCl and autoclave at 121°C, 30 minutes. Keep at 4°C until use. Following the fermentation from 24 to 60 hours, preferably 40, at 30°C, the resulting culture can be filtrated or centrifuged or other treatment without any addition in order to retrieve the proteins and the bacteria forming agglomerates.

For the same application, *Lactobacillus kefirgranum* can be cultured in Rogosa Cheese Whey (RCW) medium prepared as follows: RCW is prepared from the Rogosa S1 Broth (Difco # 0478-17-4) and prepared according to the manufacturer's protocols except that distilled water is replaced by whey permeate which is prepared from a powder of whey permeate (62.5 g/L) and the proteins are denatured thermically (Sterilization 121°C for 15 minutes) and fractionated by filtration before use. *Lactobacillus kefiranofaciens* is grown at 30°C for 24 to 60 hours, preferably 40, to allow the retrieval of bacteria and part of the proteins. The culture can be filtered or centrifuged without any other treatment or addition to retrieve the proteins and the bacteria forming agglomerates.

Also, a 400-ml culture in PL-salt, for 24 hours of fermentation at 30°C, is used to inoculate 10 litres of sterile whey. Fermentation is controlled at pH 5,5, 30°C for 24 hours and the fermented solution is centrifuged without any other treatment or addition to retrieve the proteins and the bacteria forming agglomerates. The product retrieved is a MPM at pH 5,5. Another application is to adjust the pH of the fermented solution between 5,5 to 8, preferably at 7,5, before the retrieval of the agglomerates. Finally, another application is to adjust $CaCl_2$ between 0,1 and 1,5 %, preferably at 1%, to the fermented solution before adjusting the pH between 5,5 and 8, preferably at 7,5, and to retrieve the agglomerates.

EXAMPLE 18

MPM as a flavor enhancing agent

In a panel of tasting, the majority of people concluded that the product containing the MPMs had an enhanced flavor. This was reported in butter (the salty taste) and in chocolate prepared drinks (the chocolate taste). Thus, MPM can be used to help increase the flavor of certain product.

EXAMPLE 19

Anti-inflammatory effect of MPMs (Reduction of TNF-α in blood cells)

Female Wistar rats weighing 150 g and fed *ad libitum* were submitted to 3 sessions of gavage (each session lasted a week for 7 repeated gavages) with a week of rest between each session. Blood samples were

collected during and the course of the experiment and the rats sacrificed 2 h after the last gavage for a total of 21 gavages. The samples were harvested (blood cells), on dry ice, and frozen at –80°C. RNA was isolated using TRIZOL reagent (Gibco) as per manufacturers specifications. Ten micrograms of total RNA was reverse transcribed using Superscript RT (Gibco), 500ng oligodT primers (Gibco), and 250ng Random Hexamer primers (Gibco) for 20 minutes at room temperature, followed by 2 hours at 42°C.

TNF-α was amplified from 2ul of reverse transcription reaction using Titanium PCR kit (Clontech). Amplification primers were as follows:

Rat TNF-α forward: CCCAACAA GGAGGAGAGTTCCC (SEQ ID NO:7)

Rat TNF-α reverse: ATGACTCCAAAGTAGACCTGCCC (SEQ ID NO:8)

The PCR reaction was performed for 30 cycles using 100pmole for each primer, with an annealing temperature of 68°C. The PCR products were analysed on a 1.5% agarose gel. The data showed that the MPMs can reduce the level of TNF-α at the RNA level in blood cells after 7 days in the group of rats fed with MPMs in contrast to that observed in the groups fed with saline (-control), HMS90™ (a whey protein isolate sold by Immunotec), yogurt (Danone reduced in calories) therefore suggesting that an anti-inflammatory effect is taking place in blood cells.

EXAMPLE 20

Immunomodulatory effect of MPMs (production of IL-18)

Female Wistar rats weighing 150 g and fed *ad libitum* were submitted to 3 sessions of gavage (each session lasted a week for 7 repeated gavages) with a week of rest between each session. Blood samples were collected during and the course of the experiment and the rats sacrificed 2 h after the last gavage for a total of 21 gavages. The samples were harvested (blood cells), on dry ice, and frozen at –80 degrees. RNA was isolated using TRIZOL™ reagent (Gibco) as per manufacturers specifications. Ten micrograms of total RNA was reverse transcribed using Superscript RT (Gibco), 500ng oligodT primers (Gibco), and 250ng Random Hexamer primers (Gibco) for 20 minutes at room temperature, followed by 2 hours at 42°C.

IL-18 was amplified from 2ul of reverse transcription reaction using Titanium PCR kit (Clontech). Amplification primers were as follows:

IL-18 forward: ATGCCTGATATCGACCGAACA GCC (SEQ ID NO; 9)

IL-18 reverse: CAAATTCCATTTTGTTGTGTCCTG G (SEQ ID NO: 10)

The PCR reaction was performed for 30 cycles using 100pmole for each primer, with an annealing temperature of 68°C. The PCR products were analysed on a 1.5% agarose gel. The data showed that the MPMs increase the levels of IL-18 at the RNA level in blood cells after 24 hours in the group of rats fed with MPMs in contrast to that observed in the groups fed with saline (-control), HMS90 (a whey protein isolate sold by Immunotec), yogurt (Danone reduced in calories) therefore suggesting a stimulation of the mucosal immunity.

EXAMPLE 21

Stimulation of PBMC in rats fed with MPMs

Female Wistar rats weighing 150 g and fed *ad libitum* were submitted to 3 sessions of gavage (each session lasted a week for 7 repeated gavages) with a week of rest between each session. Blood samples were collected during and the course of the experiment and the rats sacrificed 2 h after the last gavage for a total of 21 gavages. Blood samples were measured with Unopett (BD) to count the total peripheral monocular blood cells. The data are shown below and suggest that rats fed with MPMs have a tendency to see their count of PBMC to increase, almost doubled after 4 days post-gavage.

Table 11

PBMC count

Group test	PBMC count increase relative the saline
Saline	1
MPM	1.8
HMS 90	1.2
Yogurt	0.6
Butter	1.2
Butter/MPM (40%MPM)	1.6

EXAMPLE 22

Anti-triglyceridemia effect of MPMs

Female Wistar rats weighing 150 g and fed *ad libitum* were submitted to 3 sessions of gavage (each session lasted a week for 7 repeated gavages) with a week of rest between each session. Blood samples were collected during and the course of the experiment and the rats sacrificed 2 h after the last gavage for a total of 21 gavages. Serum were measured by the method of Wahlefeld (GPO-PAP) to determine the levels of circulating triglycerides. The data of table 12 below shows that MPMs affect the basal level of TG.

Table 12

Triglycerides levels after 24 hours and 3 days

	TG after 24 hours mg/dl serum (SEM)	TG after 3 days mg/dl serum (SEM)
Saline	62 (15)	51 (4)
MPM	33 (5)	32 (10)
HMS 90	31 (4)	101.(15)
Yogurt	118 (38)	76 (27)
Butter	64 (30)	128 (46)
Butter/MPM	83 (9)	156 (22)

Table 13

Triglycerides levels after 24 hours, 16 days and 21 days

Test groups	TG after 24 hours mg/dl serum (SEM)	TG after 16 days mg/dl serum (SEM)	TG after 21 days mg/dl serum (SEM)
Saline	71(14)	80(19)	43(14)
MPM	50(15)	52(1)	35(1)
HMS 90	56(6)	102(34)	92(21)
Yogurt	57(4)	56(13)	99(24)

EXAMPLE 23

Analysis of the amino acid content of MPMs

MPMs were analyzed by Bodycote Canada in order to determine the comparative amino acid content of various whey protein-based product. WPH917 is a high quality whey protein hydrolysate produced by a controlled enzyme treatment of whey protein which provides amino acids, peptides, and polypeptides. Power Pro80 is 80% whey protein concentrate produced by an ultrafiltration process that concentrates native whey proteins.

Table 14

Analysis of the amino acid content

Amino acid	MPM (batch 15)	WPH 917	PowerPro 80
Glutamic Acid	22,92	18,3	10,1
Alanine	5,73	5,2	4,1
Arginine	4,01	3	1,9
Cystine	10,32	2,9	2
Glycine	13,61	2,3	1,5
Histidine	11,17	1,9	1,6
Isoleucine	6,45	5,5	5,1
Leucine	11,89	14,2	9
Serine	7,02	5	3,9
Thryptophane	Not determined	2,3	1,4

EXAMPLE 24

Content analysis of the MPMs

MPMs were analyzed by Bodycote Canada in order to determine the overall content of various whey protein-based products. WPH917 is a high quality whey protein hydrolysate produced by a controlled enzyme treatment of whey protein which provides amino acids, peptides, and polypeptides.

Table 15

Content analysis

Content	Methods	MPM %(g/100g)	WPH917 %(g/100g)
Humidity	AC-HUM 04	80	4
Proteins	AC-PRO01AOAC	8	89
Ashes	AC-CEN01AOAC	6	3.1
Carbohydrates	AC-SUB01AOAC	5	Not determined
Lactose	AC-LAB01AOAC	2.5	0.3
Fat	AC-GRA 01	1.3	3.5
Minerals (Na, Ca, K)	SAA	0.1, 1.8, 0.2	1.3,0.1,002

EXAMPLE 25

Resulting biological effect of pasteutized MPM-INIX on Gluthathione

Female Wistar rats weighing 150 g and fed *ad libitum* were submitted to 3 sessions of gavage (each session lasted a week for 7 repeated gavages) with a week of rest between each session. Blood samples were collected during and the course of the experiment and the rats sacrificed 2 h after the last gavage for a total of 21 gavages. Blood samples were measured after 3 weeks according to a method modified from Anderson designed to measure glutathione levels in plasma.

Table 16

GSH levels in rats

Test groups	Levels of GSH In ug GSH/ml plasma (+/-SD)
Saline	0.05 (0.05)
MPM 1*	0.08 (0.08)
MPM**	1.08 (0.31)

* Plain MPM produced with R2C2 ** Pasteurized MPM produced with INIX

EXAMPLE 26

Solubilization of pyrene using MPM

50 µM pyrene stock solution in acetone is prepared and 20 µL of the stock solution is brought into borosilicate tubes (10 x 13 mm). The tubes are standing at room temperature in a fumehood for 20-30 minutes until complete evaporation of acetone. 2.0 ml of MPM dissolved in 0,1 M phosphate buffered saline (PBS) at pH 7,2 are inserted into the borosilicate tubes. The concentration of MPM used is inside the range of 0,001 to 1,0% (w/v). Let the solution stand at room temperature, protected from the light and fluorescence readings are performed after 24, 48 and 69 h of incubation. Fluorescence reading are performed on a Varian Cary Eclipse at 37°C with an excitation wavelength set at 340 nm and emission scan from 350 to 600 nm are recorded. The degree of solubilization of pyrene is measured by plotting the I_3/I_3(aqueous) of the compound studied as a function of the concentration of the compound. The inflexion point of the plot correspond to the critical micellar concentration value. The data of Table 17 show the ratio of fluorescence intensity of peak I_3 over intensity of peak I_3 from PBS solution as a function of the concentration of MPM and P85.

Table 17

ratio of fluorescence intensity of peak I3 over intensity of peak I3 from PBS solution

	P85	MPM lot 78
0.001%	1,107	1,123
0.01%	1,168	1,328
0.1%	2,564	2,416
1%	8,813	3,687

The data of Table 18 show the ratio of fluorescence intensity of peak I_e over intensity of peak I_e from PBS solution as a function of the concentration of MPM and P85. The data show that MPMs promote the formation of excimers (hydrophobic microdomains).

Table 18

ratio of fluorescence intensity of peak Ie over intensity of peak Ie from PBS solution

	P85	MPM lot 78
0.001%	0,9619	1,567
0.01%	1,233	4,834
0.1%	2,443	30,88
1%	4,829	25,24

EXAMPLE 27

Incorporation of MPM in body lotion

In order to validate the functional properties of the MPMs in a cosmetic formulation, a test was done to formulate a commercial body lotion. The commercial product was a lotion made out goat milk containing 0.3% goat milk extract. The MPM was incorporated in a 10% (w/w). The vessel was closed before mixing the component and agitated by hand and stored at room temperature. After 24 hours, the resulting mixture exhibited fines particles on the vessel walls and was more liquid than the original commercial body lotion. However, the resulting mixture did not show signs of degradation or separation of emulsion and the smell remained similar to the commercial product. Viscosity of MPMs was evaluated using spreading techniques according to the principle of consistometry USDA (Adams & Birdsall, 1946) allowing the measurement, in cm, of the distance of spreading of a semi-fluid food performed on a plate equipped with a series of concentrated circles in predetermined and standardized time. The resulting mixture of body lotion + MPM recorded 13 cm on the spreading scale but get back to its original spreading e.g. 8cm over a period of 3 weeks. The data indicated that the addition of 10% de MPM triggered a liquefaction of the matrice. In terms of shelf-life preservation the resulting mixture was found stable and not contaminated for a period of 3 weeks with the same particle in suspension with the same characteristic smell than the original product.

EXAMPLE 28

Light butter

Two types of light butter were prepared containing 25 and 40% MPM, respectively. In general, the MPM-butter is creamier at room temperature, solidifies less at 4°C, and exhibit a texture which is more breakable than regular butter. It is important to note that the salty taste of butter was enhanced by the MPMs allowing a reduction of the usual amount of salt (NaCl) in the butter by 33 to 50%. The butter containing 25 % MPM is useable for frying and turn brown like plain butter while the 40% butter does not allow frying and is used only as a spread. Also, the conservation time of MPM-butter was acceptable for 45 days and the MPM-containg butter could be frozen without any change of organoleptic properties.

EXAMPLE 29

MPM in whipped cream

Light cream formulations were prepared from 40% cream, milk and MPM to obtain a cream with 21% fat that exhibit a nice and thick texture that can be whipped. The resulting whipped cream is firm and can be stored for 15 days in the fridge without alteration. The odor and taste are similar than the plain cream. The recipe is as follows: 200 ml of cream at 40%, 85 ml milk, 95 g of MPM and sugar and /or aroma.

EXAMPLE 30

Mayonnaise and salad dressing

The same type of MPM used previously was used to prepare Mayonnaise and salad dressing according to the following formula:

Table 19

Salad dressing preparation

Ingredients :	Amount (grams)
MPM	19.6
oil	19.4
Corn syrup	1.9
sugar	0.8
aroma	To taste
spice	To taste
vinegar	10.3

EXAMPLE 31

Chocolate drink

The same type of MPM used previously was used to prepare chocolate drinks according to the following formula:

Table 20

Chocolate drink preparation

Ingredient	Amount in grams
MPM	60
milk	100 ml
cacao	0.16
sugar	7
Chocolate aroma	0.25
Flavor enhancer	To be adjusted

This formulation allows the enrichment of milk of 3,6 g of proteins which is practically doubled as compared to plain milk. The MPM gives a

better viscosity and a creamier texture which is appreciated in mouth. MPMs were also added to a weight control drink (Slim fast™) and the resulting product was found to have a reduced taste of the soy proteins found in that drink.

EXAMPLE 32

Yogurt

Four attempts were made with the formulation of yogurt. (1) control with 3,25% milk and (3) attempts with 1% milk all supplemented with 3% skim milk powder. The steps for the preparation of yogurt included heating the milk at 82-85°C, agitatingh for 30 min, followed with a cooling step at 44-45°C. The culture (yogourmet, LYO-SAN Co) containing *L. Bulgaricus*, *S. Thermophilus* and *L. Acidophilus* were added to get 5 g per liter of milk, followed by an incubation at 45°C for 4 hours, without agitation.

Control test made from 3,25% milk.

Test 2: simultaneously heating of the milk and the MPM (100g/l) followed by the addition of the ferment.

Test 3: the milk is heated first followed by the addition of the MPM (100 g/l) and the ferment.

Test 4: addition of the MPM at the end of the production, at the same time than the addition of the fruits like in an industrial production.

Following the incubation, the product obtained by the Test 2 is comparable to the control test while test 3 and 4 did not allowed the coagulation. After 6 weeks of storage, the control test exhibits a significant drainage and was contaminated with yeast while test 2 did not show neither signs of destabilization nor microbiological deterioration since its preparation. Thus, MPM play a role of preservative on the formulation and helps to obtain very good and typical texture for a yogurt while reducing the amount fat in the final formulation without the addition of starch or other thickening agents.

EXAMPLE 33

The use of different strains affect the analytical profile of the resulting MPMs produced in batches of 10 litres

Concentration of various carbohydrates (glucids), lactic acid and the yields of MPM vary according to the strain used. Also, the composition of the fermented solution influence the composition of the matrix either directly and proportionally like in the case of the carbohydrates found in the aqueous and liquid phase of the matrix, or according to a concentrating effect like in the case of lactic acid that precipitates in part during the retrieval of the matrix.

Table 21

MPM analytical profile in function of different strains used to produce them

	INIX	Lactobacillus rhamno 7469	Lactobacillus zeae	Lactobacillus kefirgranum	Lactobacillus helveticus
***bacterial count**	2,47 x 10^8 b/ml	5,1 x 10^8 b/ml	1,34 x 10^8 b/ml	Not determined	6,77 x 10^7 b/ml
Lactic acid ; t0	0,211g/L	0,439g/L	0,143 g/L	0,234 g/L	0,157 g/L
Lactic acid, End of fermentation	2,414g/L	1,67 g/L	0,821 g/L	2,22 g/L	3,5 g/L
Lactic acid in Residual Solution	1,122 g/L	0,215 g/L	0,384 g/L	1,11 g/L	1,53 g/L
MPM	9,57 g/kg	15,82 g/kg	9,85 g/kg	2,94 g/kg	5,23 g/kg
Total glucide, t0	45,0 g/L	59,25 g/L	41,30 g/L	54,22 g/L	47,06 g/L
Glucide at the end	41,25 g/L	51,0 g/L	55,01 g/L	51,55 g/L	42,67 g/L
Glucide in Residual solution	42,0 g/L	48,0 g/L	53,86 g/L	46,54 g/L	54,24 g/L
Glucide in MPM	36,50 g/kg	29,35g/kg	39,86g/kg	38,593g/kg	41,197g/kg
Lactose at T0	47,95 g/L	45,52 g/L	53,46 g/L	50,99 g/L	52,58 g/L
Lactose at the end	42,95 g/L	39,68 g/L	51,74 g/L	47,28 g/L	42,65 g/L
Lactose in Residual solution	42,57 g/L	42,40 g/L	47,31 g/L	44,92 g/L	41,55 g/L
Lactose in MPM	24,78g/kg	20,70g/kg	27,70g/kg	37,02g/kg	35,17g/kg
GALACTOSE at T0	0	0	0	0,13 g/L	0
Galac. At the end	0,75 g/L	0	0	0,75 g/L	2,51 g/L

Table 21

MPM analytical profile in function of different strains used to produce them

	INIX	Lactobacillus rhamno 7469	Lactobacillus zeae	Lactobacillus kefirgranum	Lactobacillus helveticus
Galac. in Residual solution	0,70 g/L	0	0	0,36 g/L	2,02 g/L
MPM	1,72g/kg	0	0	1,55g/kg	2,22g/kg
GLUCOSE at T0	0	0	0	0	0
Glucose at the end	0	0	0	0,49 g/L	1,49 g/L
Glucose in Residual solution	0	0	0	0	0,88 g/L
MPM	0	0	0	1,48 g/kg	1,74 g/kg
Yield of MPM	41,2 g/L	28,69 g/L	27,5 g/L	40,6 g/L	40,12 g/L
% humidity	85,98%	84,67%	85,23%	85,11%	
% organic matter	9,49%	9,01%	8,66%	9,87%	
% minerals	4,53%	4,95%	6,12%	5,03%	

* from the culture before retrieval of the MPMs. The count in the MPMs is 5 times higher.

EXAMPLE 34

Characteristics of various MPMs produced from different strains

The use of different strains during the fermentation process affect texture, taste, and smell of resulting MPMs as shown in Table 22.

Table 22
Characteristics of MPM produced from different strains

Parameters	Lactococcuslactis	Lactobacillus helveticus	INIX	Lactobacillus rhamnosus	R2C2
pH	5.69	6.99	6.58	5.06	6,26
Color	7039-12	7042-22	7042-12	7039-12	parsnip
taste	Acre	Acre	Acre	nd	Neutral
smell	Yogurt plain	Condensed milk	Algeas	Strong whey	Cooked cauliflower
Appearence	synerisis important	Nice homogenous texture	Nice soft and viscous texture	bubbles agglomerates	Heterogenous agglomerates
%synerisis	0.07	0.08	0.1	0	0,23
Drainage	nul	nul	nul	nul	nul
***Spreading**	1.25	2	2	5.5	0
** **Emulsifying activity**	Mayonnaise	Vinaigrette	Vinaigrette	Vinaigrette	Vinaigrette

* Viscosity of MPMs was evaluated using spreading techniques according to the principle of consistometry USDA (Adams & Birdsall, 1946) allowing the mesureament, in cm, of the distance of spreading of a semi-fluid food performed on a plate equiped with a series of concentrated circles in predetermined and standadized time.

** The emulsifying activity is evaluated by means of model system representing a good capacity for the formation of a vinaigrette and of and of mayonnaise.

EXAMPLE 35

Characterization and composition of the MPMs produced in 10-litres in fermentors by the R2C2 strain.

Table 33

Characteristics of the MPMs vary according to the growth rate of the strain

Parameters	Sample 1	Sample 2	Sample 7	Sample 8	Sample 7 pasteurized
pH	nd	nd	7.17	7.21	7.06
Color	7042-22	7042-22	7039-12	7039-12	7041-22
Taste	bitter	bitter	bitter	bitter	nd
Odor	Cooked cauliflower	Cooked cauliflower	Cooked cauliflower	Cooked cauliflower	Algae
Appearence	Firm, no synerisis	Yogurt-like synerisis	homogenous soft	homogenous soft	homogenous soft Yogurt-like
%synerisis	0	0.01	0.1	0	0
Drainage	nil	nil	nil	nil	nil
Spreading	nd	nd	8.25	plus de 12	6.5
Emulsifying activity	Vinaigrette	Vinaigrette	Vinaigrette	Vinaigrette	Vinaigrette
Bacterial count in MPM	2×10^9 b/g	$1,4 \times 10^9$ b/g	5×10^8 b/g	$6,7 \times 10^8$ b/g	5×10^8 b/g
Lactic Acid	16,39 mg/g	24,38 mg/g	7,02 mg/g	5,61 mg/g	7,02 mg/g
Total sugars	15,70g/kg	18,30 g/kg	45,19g/kg	43,15g/kg	45,19g/kg
Lactose	0	0	49,88g/kg	48,57g/kg	49,88g/kg
Galactose	14,9g/kg	18,1g/kg	0,66 g/kg	0,77 g/kg	0,66 g/kg
Yield	27,32 g/L	33,85 g/L	41,10 g/L	44,10 g/L	41,10 g/L
% humidity	82,26%	84,82%	86,05%	85,86%	86,05%
% organic matter	10,92%	9,37%	9,01%	9,86%	9,01%
% minerals	6,83%	5,82%	5,08%	4,28%	5,08%

Legend:
Nd, not determined
7039-12 white asparagus, off-white with greenish tendency.
7041-12 whiter and less green than 7039-12 (canevas)
7042-12 cauliflower, more beige than white
7042-22 White but with orange background

EXAMPLE 36

Production of MPMs containing proteins from plant origin

The preculture medium is a medium composed of de whey permeat (62,5 g/l) containing 10g/l yeast extract (1%). Sterilized water is used to reconstitute powder whey. Powder of whey permeat is added along with the yeast extract. The medium is pasteurised at 90°C for 30 minutes avoiding the caramel-formation phenomenon and allowing a better ferment growth.

The preculture is generated in fermentor in the following conditions: 39°C, initial pH of 5 is controlled at 4,3 during fermentation, minimal agitation (50 RPM) for 18 to 20 hours with a ratio of initial inoculation between 1 to 15%, but preferably 10% (10^8 bacteria/ml). The inoculation is performed from frozen ferments. The same conditions are used to ferment whey (supplemented with commercial soy proteins 1 to 10% w/v) in order to produce the MPMs, but 1% $CaCl_2$ is added to crude whey, just before the pH adjustment at 5 with HCl. This minimizes the risks of contamination and brings the starting pH close the optimal growth of the bacterial strain used. Once the pH adjusted, whey is pasteurized in the fermentor at 70°C for 40 minutes, temperature is brought to 39°C and whey is inoculated with 0.5 to 5%, but preferably 2,5% of preculture as defined previously. The fermentation of whey lasts 16 hours with the R2C2 strain. The pH is controlled by adding NaOH. Agitation is maintained to a minimum to allow a uniform distribution without causing an excessive aeration. Readjustment of the pH at 7,5 and the retrieval of the MPMs is performed with a, industrial clarifier Westfalia model NA-7. The yields obtained depend on the desired firmness and vary between 30 to 50 g/l of fermented solution. The MPM retrieval trigger the recuperation practically of all bacteria found in suspension.

EXAMPLE 37

Formulation of 5-Fluoro Uracile with the MPMs

MPMs can be used to formulate various bioactive materials. In this example 5-Fluoro Uracile was formulated with the typical MPM described in the above examples and given orally by gavage to mice. The efficacy on

this new formulation was compared to free 5FU and tested in animal models in which colon cancer cells were implanted or in which colon cancer was chemically inducted. The finding was that the 5FU/MPM formulation was improving the therapeutic index of 5FU as shown by reduction of tumor growth.

EXAMPLE 38

Basic formulation of a firm yogurt

1. Add 3 to 5% of mild solids to the milk

2. Heat milk to 85°C and maintain for 30 minutes

3. Reach ebullition and add 0.5% of gelatine, pectine or corn starch to the milk

4. Cool milk to 44-46°C and add bacterial strains converting part of the lactose into lactic acid such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus*

5. Add 20% of MPM

6. Place in containers and incubate between 40-46°C until 80°D is reach, for 4-6 hours

7. Refrigerate at 4°C for at least 24 hours.

EXAMPLE 39

Composition of vanilla pudding

A vanilla pudding is produced by mixing milk and MPM, adding dry ingredient to the milk-MPM mixture in mixing well, adding slowly oil to the mixture by using ultra-turrax homogenizer, cooking between 60-70°C until the mixture thickens and refrigerating at 4°C for at least 24 hours. The ingredients are listed in Table 34.

Table 34

Composition of vanilla pudding

	%
MPM	50
Milk	36.1
Sugar	14.9
Vegetal oil	3.3
Na_2HPO_4	0.2
Vanilla	0.5

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

SEQUENCE LISTING

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Pilote, Dominique
Dupont, Claude
Lajoie, Nathalie
Paquet, Michel
Lemieux, Pierre
Goyette, Philippe
Technologies Biolactis Inc.

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WHAT IS CLAIMED IS:

1. A malleable protein matrix comprising:

- a precipitate of a protein of interest in solution;

- at least one microorganism capable of fermenting said solution containing said protein; and

-a matrix carrier allowing fermentation of said protein and said microorganism.

2. The matrix of claim 1, wherein said fermentation is promoted by co-culture of at least two microorganisms simultaneously or successively.

3. The matrix of claim 1, further comprising a fermentation by-products of the fermentation of said solution containing said protein by said bacterial strain.

4. The matrix of claim 1, further comprising peptide.

5. The matrix of claim 4, wherein said peptide comprises at least two amino acid residues.

6. The matrix of claim 4, wherein said peptide comprises more than one hundred amino acid residues.

7. The matrix of claim 1, further comprising components obtained during agglomeration of said protein.

8. The matrix of claim 1, further comprising components present in aqueous phase.

9. The matrix of any one of claims 1 to 8, wherein said protein is selected from the group consisting of natural protein, plant protein, animal derived protein and synthetic protein.

10. The matrix of any one of claims 1 to 8, wherein said protein is selected from the group consisting of albumen, amylase, amyloglucosidase, arginine/lysine polypeptide, casein, catalase, collagen, crystalline, cytochrome C, deoxyribonuclease, elastin, fibronectin, gelatin, gliadin, glucose oxidase, glycoproteins, hexyldecyl ester of hydrolyzed collagen, human placental protein, human placental enzymes, iodized corn

protein, keratin, lactalbumine, lactoferrin, lactoglobulin, lactoperoxidase, lipase, milk protein, hyristoyl glycin/histidine/lysin polypeptide, nisin, oxido reductase, pancreatin, papaïne, pepsin, placental protein, protease, saccharomyces polypeptides, serum albumin, serum protein, silk, sodium stearoyl lactalbumin, soluble proteoglycan, soybean palmitate, soy, egg, peanut, cottonseed, sunflower, pea, whey, fish, seafood, subtilisin, superoxide dismutase, sutilains, sweet almond protein, urease, wheat germ protein, wheat protein, whey protein, zein and hydrolyzed vegetable protein.

11. The matrix of any one of claims 1 to 8, wherein said protein is whey protein.

12. The matrix of claim 3, wherein said fermentation by-products is polysaccharide.

13. The matrix of claim 12, wherein said polysaccharide is selected from the group of exopolysaccharide and anionic polysaccharide.

14. The matrix of claim 12, wherein said polysaccharide contains at least four saccharide moieties.

15. The matrix of claim 14, wherein said saccharide moieties are selected from the group consisting of D and L forms of glucose, fructose, xylose, arabinose, fucose, galactose, pyruvic acid, succinic acid, acetic acid, 3,6-anhydrogalactose sulfate, galactose-4-sulfate, galactose-2-sulfate, galactose-2, 6-disulfate, mannose, glucuronic acid, mannuronic acid, guluronic acid, galactouronic acid, and rhamnose.

16. The matrix of claim 12, wherein said polysaccharide have molecular weight ranging from about 500 to about 15,000,000 daltons.

17. The matrix of claim 12, wherein said molecular weight is ranging from about 5,000 to 6,000,000 daltons.

18. The matrix of claim 12, wherein said molecular weight is ranging from about 25,000 to 1,000,000 daltons.

19. The matrix of claim 12, wherein said polysaccharide is selected from the group consisting of heteropolysaccharide, homopolysaccharide and mixture thereof.

20. The matrix of claim 19 wherein said heteropolysaccharide is selected from the group consisting of gellan, welan, gum arabic, karaya gum, okra gum, aloe gum, gum tragacanth, gum ghatti quicessed gum , psyllium, galactans, galactomannans, glucomannans, polyuronic acids, dextran sulfate, heparin, pectin, sodium alginate and starch arabinogalactan.

21. The matrix of claim 20, wherein said galactan is selected from the group consisting of agar, agarose, kappa, carageenan, iota carageenan and lambda carageenan.

22. The matrix of claim 20, wherein said galactomannan is selected from the group consisting of locust bean gum and guar.

23. The matrix of claim 20, wherein said glucan is selected from the group consisting of cellulose and derivatives thereof, starch and derivatives, dextrans, pullulan, beta 1,3-glucans, chitin, xanthan and tamatind.

24. The matrix of claim 20, wherein said glycomannan is konjac.

25. The matrix of claim 20, wherein said polyuronic acid is selected from the group consisting of algin, alginate and pectin.

26. The matrix of claim 19, wherein said homopolysaccharide is cellulose.

27. The matrix of claim 1, wherein said microorganism is selected from the group consisting of *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium animalis*, *Bifidobacterium asteroides*, *Bifidobacterium bifidum*, *Bifidobacterium boum*, *Bifidobacterium breve*, *Bifidobacterium catenulatum*, *Bifidobacterium choerinum*, *Bifidobacterium coryneforme*, *Bifidobacterium cuniculi*, *Bifidobacterium dentium*, *Bifidobacterium gallicum*, *Bifidobacterium gallinarum*, *Bifidobacterium indicum*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium longum* DJO10A, *Bifidobacterium longum* NCC2705, *Bifidobacterium magnum*, *Bifidobacterium merycicum*, *Bifidobacterium minimum*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium pseudolongum*, *Bifidobacterium pseudolongum subsp. globosum*, *Bifidobacterium pullorum*, *Bifidobacterium ruminantium*, *Bifidobacterium saeculare*,

Bifidobacterium scardovii, *Bifidobacterium subtile*, *Bifidobacterium suis*, *Bifidobacterium thermacidophilum*, *Bifidobacterium thermacidophilum subsp. suis*, *Bifidobacterium thermophilum*, *Bifidobacterium urinalis*, *Lactobacillus acetotolerans*, *Lactobacillus acidipiscis*, *Lactobacillus acidophilus*, *Lactobacillus agilis*, *Lactobacillus algidus*, *Lactobacillus alimentarius*, *Lactobacillus amylolyticus*, *Lactobacillus amylophilus*, *Lactobacillus amylovorus*, *Lactobacillus animalis*, *Lactobacillus arizonensis*, *Lactobacillus aviarius*, *Lactobacillus bifermentans*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus cellobiosus*, *Lactobacillus coleohominis*, *Lactobacillus collinoides*, *Lactobacillus coryniformis*, *Lactobacillus coryniformis subsp. coryniformis*, *Lactobacillus coryniformis subsp. torquens*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus cypricasei*, *Lactobacillus delbrueckii*, *Lactobacillus delbrueckii subsp. bulgaricus*, *Lactobacillus delbrueckii subsp. delbrueckii*, *Lactobacillus delbrueckii subsp. lactis*, *Lactobacillus durianis*, *Lactobacillus equi*, *Lactobacillus farciminis*, *Lactobacillus ferintoshensis*, *Lactobacillus fermentum*, *Lactobacillus fornicalis*, *Lactobacillus fructivorans*, *Lactobacillus frumenti*, *Lactobacillus fuchuensis*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus graminis*, *Lactobacillus hamsteri*, *Lactobacillus helveticus*, *Lactobacillus helveticus subsp. jugurti*, *Lactobacillus heterohiochii*, *Lactobacillus hilgardii*, *Lactobacillus homohiochii*, *Lactobacillus intestinalis*, *Lactobacillus japonicus*, *Lactobacillus jensenii*, *Lactobacillus johnsonii*, *Lactobacillus kefir*, *Lactobacillus kefiri*, *Lactobacillus kefiranofaciens*, *Lactobacillus kefirgranum*, *Lactobacillus kimchii*, *Lactobacillus kunkeei*, *Lactobacillus leichmannii*, *Lactobacillus letivazi*, *Lactobacillus lindneri*, *Lactobacillus malefermentans*, *Lactobacillus mali*, *Lactobacillus maltaromicus*, *Lactobacillus manihotivorans*, *Lactobacillus mindensis*, *Lactobacillus mucosae*, *Lactobacillus murinus*, *Lactobacillus nagelii*, *Lactobacillus oris*, *Lactobacillus panis*, *Lactobacillus pantheris*, *Lactobacillus parabuchneri*, *Lactobacillus paracasei*, *Lactobacillus paracasei subsp. paracasei*, *Lactobacillus paracasei subsp. tolerans*, *Lactobacillus parakefiri*, *Lactobacillus paralimentarius*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus perolens*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus psittaci*, *Lactobacillus reuteri*, *Lactobacillus*

rhamnosus, *Lactobacillus ruminis*, *Lactobacillus sakei*, *Lactobacillus sakei* L45, *Lactobacillus salivarius*, *Lactobacillus salivarius subsp. salicinius*, *Lactobacillus salivarius subsp. salivarius*, *Lactobacillus sanfranciscensis*, *Lactobacillus sharpeae*, *Lactobacillus sp.* NGRI 0001, *Lactobacillus suebicus*, *Lactobacillus thermotolerans*, *Lactobacillus vaccinostercus*, *Lactobacillus vaginalis*, *Lactobacillus vermiforme*, *Lactobacillus versmoldensis*, *Lactobacillus zeae*, *Lactococcus garvieae*, *Lactococcus lactis*, *Lactococcus lactis subsp. cremoris*, *Lactococcus lactis subsp. hordniae*, *Lactococcus lactis subsp. lactis*, *Lactococcus lactis subsp. lactis bv. diacetylactis*, *Lactococcus piscium*, *Lactococcus plantarum*, *Lactococcus raffinolactis*, *Leuconostoc argentinum*, *Leuconostoc carnosum*, *Leuconostoc citreum*, *Leuconostoc fallax*, *Leuconostoc ficulneum*, *Leuconostoc fructosum*, *Leuconostoc gasicomitatum*, *Leuconostoc gelidum*, *Leuconostoc inhae*, *Leuconostoc kimchii*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Leuconostoc mesenteroides subsp. cremoris*, *Leuconostoc mesenteroides subsp. dextranicum*, *Leuconostoc mesenteroides subsp. mesenteroides*, *Leuconostoc mesenteroides subsp. mesenteroides* ATCC 8293, *Leuconostoc pseudomesenteroides*, *Propionibacterium acidipropionici*, *Propionibacterium acnes*, *Propionibacterium australiense*, *Propionibacterium avidum*, *Propionibacterium cyclohexanicum*, *Propionibacterium freudenreichii*, *Propionibacterium freudenreichii subsp. freudenreichii*, *Propionibacterium freudenreichii subsp. shermanii*, *Propionibacterium granulosum*, *Propionibacterium jensenii*, *Propionibacterium lymphophilum*, *Propionibacterium microaerophilum*, *Propionibacterium propionicum*, *Propionibacterium thoenii*, *Saccharomyces delbrueckii*, *Saccharomyces cerevisiae*, *Saccharomyces unisporus*, *Saccharomyces globosus*, *Saccharomyces carlsbergensis*, *Kluyveromyces fragilis*, *Kluyveromyces bulgaricus*, *Kluyveromyces lactis*, *Torula holmii*, *Candida tenuis*, R2C2, INIX, ES1 and K2.

28. The matrix of claim 1, wherein said microorganism is selected from the group consisting of *L. rhamnosus, L. acidphilus, L. casei, L. lactis, L. plantarum, L. Kefirgranum,* R2C2, INIX, ES1 and K2.

29. The matrix of claim 1, wherein said microorganism is R2C2 under NML accession number 041202-3.

30. The matrix of claim 1, wherein said microorganism is INIX under NML accession number 041202-4.

31. The matrix of claim 1, wherein said microorganism is *L. Kefirgranum.*

32. The matrix of claim 1, wherein said microorganism is *bacillaceae*, *bifidobacteriaceae*, *enterobacteriaceae*, *enterococcaceae*, *lactobacillaceae*; *propionibacteriaceae* and yeast.

33. The matrix of claim 32, wherein said bacillaceae is *Bacillus subtilis.*

34. The matrix of claim 32, wherein said bifidobacteriaceae is one selected from the group consisting of *Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis* and *Bifidobacterium lactis.*

35. The matrix of claim 32, wherein said *enterobacteriaceae* is *Escherichia coli* Nissle 1917.

36. The matrix of claim 32, wherein said *enterococcaceae* is *Enterococcus faecium.*

37. The matrix of claim 32, wherein said *lactobacillaceae* is one selected from the group consisting of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus crispatus*, *Lactobacillus fermentum*, *Lactobacillus johnsonii*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobaillus reuteri*, *Lactobacillus rhamnosus* and *Lactobacillus salivarius.*

38. The matrix of claim 32, wherein said yeast is *saccharomyces cerevisiae boulardii.*

39. A microorganism R2C2 isolated from a consortium obtained from Kefir grain under NML accession number 041202-3.

40. A microorganism K2 isolated from a consortium obtained from Kefir grain under NML accession number 041202-1.

41. A microorganism ES1 isolated from a consortium obtained from Kefir grain under NML accession number 041202-2.

42. A microorganism INIX isolated from ATCC 43761 strain.

43. A process for manufacturing the matrix of claim 1, said process comprising the steps of:

a) fermenting a protein solution with bacteries in a medium;

b) precipitating proteins from the protein solution of step a); and

c) isolating precipitated proteins from supernatant.

44. The process of claim 43, wherein said fermenting step is promoted by co-culturing at least two microorganisms simultaneously or successively.

45. The process of claim 43, wherein said process further comprises a step between steps a) and b) for addition of a polysaccharide.

46. The process of claim 43, wherein said process further comprises a step between steps b) and c) for addition of a polysaccharide.

47. The process of claim 43, further comprising a step of pasteurization of said proteins solution before step a).

48. The process of claim 47, wherein said step of pasteurization is followd by a step of sterilization.

49. The process of any one of claims 43 to 48, wherein precipitation of fermented proteins is effected by at least one method selected from the group consisting of salt addition, pH modulation, thermal treatment, proteolytic enzymes addition and floculent addition.

50. The process of claim 49, wherein said flocculent is a bacterial flocculent.

51. The process of claim 50, wherein said bacterial flocculent is *L. Kefirgranum.*

52. The process of any one of claims 43 to 51, wherein separation of precipitated proteins from supernatant is effected by a method selected from the group of centrifugation and filtration.

53. A composition comprising the matrix of any one of claims 1 to 37 in association with a pharmaceutically acceptable carrier.

54. Use of the matrix of any one of claims 1 to 37, wherein said use is for the manufacture of a product selected from the group of food product, medical product, pharmaceutical product, cosmetic product and nutraceutical.

55. Use of the matrix of any one of claims 1 to 37, wherein said use is for the manufacture of a food product.

56. The use as claimed in claim 55, wherein said matrix is used as an emulsion stabilizer or thickening agent.

57. The use as claimed in any one of claims 55 and 56, wherein said food product is selected from the group consisting of mayonnaise, dressing, margarine, spread, butter, whipped cream and low-fat substitute.

58. The use as claimed in claim 54, wherein said matrix is used as a delivery vehicle.

59. Use of the matrix of one of claims 31 to 37 for the preparation of a probiotic.

60. Use of the matrix of any one of claims 1 to 37, wherein said use is for cosmetic product.

61. The use as claimed in claim 60, wherein said cosmetic product is selected from the group consisting of skin lotion, cream, sunscreen, blush, mascara, eyeshadow, shampoo and conditionner.

62. Use of the matrix of any one of claims 1 to 37 for increasing immune response in a subject.

63. A method of increasing immune response in a subject, comprising administering an effective amount of the matrix of any one of claims 1 to 37 to said subject.

64. Use of the matrix of any one of claims 1 to 37 for reducing triglyceride level in a subject.

65. A method for reducing triglyceride level in a subject, comprising administering an effective amount of the matrix of any one of claims 1 to 37 to said subject.

66. Use of the matrix of any one of claim 1 to 37 for reducing TNF-α level in a subject.

67. A method for reducing TNF-α level in a subject, comprising administering an effective amount of the matrix of any one of claims 1 to 37 to said subject.

68. Use of the matrix of any one of claims 1 to 37 for increasing gluthatione level in a subject.

69. A method for increasing gluthatione level in a subject, comprising administering an effective amount of the matrix of any one of claims 1 to 37 to said subject.

4.3 Discussion et conclusion du chapitre

Cette discussion ne reprendra pas l'ensemble des éléments de discussion présentés dans le manuscrit et le brevet. Les principaux éléments de discussion seront rappelés et complétés. L'objectif est de bien dégager l'essentiel des connaissances et des constats que les travaux réalisés ont permis de mettre en lumière tout en terminant par des ouvertures sur le domaine scientifique traité.

Tel que mentionné précédemment, les MPM allient des avantages fonctionnels à la fois technologiques et biologiques. D'un point de vue technologique, ces matrices protéiques ont une grande capacité à retenir de l'eau tout en permettant l'incorporation de leur équivalent en poids d'huile et ce, en demeurant stables. Pour obtenir des fonctionnalités technologiques et biologiques intéressantes, les protéines du lactosérum doivent être hydrolysées (propriétés hydratantes, émulsifiantes, gélifiantes et santé). L'hydrolyse et la récupération des peptides générés sont des étapes coûteuses. Le procédé de production des MPM permet de réaliser ces étapes directement à partir du lactosérum durant sa fermentation et de récupérer les peptides et protéines par centrifugation comparativement à l'utilisation de concentrés protéiques et de procédé membranaire ou par échange d'ions utilisés par les autres technologies. Les MPM sont ainsi le seul produit partiellement hydrolysé et fermenté, de protéines de lactosérum contenant un haut taux de bactéries lactiques (60X10E10 par 100gr versus 1 à 10 X10E10 pour des produits laitiers fermentés conventionnels).

La structure du brevet permet de protéger le concept en définissant différentes méthodes de récupération, les différentes façons d'influencer l'agglomération, les différentes souches et espèces de bactéries lactiques pouvant être utilisées ainsi que les différentes applications. La caractérisation plus fine des composantes et leur relation avec les effets santé identifiés permettront d'orienter le développement de nouveaux MPM par la modification des conditions de fermentation, par le choix de la souche bactérienne utilisée, par l'ajout d'enzymes protéolytiques, par l'utilisation de différents minéraux ou floculants, etc. Ainsi, la production des MPM doit être vue comme une nouvelle voie technologique permettant la production d'une grande variété d'ingrédients bioactifs de fermentation.

Chapitre 5 : Les souches bactériennes; caractérisation, identification et implications probiotiques.

Contenu du chapitre : manuscrit en préparation et brevet en dépôt international.

Manuscrit en préparation.
Un grand nombre de résultats furent compilés dans le but de préparer une publication sur la description de ces souches particulières de *L. kefiranofaciens*. L'ensemble des résultats ne sera pas nécessairement présenté dans la version finale du manuscrit.

Simard, E., Pilote, D., Précourt, L.P., J., Goyette, P., Dubuc, R., Lemieux, P., Shareck, F., Dupont, C. 2007.* Description de nouvelles souches de *Lactobacillus kefiranofaciens* ; extension des caractéristiques de l'espèce. Journal à définir. Contenu de 27 pages.

Brevet en dépôt international :

Simard, E., Précourt, L.P., Lemieux, P. 2005. International 2006. Use Lactobacillus kefiranofaciens as a probiotic. US60/651 657. 38 pages.

Contributions : J'ai personnellement défini les différents projets de recherche depuis 1995, avec la collaboration de Claude Dupont à l'INRS-Institut Armand-Frappier depuis 1999. J'ai effectué l'isolement des souches bactériennes durant ma maîtrise à partir de grains de kéfir. J'ai effectué la caractérisation morphologique et l'élaboration du profil de fermentation des sucres avec l'implication de stagiaires. Pour ces stages, j'ai participé à l'élaboration des projets, à l'aide technique, à l'encadrement et à l'analyse des résultats. J'ai personnellement compilé et rédigé l'ensemble de l'information présenté dans le but de préparer une publication.

L'isolement de l'ARN 16S a été effectué avec monsieur Roger Dubuc, agent de recherche du laboratoire de Claude Dupont. J'ai effectué la compilation et l'analyse des séquences en fonction de toutes les séquences connues déposées d'ARN 16S de lactobacilles. Ces travaux d'alignement de séquences m'ont permis, par l'analyse de la complexité totale, d'élaborer des amorces spécifiques à l'espèce et de démontrer leur efficacité en collaboration avec trois autres chercheurs : Jean-François Brodeur (stagiaire : participation à l'encadrement), Philippe Goyette (chercheur senior Biolactis) et Louis-Philippe Précourt (étudiant à la maîtrise).

J'ai participé de façon majeure à la rédaction du brevet, à la réalisation complète de la revue des brevets existants et à la revue de littérature nécessaire à la prise du brevet. J'ai été en charge de la révision du brevet pour son dépôt international.

5.1 Manuscrit en préparation.

Simard, E., Pilote, D., Précourt, L.P., J., Goyette, P., Dubuc, R., Lemieux, P., Shareck, F., Dupont, C. 2007.* Description de nouvelles souches de *Lactobacillus kefiranofaciens* ; extension des caractéristiques de l'espèce. Journal à définir. Contenu de 27 pages.

5.1.1 Introduction

En 1967, La Rivière et Kooiman avaient décrit l'espèce bactérienne produisant le kéfiran, les exopolysacharides des grains de kéfir, comme étant *Lactobacillus brevis*. Ce n'est qu'en 1986 que le développement d'un nouveau milieu de culture permit d'étudier plus en détails la composition de la microflore des grains de kéfir (milieu KPL; Toba et al., 1986). Ce milieu de culture très riche fut amélioré en 1987 pour la production de kéfiran (Toba et al., 1987). Les souches de lactobacilles produisant des exopolysaccharides ainsi isolées demandaient tout de même 7 jours d'incubation pour produire des colonies de taille raisonnable (Toba et al., 1986). Cela démontre bien le caractère exigeant de cette espèce bactérienne.

L'espèce fut décrite en 1988. Selon Fujisawa et ses collaborateurs (1988), l'espèce *Lactobacillus kefiranofaciens* peut croître jusqu'à un de pH 3,9 à 3,5. Aucune croissance n'a été observée sous 15 °C et au-dessus de 45 °C. La souche croît sur milieu KPL modifié (pH 5,5) à 30 °C en 10 jours et elle est encapsulée. L'espèce est anaérobie facultative et produit la forme « L » et « D » de l'acide lactique, majoritairement « D ». Elle est aussi catalase négative, n'hydrolyse pas l'esculine et l'amidon. Elle forme un caillé dans le lait. Elle ne produit pas de gaz à partir du glucose. Son profil de fermentation des sucres est positif pour le glucose, fructose, galactose, sucrose, maltose, lactose, melibiose et raffinose, mais négatif pour l'arabinose, xylose, rhamnose, ribose, cellobiose, tréhalose, mélézitose, dextrine, mannitol, sorbitol, esculine, salicine ou amygdaline. Les grains de kéfir constituent l'habitat naturel de l'espèce. L'espèce fut décrite comme étant non cultivable sur milieu MRS (souche WT-2B). Toba et ses collaborateurs ont rapporté une faible croissance après 18 heures dans du lait écrémé et le maintien de la souche en milieu KPL ou MRS en 1991 (souche K1 : Toba et *al.*, 1991).

Kojima et ses collaborateurs (1993) ont rapporté des résultats intéressants sur des nouvelles souches productrices d'exopolysaccharides isolées des grains de kéfir (*Lactobacillus sp.*) qui pourraient croître en milieu MRS. Toutefois, les souches de *L. kefiranofaciens* utilisées à titre de référence n'y présentaient aucune croissance. Le groupe décrit aussi un nouveau milieu plus efficace pour l'isolement de souches productrices d'exopolysaccharides (le milieu RCW).

La définition de l'espèce fut amendée en 2004 par Vancanneyt et ses collaborateurs. Par la même occasion, une espèce rapprochée (*Lactobacillus kefirgranum*) fut reclassée comme une sous-espèce de *L. kefiranofaciens*. Les principales différences évoquées par l'auteur sont le type de colonies, le profil de fermentation des sucres et la présence de flocons en culture. Le milieu de culture utilisé fut le MLR (gélose lait) et le MRSfp (MRS à pH 5,4 ajusté à l'acide acétique). Les colonies de *L. kefiranofaciens subsp kefirgranum* furent blanches, compactes, sèches, mattes et celles de *L. kefiranofaciens subsp kefiranofaciens* furent bosselées et transparentes, vitreuses, convexes et très visqueuses. La formation d'agglomérats en culture liquide fut notée pour *L. kefiranofaciens subsp kefirgranum* et l'hydrolyse de l'esculine, l'utilisation du tréhalose et de la salicine fut différente de l'autre sous-espèce. Sept souches de chaque sous-espèce furent analysées et comparées.

Le présent article présente la description de 4 nouvelles souches de l'espèce dont la souche la plus robuste décrite jusqu'à maintenant. Les difficultés de différentiation des deux sous-espèces seront discutées et un phénotype d'élongation cellulaire est décrit en milieu naturel et *in-vitro*. Ces souches furent isolées de grains de kéfir, dans le but de développer une nouvelle technologie de valorisation du lactosérum.

5.1.2 Matériel et méthodes

5.1.2.1 Souches bactériennes utilisées

Parmi les souches utilisées, 3 souches furent isolées de grains de kéfir en croissance dans du lactosérum depuis plusieurs mois (R2C2, K2 et ES1) et une souche est dérivée de la souche de référence de l'ATCC 43761 (souche INIX). La souche INIX provient d'une culture initiée en milieu KPL (Toba et al., 1986) liquide à partir d'une ampoule de la souche ATCC 43761. Après quatorze jours d'incubation à 37 °C et plusieurs essais de repiquage du tube initial, un tube montrant une faible croissance fut sélectionné. Cette culture fut conservée par repiquage en milieu KPL liquide et lyophilisée. Lors de travaux sur l'utilisation des souches en lactosérum, une différence fut notée entre les colonies d'une même gélose; certaines colonies présentaient un aspect visqueux. Ces colonies furent isolées et repiquées pour donner la souche INIX actuelle.

5.1.2.2 Isolement des souches provenant des grains de kéfir

Des grains de kéfir adapté à la croissance en lactosérum furent fournis par Technologie Biolactis Inc. Les grains furent homogénéisés (5 gr dans 100 ml) dans une solution isotonique de NaCl (0,7%) stérile en utilisant un homogénéisateur de cuisine à la vitesse minimale. La solution fut ensuite répartie sur 100 géloses de milieu KPL (Toba et al., 1986). Les géloses furent incubées à 37 °C en anaérobie (atmosphère de CO_2) durant 14 jours. Une attention particulière fut portée aux colonies blanches-beiges à aspect visqueux. Après vérification microscopique de la morphologie bacille, les colonies sélectionnées furent repiquées sur milieu KPL pour s'assurer de leurs puretés. Le caractère homofermentaire fut vérifié par l'utilisation d'un tube de Durant (Prescott et *al.*, 1995) dans un milieu KPL liquide.

5.1.2.3 Rogosa Cheese Whey (RCW)

Des milieux Rogosa Cheese Whey (RCW), gélosé ou liquides, ont été utilisés pour la culture subséquente des souches (Kojima et al., 1993). Le milieu RCW est préparé à partir du milieu Rogosa SL Broth (Difco # 0478-17-4) pour les milieux liquides et à partir du milieu Rogosa Agar (BDH #1.05413) pour les milieux solides.

Les milieux sont préparés selon les directives du fabricant à l'exception que l'eau distillée est remplacée par du perméat de lactosérum déprotéiné.

Le perméat de lactosérum déprotéiné est reconstitué à partir de poudre de perméat de lactosérum (62,5 g/L) dissout dans de l'eau distillée. Les protéines sont dénaturées thermiquement par un passage à l'autoclave de 10 minutes à 121 °C et retirées par filtration sur une membrane qualitative Whatman n° 1.

5.1.2.4 Lyophilisation et dépôt des souches

Une culture de la souche sur gélose RCW est utilisée pour inoculer 500 ml de milieu liquide RCW. L'incubation est réalisée à 30°C, sans agitation durant 40 heures. Le milieu est ensuite centrifugé durant 15 minutes à 8000 x g à 24 C. Le culot bactérien est suspendu dans une préparation de lait écrémé stérile 10% (p/V) contenant 1,3% (p/v) d'acide ascorbique et 4% (p/v) de sucrose (Champagne, 1991; Couture, et al., 1991). La suspension bactérienne est ensuite distribuée en volume de 0,4 ml dans des ampoules de verre stériles pour la lyophilisation. Les ampoules sont congelées dans un mélange de glace sèche et de méthanol (-80°C). Les ampoules sont lyophilisées durant 24 heures puis scellées sous vide. Elles sont identifiées par le numéro de lot et le nom de la souche pour être finalement conservées à 4°C.

Les souches furent déposées au Bureau de la microbiologie du « International Depositary Authority of Canada (IDAC) », de Santé Canada. Le tableau 17 présente les numéros de dépôt.

Tableau 14 : Numéros de dépôt des souches bactériennes attribués par l'IDAC.

Souches	**Numéro**
K2	041202-1
ES1	041202-2
R2C2	041202-3
INIX	041202-4

5.1.2.5 Profil de fermentation des sucres

Les profils de fermentation des sucres furent effectués en utilisant les galeries API 50 CH selon les indications du fabriquant (bioMérieux REF 50 410 et 50 300). Les résultats présentés sont le consensus de plusieurs essais avec des lectures effectuées à différents temps de fermentation (24, 48 72 et 96 heures). Les résultats furent compilés selon s'il s'agit d'une utilisation forte du sucre (rapide et résultat très claire), une utilisation moyenne (tardive ou moins prononcée, mais évidente) ou encore d'une utilisation faible (tardive et/ou de faible réaction). L'absence de réaction fut notée pour tous les autres cas.

5.1.2.6 Séquençage du gène de l'ARN ribosomal 16S

L'ADN bactérien fut isolé selon Chagnaud (2001). L'amplification universelle, pour l'identification des bactéries, du gène codant pour l'ARN ribosomal 16S des eubactéries est effectuée en utilisant les amorces :

PA : 5' AGA GTT TGA TC(C/A) TGG CTC AG 3' = 20 nt.
PH: 5' AAG GAG GTG ATC CA(G/A) CCG CA 3' = 20 nt.

Ces amorces se positionnent respectivement aux nucléotides 8 à 28 et 1522 à 1542 du gène selon la numérotation du gène de *E. coli* (Edward et al.; 1989). Une vérification de la présence et de la quantité approximative d'ADN du produit de PCR est effectuée en chargeant 10 µl des produits de PCR sur un gel d'agarose 0,8% (p/v). Le gel est incubé dans un tampon TBE + EtBr (100 V, environ 1 heure). La quantité d'ADN est estimée en comparant la bande présente avec le marqueur (5 µl du marqueur 1 Kb+ : Pharmacia) qui contient une bande à 1650 pb à une concentration connue pour une quantité sur le gel de 40 ng d'ADN. La bande observée à 1500 pb est excisée pour être purifiée.

La trousse commerciale QIAEX II est utilisée selon le protocole fourni par la compagnie. Chaque produit purifié est recueilli dans 100 µl de tampon Tris-HCl 10 mM à pH 8.0. Une vérification – quantification approximative est ensuite réalisée sur gel d'agarose dans les mêmes conditions que celles décrites précédemment (figure

19). Le produit est ensuite envoyé pour séquençage à la compagnie Bio S&T inc. (23-03-2002: DNA Sequencing and Oligo Synthesis Center of Bio S&T Inc., 5020 Fairway Street, suite 220, Montréal (Québec) H8T 1B8, Canada, tel: 514-633-6006, www.biost.com).Les gènes furent séquencés à partir des deux extrémités vers leur centre jusqu'à l'obtention de séquences complémentaires entre les deux séquençages.

5.1.2.7 Analyse des séquences

L'analyse des séquences fut effectuée en utilisant les logiciels Clone Manager Suite 7 et Vector NTI 7.0. Cette analyse fut complétée par une comparaison aux banques de données et par la réalisation d'un alignement de toutes les séquences déposées du genre *Lactobacillus.* La banque de données utilisée pour la comparaison des séquences est la banque du National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Le logiciel BLAST est utilisé sur ce site pour produire la comparaison (Nucleotide-nucleotide BLAST (blastn)).

5.1.3 Résultats

5.1.3.1 Comparaison des souches.

Les souches décrites présentent des caractéristiques particulières permettant de les différencier entre elles. Les caractéristiques phénotypiques de ces souches seront décrites par comparaison avec celles de la souche R2C2 qui est la souche la mieux connue. À titre de comparaison, deux souches de référence seront aussi présentées : la souche *L .kefiranofaciens kefiranofaciens* ATCC 43761 et la souche *L. kefiranofaciens kefirgranum* ATCC 51647. Les caractéristiques décrites sont celles généralement observées pour des cultures en milieux RCW ou en lactosérum. Les souches sont des bacilles Gram positif, non-mobiles, ne formant pas de spore, catalase négative et homofermentaire. Elles sont anaérobies facultatives, sans croissance à 15 et 55 C. Les bacilles sont de longueur variable, généralement allongés, plutôt minces et ils peuvent former des chaînes. Le tableau 14 présente les caractéristiques émanant des comparaisons entre elles.

5.1.3.2 Polymorphisme cellulaire.

Lors de l'isolement des souches, il fut possible d'observer la forme allongée des bactéries dans leur milieu naturel (figure 18).

Tableau 15 : Comparaisons variées des différentes souches de l'espèce *L. kefiranofaciens*.

Souche	Caractéristiques
R2C2	♦ Bacille généralement court, épaisseur moyenne, ne produisant pas ou peu d'exopolysaccharides (figure 15). ♦ La longueur et l'épaisseur des bacilles varient beaucoup selon le milieu de culture utilisé et le milieu de conservation avant repiquage (figure 16). ♦ Colonies typiques : blanches au centre beige, convexes, au dessus bosselé, lisse et brillant (figure 17). ♦ Souche très robuste; bonne croissance dans des milieux pauvres (par exemple en lactosérum non supplémenté). ♦ Souche sélectionnée pour la production industrielle des MPM. ♦ Présente très rarement des agglomérats de petites tailles en culture (environ 10 bactéries et moins). ♦ Participe très peu à la formation des agglomérats de protéines lors de la production des MPM.
INIX	♦ Bacilles plus longs et plus minces. ♦ Colonies d'apparence visqueuse. ♦ Souche peu robuste, croissance très faible en milieux pauvres. ♦ Produit des EPS en grande quantité en milieu RCW. ♦ Présente à l'occasion des agglomérats de petite et moyenne taille (environ 10 à 50 bactéries). ♦ Les cellules se retrouvent entrappées dans la matrice protéique lors de la production de MPM.
ES1	♦ Similaire à la souche R2C2, mais présente une moins bonne croissance en milieux pauvres.
K2	♦ Bacilles de longueur moyenne, légèrement plus épais que R2C2. ♦ Colonies typiques semblables à R2C2. ♦ Produit pas ou peu d'exopolysaccharides. ♦ Croissance en milieu pauvre, mais atteint la phase stationnaire à un niveau inférieur aux autres souches pour tous les milieux de culture (RCW : 5 à 7 X 10^8 bactéries comparativement à 1,5 à 3 X 10^9 pour R2C2 et ES1). ♦ Présente des agglomérats de taille moyenne (environ 50 à 100 bactéries). ♦ Jamais été utilisé pour la production de MPM.
ATCC 43761	♦ Apparence similaire à INIX, mais croissance plus difficile. ♦ Faible production d'exopolysaccharides. ♦ Colonies similaires à R2C2.
ATCC 51647	♦ Bacilles de longueur moyenne, plus épais que toutes les autres souches, ne produisant pas ou peu d'exopolysaccharides. Surface des cellules irrégulière. ♦ Colonies blanches, convexes, au dessus plus uniforme, mais présentant de fins points blancs en surface. ♦ Croissance dans des milieux pauvres, mais fortement réduite en présence de forte concentration de calcium (1% $CaCl_2$ p/v). ♦ Présente toujours des agglomérats de grande taille contenant aussi des protéines précipitées (plus de 100 bactéries). ♦ Participe toujours à la formation des agglomérats avec les protéines lors de la production des MPM.

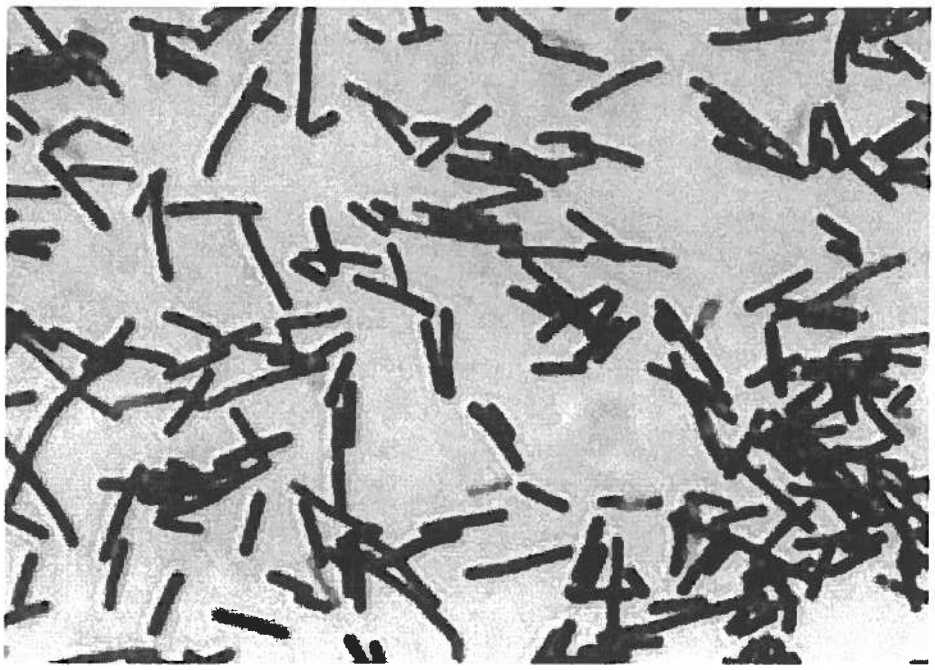
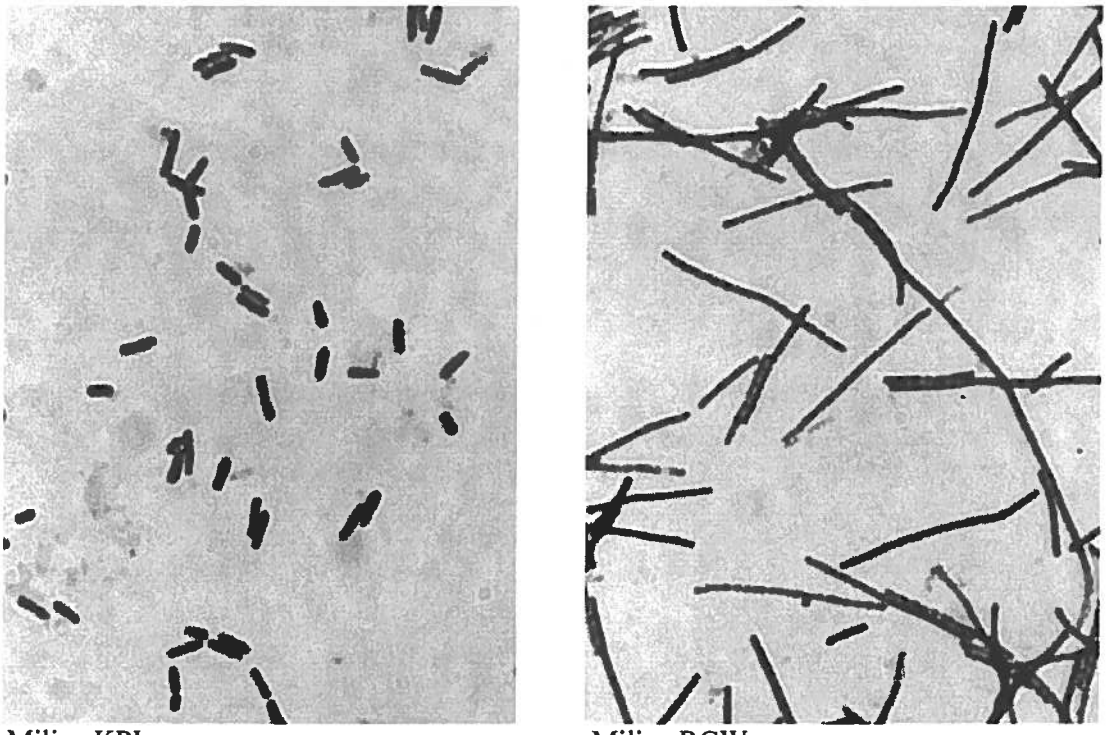


Figure 15 : Apparence typique de la souche R2C2 en coloration de Gram (400X).



Milieu KPL Milieu RCW

Figure 16 : Apparence de la souche R2C2 en coloration de Gram lors de repiquages à partir de géloses KPL sur différents milieux (400X).

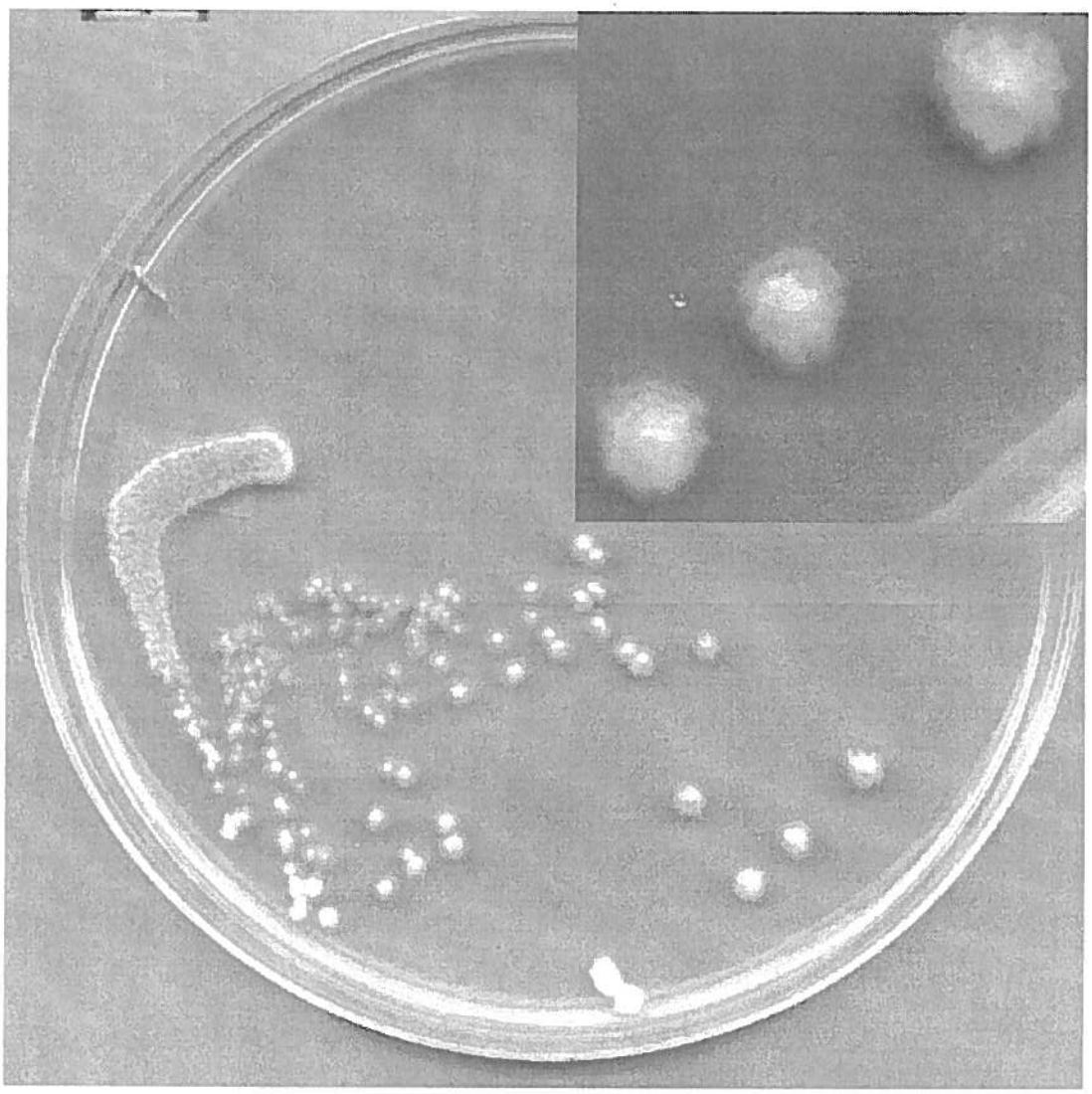


Figure 17 : Apparence des colonies de la souche R2C2 sur milieu RCW (1 et 4X).

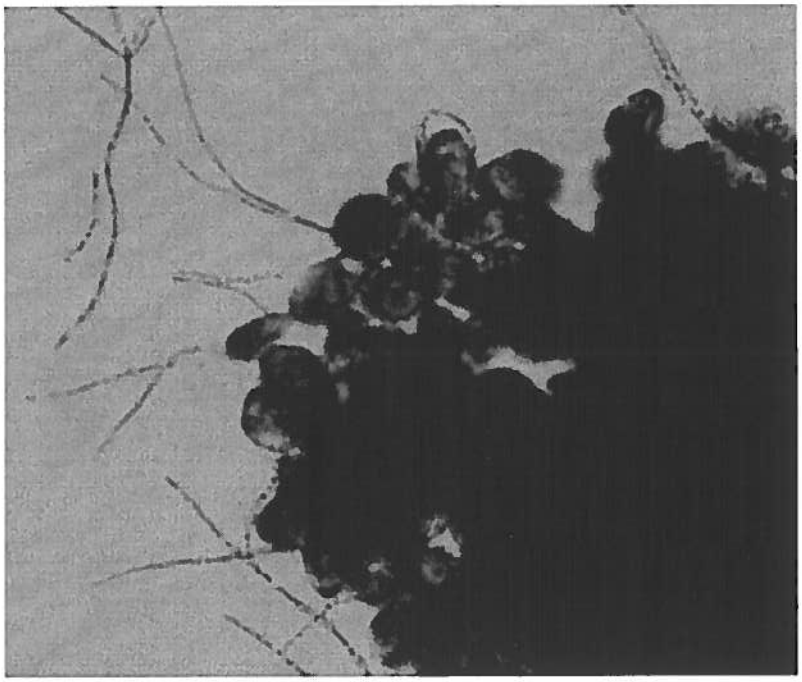


Figure 18 : Type long des cellules bactériennes observé en association avec les grains de kéfir (400X).

5.1.3.3 Limite et optimum de croissance de la souche R2C2

Une grande expérience de culture fut acquise avec la souche *L. kefiranofaciens* R2C2. Cette souche fut le sujet d'un mémoire de maîtrise en microbiologie appliquée de l'INRS-Institut Armand-Frappier en 2003 : Optimisation des conditions de fermentation de la souche ES R2C2 pour la valorisation du lactosérum (Pilote, 2002). Durant ces travaux de maîtrise et les différentes productions industrielles effectuées, il fut possible de déterminer les limites de croissance de cette souche. Le tableau 15 présente les limites de pH et de température avec la valeur optimale.

Tableau 16 : Limites de croissance en fonction de la température et du pH pour la souche *L. kefiranofaciens* R2C2.

Paramètres	Limites inférieures	Optimums	Limites supérieures
Température (C)	22	39	49
pH	3.3	5.0	6.4

Les recherches ont montré qu'aux valeurs limites de température la croissance de la souche est très faible ou nulle. La limite inférieure de pH de croissance de la souche a été déterminée en culture sans contrôle du pH, où le pH devient le facteur limitant avant l'épuisement des éléments nutritifs. Une culture de la souche R2C2, à pH non contrôlé, peut atteindre un pH de 3,3 si aucun facteur ne devient limitant. Les cultures industrielles ont aussi démontré la capacité de croître dans une solution concentrée à 13% de solides (lactosérum dont la charge en solide fut concentrée deux fois par osmose inverse) où la concentration en lactose était d'environ 10%. La croissance fut nulle dans une solution de lactosérum possédant trois fois la charge normale en solide. Cette souche semble aussi démontrer une préférence pour un pH de culture qui fluctue, oscillant entre des valeurs de 4 à 5,5.

5.1.3.4 Les agglomérats en culture

La formation des agglomérats de bactéries en culture liquide est une caractéristique discriminante des souches de *L. kefiranofaciens* peu décrite dans la littérature. La figure 19 présente cette caractéristique pour quatre des souches comparées précédemment. Les travaux effectués ont démontré la dissolution totale des agglomérats de bactéries en culture liquide lorsque chauffés à 55 C. Cela permet un décompte précis de la quantité de bactéries par cytométrie.

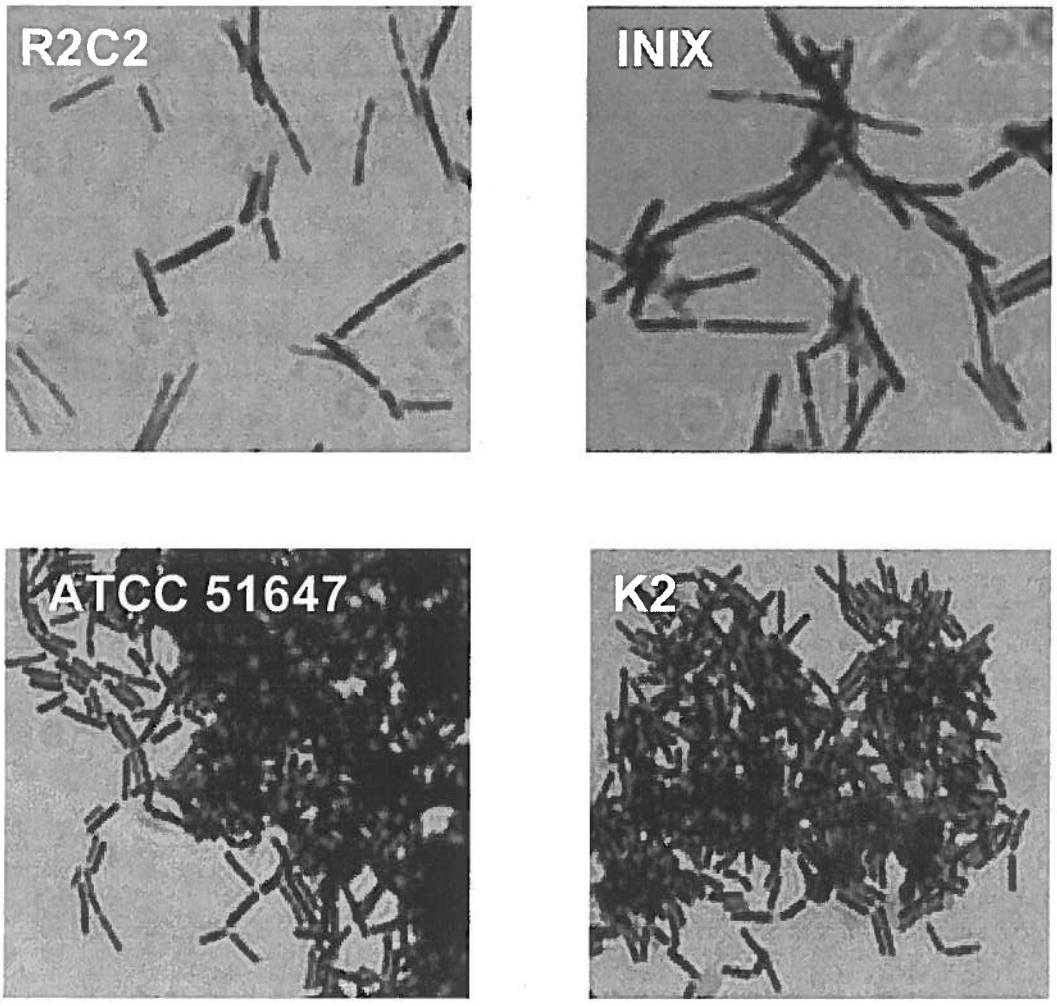


Figure 19 : Apparence des agglomérats bactériens de différentes souches de *L. kefiranofaciens* en culture liquide RCW (coloration de Gram R2C2 et INIX à 400X, ATCC 51647 et K2 à 200X).

5.1.3.5 Profil de fermentation des sucres

Ces résultats sont le consensus de plusieurs essais avec des lectures effectuées à différents temps de fermentation (24, 48 72 et 96 heures). Le nombre d'essais est indiqué et une couleur noire indique une forte utilisation du sucre, une couleur grise foncée une utilisation moyenne et une couleur grise pâle, une utilisation faible. Les cases vides représentent l'absence de réaction. Les résultats obtenus sont comparés à ceux de Fujisawa et *al.*, 1988, selon les critères de différentiation des sous-espèces de Vancanneyt et *al.*, 2004.

5.1.3.6 Séquençage et analyse du gène de l'ARN 16S

La figure 20 présente la bande observée à 1500 pb et excisée pour être purifiée et séquencée pour les souches *L. kefiranofaciens* R2C2 et *L. kefiranofaciens* INIX.

5.1.3.7 Analyse des séquences

Les résultats de l'analyse des gènes séquencés sont pratiquement identiques pour les 6 souches (figure 21) et donne 99% d'homologie pour quatre séquences déposées de l'espèce *L. kefiranofaciens sp.*, deux de L. *kefiranofaciens subsp. kefirgranum* et une de *L. kefiranofaciens subsp. kefiranofaciens.* L'homologie chute ensuite à 97% pour une espèce non identifiée isolée de la flore buccale humaine et quinze souches de l'espèce *L. crispatus*. Les autres espèces apparentées sont dans l'ordre d'importance de l'homologie : *L. kitasatonis*, *L. ultunensis*, *L. amylovorus*, *L. acidophilus*, *L. hamsteri*, *L. amyloliticus* et *L. kalixensis*.

Les résultats ont montré une homologie de séquence de 99,8% des souches entre elles. L'analyse confirme que pour toutes les souches il s'agit de *Lactobacillus kefiranofaciens*. L'espèce *Lactobacillus kefirgranum* fut récemment reclassée comme appartenant à l'espèce *L. kefiranofaciens* à titre de sous-espèce (Vancanneyt et *al.*, 2004).

Tableau 17 : Profil de fermentation des sucres (galerie API 50CH) : compilation des données expérimentales et théoriques. Éléments de différenciation encadrés (Vancanneyt et *al.*, 2004).

Substrats	R2C2	INIX	K2	ES1	ATCC 43761	Fujisawa et al.,1988.	Vancanneyt et al., 2004. Kefirrano.	Vancanneyt et al., 2004. Kefirgra.
Nombre d'essais	4	2	3	3	3			
Glycérol							-	-
Erythritol							-	-
D-Arabinose						-	-	-
L-Arabinose							-	-
Ribose							-	-
D-Xylose						-	-	-
L-Xylose							-	-
Adonitol							-	-
β-Methyl xyloside							-	-
Galactose						+	+	+
D-Glucose						+	+	+
D-Fructose						+	+	+
D-Mannose						+/-	-	-
L-Sorbose							-	-
Rhamnose						-	-	-
Dulcitol							-	-
Inositol							-	-
Mannitol						-	-	-
Sorbitol						-	-	-
α-Methyl-D-Mannoside							-	-
α-Methyl-D-Glucoside							-	-
N-acetyl glucosamine							+/-	+/-
Amygdaline						-	-	+/-
Arbutine							-	+/-
Esculine						-	-	+
Salicine						-	-	++/-
Cellobiose						-	-	+/-
Maltose						+	+/-	+
Lactose						+	+	+
Melibiose						+	+/-	+
Surose						+	+	++/-
Trehalose						-	-	++/-
Inuline							-	+/-
Melezitose						-	-	-
D-Raffinose						+	+/-	++/-
Amidon						-	-	-
Glycogene							-	-
Xylitol							-	-
β-Gentibiose							-	+/-
D-Turanose							-	+/-
D-Lyxose							-	-
D-Tagarose							-	-
D-Fucose							-	-
L-Fucose							-	-
D-Arabitol							-	-
L-Arabitol							-	-
Gluconate							-	-
2-ceto-gluconate							-	-
5-ceto-gluconate							-	-

forte moyenne faible

+/- = souches dependant
++/- = presque toutes les souches

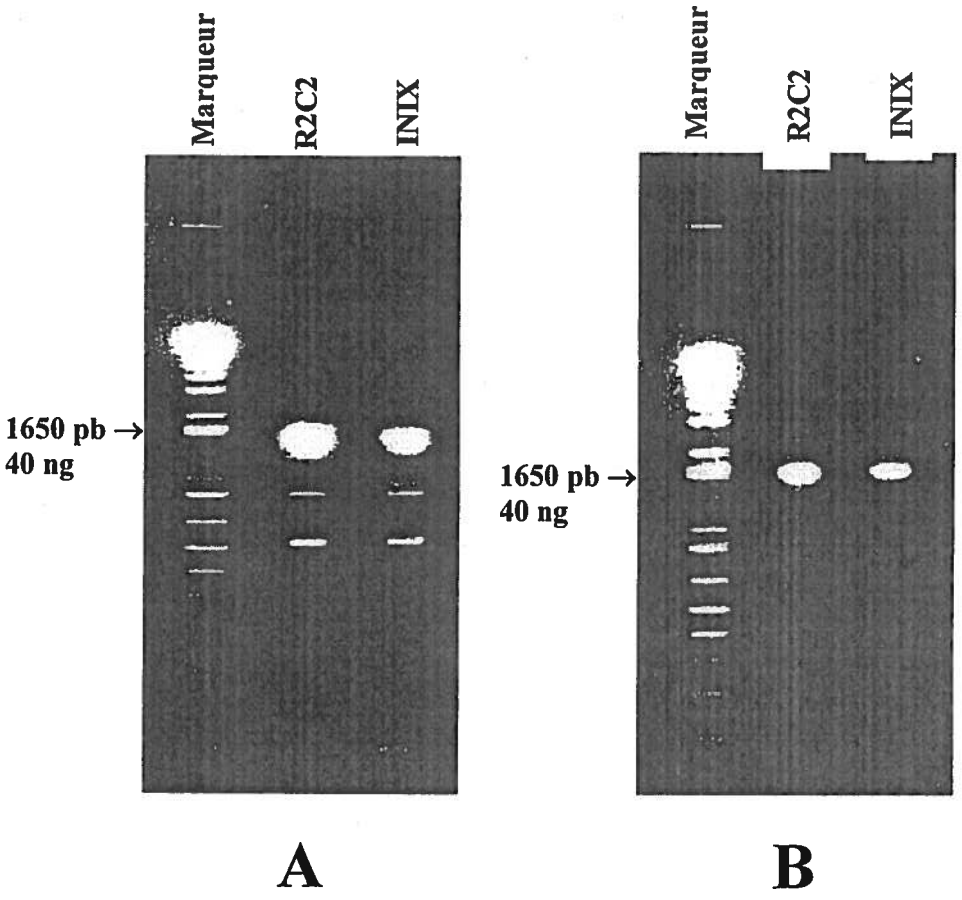
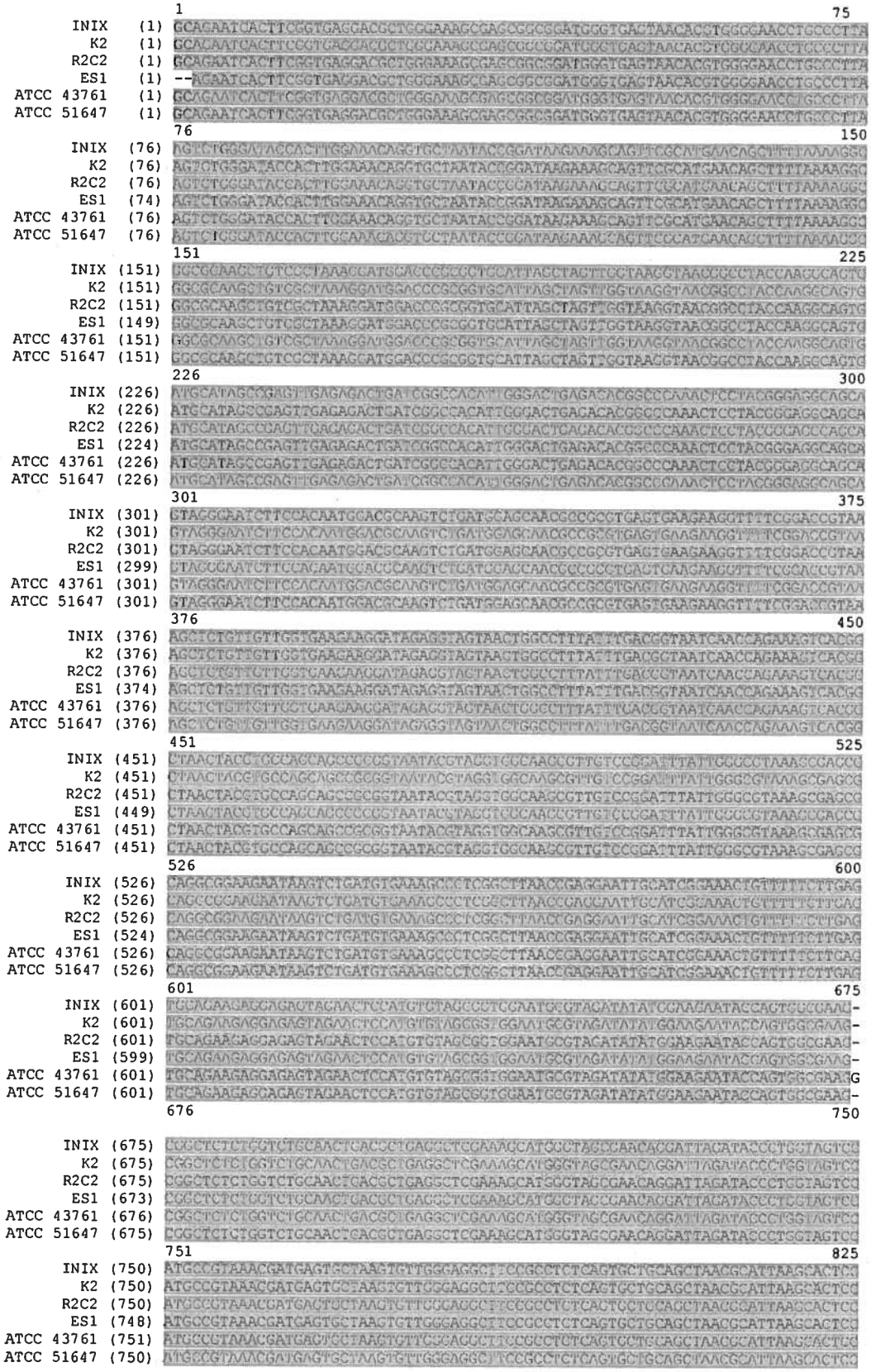


Figure 20 : Purification du produit de PCR codant pour le gène de l'ARN 16S de la souche R2C2 et INIX. Observation du produit de PCR pour l'excision (A), vérification de la pureté et quantification pour le séquençage (B).


1 75
INIX (1)
K2 (1)
R2C2 (1)
ES1 (1)
ATCC 43761 (1)
ATCC 51647 (1)
76 150
INIX (76)
K2 (76)
R2C2 (76)
ES1 (74)
ATCC 43761 (76)
ATCC 51647 (76)
151 225
INIX (151)
K2 (151)
R2C2 (151)
ES1 (149)
ATCC 43761 (151)
ATCC 51647 (151)
226 300
INIX (226)
K2 (226)
R2C2 (226)
ES1 (224)
ATCC 43761 (226)
ATCC 51647 (226)
301 375
INIX (301)
K2 (301)
R2C2 (301)
ES1 (299)
ATCC 43761 (301)
ATCC 51647 (301)
376 450
INIX (376)
K2 (376)
R2C2 (376)
ES1 (374)
ATCC 43761 (376)
ATCC 51647 (376)
451 525
INIX (451)
K2 (451)
R2C2 (451)
ES1 (449)
ATCC 43761 (451)
ATCC 51647 (451)
526 600
INIX (526)
K2 (526)
R2C2 (526)
ES1 (524)
ATCC 43761 (526)
ATCC 51647 (526)
601 675
INIX (601)
K2 (601)
R2C2 (601)
ES1 (599)
ATCC 43761 (601)
ATCC 51647 (601)
676 750
INIX (675)
K2 (675)
R2C2 (675)
ES1 (673)
ATCC 43761 (676)
ATCC 51647 (675)
751 825
INIX (750)
K2 (750)
R2C2 (750)
ES1 (748)
ATCC 43761 (751)
ATCC 51647 (750)

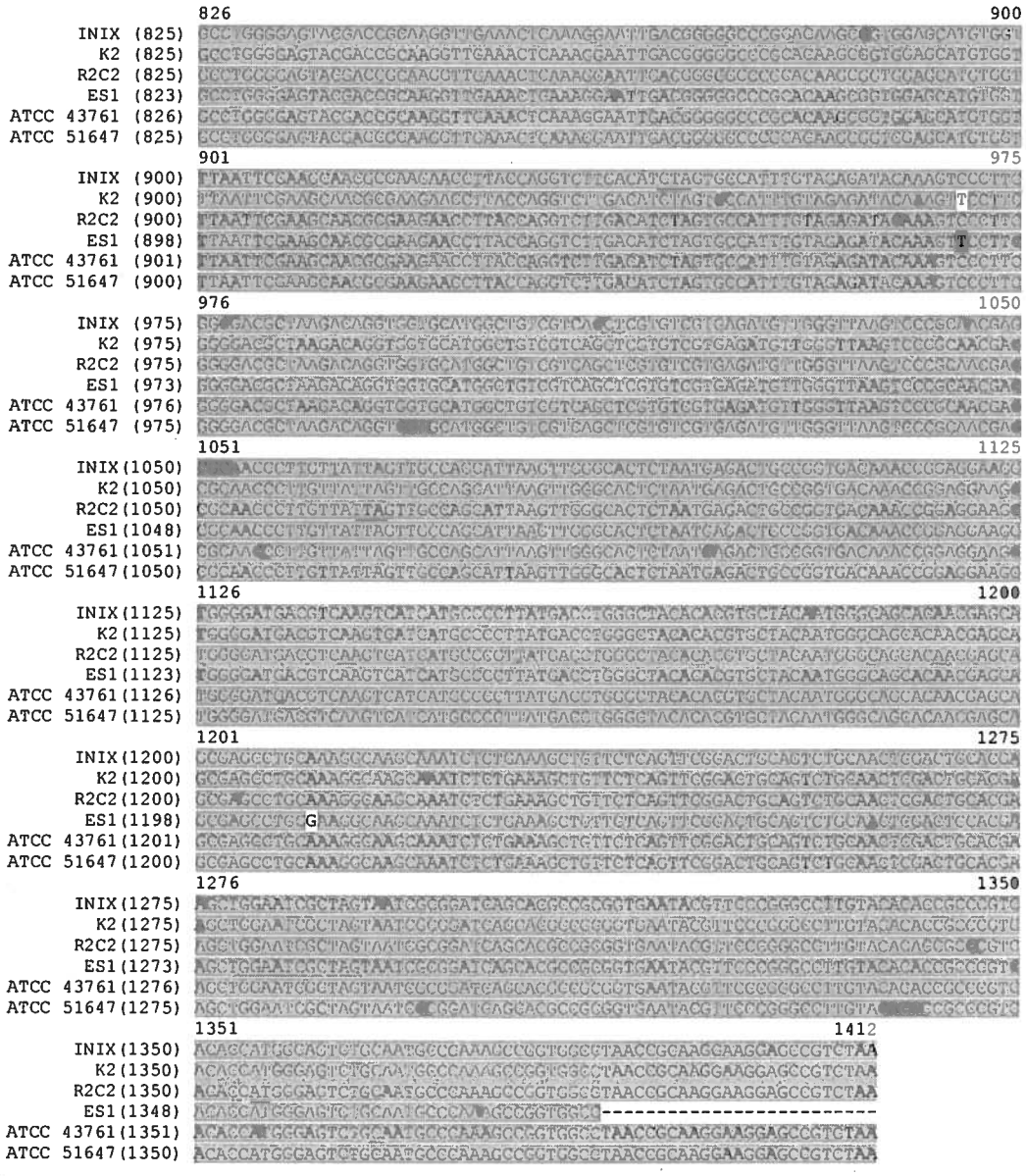


Figure 21 : Séquence du gène de l'ARN 16S de six souches bactériennes.

L'alignement des séquences déposées du genre *Lactobacillus* fut réalisé avec le logiciel Vector NTI suite 7 selon les paramètres par défaut et un arbre phylogénétique peut être visualisé sur la base des résultats de l'alignement (non présenté). Cet arbre phylogénétique ne permet pas d'analyses fines dues à l'absence d'analyses statistiques des jonctions. Toutefois, pour des séquences présentant beaucoup d'homologie, cette analyse permet de regrouper les espèces apparentées en grand groupe. Il est possible d'y distinguer les principaux groupes des *Lactobacillus* : *acidophilus*, *casei* et *fermentum*. L'espèce *L. kefiranofaciens* est classée à l'intérieur du groupe *acidophilus* avec une grande proximité avec les espèces *L. acidophilus*, *L. amyloliticus*, *L. amylovorus*, *L. crispatus*, *L. gallinarum*, *L. hamsteri*, *L. helveticus*, *L. intestinalis*, *L. kalixensis*, *L. kitasatonis* et *L. ultunensis* (en ordre alphabétique). Il s'agit de résultats en accord avec les résultats de la comparaison des séquences du gène de l'ARN 16S à la banque de données NCBI (www.ncbi.nlm.nih.gov).

5.1.3.8 Élaboration des amorces spécifiques à l'espèce

L'objectif était d'amplifier une section du gène de l'ARN 16S qui serait unique à l'espèce *L. kefiranofaciens*. L'utilisation d'amorces spécifique permet de différencier l'espèce d'autres espèces de lactobacilles afin de détecter sa présence dans des produits finis, dans des tissus, dans les selles d'animaux gavés, etc. Pour ce faire, l'alignement des séquences du gène de l'ARN 16S de différentes espèces de lactobacilles discutées auparavant fut utilisé. Par l'analyse de la complexité globale de l'alignement, il fut possible d'identifier deux régions pour lesquelles aucune autre espèce ne présentait le même nucléotide terminal, en position 3'de la séquence de l'amorce, pour les deux amorces suivantes.

Séquence des amorces spécifiques pour *L. kefiranofaciens* :
R2C2-16SF : 5' TAAGAAAGCAGTTCGCATGAACAG 3'
R2C2-16SR : 5' GGGACTTTGTATCTCTACAAATGG 3'

Afin de démontrer la spécificité des amorces, l'ADN de 8 souches de lactobacilles, provenant de 6 espèces différentes a été amplifié avec les amorces R2C2-16SF et R2C2-16SR. Seul l'ADN provenant de la souche R2C2 a donné une amplification significative et spécifique (figure 22). Pour confirmer cette spécificité, un essai *in-vivo* fut effectué (figure 23). Pour cet essai, des selles d'animaux gavés

pendant 14 jours aux bactéries R2C2 (culture en MRS pour 10^9 bactéries viables / ml) et aux MPM (R2C2), de même que d'un groupe contrôle (gavé à l'eau), ont été collectés au moment de la fin des gavages (jour 1) et 3 jours plus tard (jour 3) pour deux souris par groupe. L'ADN présent dans les selles fut isolé pour fin de détection de R2C2 par PCR. Les résultats démontrent l'absence de la bactérie dans les selles du groupe contrôle, et sa présence dans les selles des groupes gavés avec la bactérie R2C2 et aux MPM à la fin des gavages. Trois jours suivant l'arrêt des gavages, la bactérie est indétectable dans les groupes R2C2 et MPM.

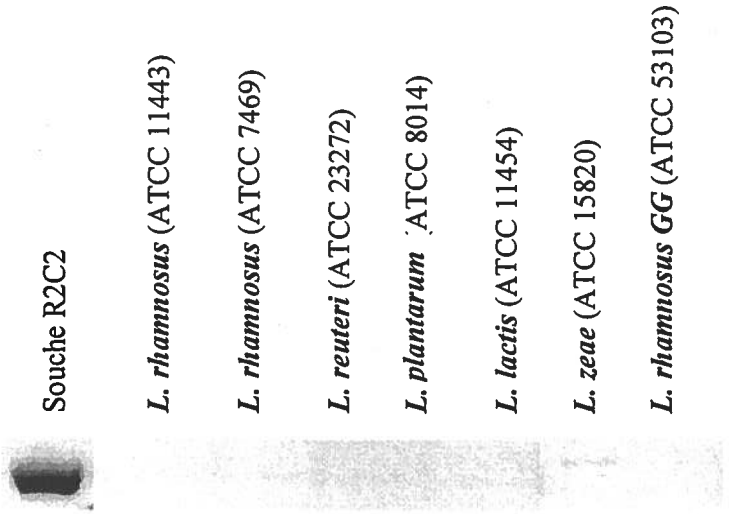


Figure 22 : Vérification de la spécificité des amorces développées pour l'espèce *Lactobacillus kefiranofaciens*.

Résultats de 10 µl des produits d'amplification sur un gel d'agarose 0,8% (p/v) visualité par coloration au bromure d'éthidium.

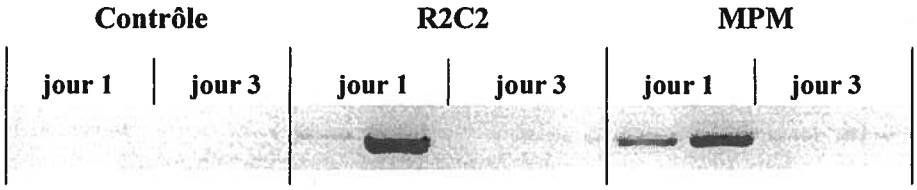


Figure 23 : Vérification *in-vivo*, dans des selles d'animaux gavés, de la spécificité des amorces développées pour l'espèce *Lactobacillus kefiranofaciens*.

Résultats de 10 µl des produits d'amplification sur un gel d'agarose 0,8% (p/v) visualité par coloration au bromure d'éthidium.

5.1.4 Discussion et conclusion

La fermentation joue un rôle primordial dans la production des MPM au niveau de l'hydrolyse protéique, du goût du produit, de ses effets santé et de ses propriétés technologiques. Tel que mentionné en avant propos (page XIII), cette thèse est la prolongation, par un passage directe au doctorat, de travaux ayant portés sur la croissance des grains de kéfir en lactosérum. La stabilité du procédé et la conservation du produit était alors problématique. Il fut décidé de développer une nouvelle version de la technologie en utilisant des souches bactériennes pures. L'hypothèse élaborée pour y arriver fut que « les microorganismes responsables de la bonne récupération des protéines peuvent être isolés du consortium et utilisés de façon séparée ». Étant donné la corrélation positive entre la croissance des grains et la récupération des protéines du lactosérum, une attention particulière fut portée aux bactéries principalement responsables de la croissance des grains : *Lactobacillus kefiranofaciens*. Quatre souches furent isolées des grains en croissance.

La vérification de l'appartenance à cette espèce fut effectuée par le séquençage du gène de l'ARN ribosomal 16S. Les résultats ont montré une homologie de séquence de 99,8% des souches entre elles et avec la souche de référence de l'ATCC. Selon Stackebrandt et Gobel (1994), une homologie de séquence de plus de 98% constitue une probabilité très élevée qu'il s'agisse de la même espèce. Le séquençage du gène et l'analyse des séquences du groupe Lactobacillus a aussi permis d'identifier deux séquences permettant la préparation d'amorces spécifiques à l'espèce. La démonstration effectuée in-vivo permet d'apprécier la présence de l'espèce lors des gavages et sont absence dans le groupe témoin ou lors de l'arrêt des gavages. Les amorces développées sont donc spécifiques à l'espèce *L. kefiranofaciens* pour l'ensemble de la flore intestinale.

Les caractéristiques phénotypiques des souches peuvent nous renseigner sur leur association avec une des deux sous-espèces tel que proposé par Vancanneyt et ses collaborateurs (2004). Premièrement, la description des colonies de *L. kefiranofaciens subsp kefirgranum* correspond aux colonies observées pour les souches R2C2, ES1 et

K2. Les colonies de la souche INIX peuvent être associées à *L. kefiranofaciens subsp kefiranofaciens.*

Le profil de fermentation des sucres donne des résultats dans le même sens (tableau 17). L'utilisation de l'esculine devrait être réservée à la sous-espèce *kefirgranum*, ce qui est en accord avec la description des colonies à l'exception de la souche de référence 43761 qui est de la sous-espèce *kefiranofaciens*.

Le fait que la souche K2 présente des agglomérats en culture et qu'elle fermente les sucres cellobiose, β-gentibiose et amygdaline semble confirmer qu'il s'agit de la sous-espèce *kefirgranum*. Pour R2C2 et ES1, la fermentation du tréhalose favorise aussi l'association de ces souches à la sous-espèce *kefirgranum*. Toutefois, la fermentation du tréhalose par INIX remet en question l'utilisation de ce sucre pour différencier les sous-espèces.

Rappelons qu'INIX produit de grandes quantités d'exopolysaccharides. Aussi, cette souche s'est montrée faiblement positive à la salicine et ce, pour toutes les lectures (huit lectures à quatre temps différents) des deux essais effectués (résultats non présentés). On pourrait s'interroger sur les méthodes utilisées, mais les résultats de Vancanneyt et ses collaborateurs (2004) ont aussi été obtenus avec les galeries API 50CH utilisées selon les instructions du fabricant. L'utilisation du mannose et la non utilisation du mélibiose furent rapportées pour certaines souches par Yokoi et ses collaborateurs (1991). Finalement, l'utilisation du mannitol et la non-utilisation du galactose sont des résultats particuliers à notre étude. Le profil de fermentation des sucres est un outil peu discriminant pour différencier les deux sous-espèces. Étant donné la forte homologie relevée par l'hybridation de l'ADN pour l'ensemble des souches étudiées par Vancanneyt et ses collaborateurs (2004; 79 à 91%), on peut se questionner sur la pertinence de maintenir ces deux sous-espèces séparées.

Notons aussi, que l'apparence des bacilles, des colonies et des cultures liquides des souches R2C2 et de ES1 sont similaire à celle de la souche de référence de l'ATCC de la sous-espèce *kefiranofaciens*. Si l'on considère la production de kéfiran, il fut observé lors de l'isolement des souches que l'apparence visqueuse des colonies et des cultures liquides de R2C2 et ES1 fut perdue après les premiers repiquages.

Cette observation est aussi rapportée dans un brevet américain (No. 5 204 247 : Adachi, S. et *al.*, 1993) où l'auteur, qui a participé à la description initiale de l'espèce un an avant le dépôt de ce brevet, mentionne que la productivité en exopolysaccharides diminue suite aux repiquages ou à la conservation de la souche isolée des grains. L'incapacité de produire l'exopolysaccharide ne peut donc pas être considérée comme une caractéristique de la sous-espèce *kefirgranum* même si la capacité à le produire est associée à la sous-espèce *kefiranofaciens*.

Pour ce qui est de la formation des agglomérats en culture, ils ont été observés de façon plus marquée pour la souche K2 et la souche de référence *kefirgranum* de l'ATCC 15 647. Toutefois, on les observe aussi dans certaines conditions de culture pour la souche R2C2 et INIX. Les travaux d'optimisation des conditions de croissance de la souche R2C2, ainsi que les fermentations industrielles, ont démontré que dans certaines conditions de culture, les bactéries en solution apparaissent majoritairement sous forme d'agglomérats. On peut facilement proposer que cette particularité soit reliée à la production d'un exopolysaccharide de surface étant donné qu'il est possible de dissoudre ces agglomérats en chauffant la solution à 55 C durant 20 à 30 minutes.

Cette caractéristique pourrait être reliée à la capacité d'adhérence des bactéries à l'épithélium intestinal. Les travaux de Santos et ses collaborateurs (2003) soulignent la capacité d'adhésion de la souche *L. kefiranofaciens* CYC 10058 qui produit des exopolysaccharides. Il est généralement accepté que la capacité d'adhésion des souches dépends de facteurs protéiques et polysaccharidiques dont leur rôle relatif à la capacité d'adhésion est variable selon l'espèce et la souche (Greene et Kleanhammer; 1994).

Ainsi, il est possible de proposer l'hypothèse que cette espèce ne produirait pas un, mais au moins deux types d'exopolysaccharides. Le kefiran serait responsable de l'augmentation de la viscosité des solutions et de la formation de la majeure partie des grains de kéfir et le second polymère, produit en faible quantité, serait responsable de la formation d'agglomérats bactériens. Farnworth (2005) mentionne la possibilité que les grains de kéfir soient aussi composés d'autres types d'exopolysaccharides. La capacité de produire ces polymères en différentes quantités serait une particularité propre à une souche donnée et elle varierait en fonction d'évènements particuliers

comme le retrait des bactéries de leur consortium original ou une mutation spontanée permettant une plus forte production (INIX). Une autre souche bactérienne surproductrice d'exopolysaccharide fut déjà isolée d'une souche de l'ATCC. Cette souche, nommé *L. rhamnosus* 9595M, est parmi les souches connues produisant les plus grandes quantités d'EPS (Dupont et *al.*, 2000).

Au niveau de la longueur des bacilles, ce phénomène est probablement dû à une modification de la division cellulaire. Il fut démontré que la présence de calcium peut augmenter le taux de division cellulaire et influencer la longueur des bacilles en culture (Wright et Klaenhammer, 1981). Ce phénomène est fortement dépendant de la souche utilisée et pour une même espèce, il est possible d'observer deux types de colonies associées aux types long ou court des bacilles (Barber et Frazier, 1945). Cette particularité fut observée pour la souche R2C2. Barber et Frazier (1945) ont démontré que le stress induit par la température, les ultraviolets ou les rayons X peut induire la formation de cellules bactériennes plus longues.

Pour la souche R2C2, il fut noté que le repiquage d'une colonie du type long en milieu KPL liquide peut produire uniquement le type long (figure 16). Ainsi, il ne s'agit pas simplement d'une modification de la division cellulaire induite par un stress, mais d'un état cellulaire pouvant être conservé lorsqu'il est induit. Neysens et ses collaborateurs (2003) ont décrit une croissance biphasique pour une souche bactérienne d'une espèce apparentée du groupe *acidophilus* (*L. amylovorus*). Pour cette espèce, la croissance biphasique est associée à une augmentation de la production d'une bactériocine et à une élongation des cellules qui deviennent aussi plus sensibles à la concentration en NaCl. Whitley et Marshall (1999) ont décrit que cet état cellulaire peut être conservé au repiquage pour cette souche et que les deux types cellulaires peuvent présenter des profils de fermentation des sucres différents. Il s'agirait donc d'un ensemble de régulation des gènes exprimés qui serait modifié par cet état cellulaire.

Récemment, Alterman et ses collaborateurs (2004) ont décrit une nouvelle protéine impliquée dans la division cellulaire chez *L. acidophilus*. Les mutants négatifs de cette protéine présentent des cellules allongées très similaires à celles observées pour R2C2. Il est intéressant de constater que ce mutant démontre un taux

adhésion grandement diminué par rapport à la souche sauvage (*L. acidophilus* NCFM : 17,19%). Tel que proposé aussi par Buck et ses collaborateurs (2005), cette diminution d'adhésion serait causée par une perte d'organisation de l'intégrité cellulaire. En comparant l'ARN messager des deux types bactériens en culture, l'affichage différentiel de l'expression des gènes pourrait permettre de mettre en lumière les gènes impliqués dans ce processus pour *L. kefiranofaciens* R2C2.

La nouvelle protéine identifiée par Alterman et ses collaborateurs (2004) ne fut pas retrouvée dans les génomes des bactéries apparentées que sont *L. gasserie et L. johnsonii.* Étant donné que ces deux espèces font partit du type "B" du groupe *acidophilus* et que *L. kefiranofaciens*, *L. amylovorus* et *L. acidophilus* font partit du type "A" (Stiles et *al.*, 1997), il serait intéressant de tenter de vérifier la présence de cette protéine par PCR dans *L. kefiranofaciens.* La présence de cette protéine pourrait expliquer ce phénomène d'élongation cellulaire et être particulier à cet important groupe probiotique.

Pour ce qui est des conditions de croissance, Adachi et ses collaborateurs (1993) décrivent un optimum de croissance pour la souche *L. kefiranofaciens* KPB167, à 30 °C et des limites de culture de 20 et 35 °C. L'optimum de pH est de 5,5 à 6 et la limite de croissance est de 5 et 7,5. Ainsi, les caractéristiques de croissance de la souche R2C2 en font une souche très particulière et la souche la plus robuste jamais décrite.

Pour ce qui est de l'identification, étant donné la difficulté d'effectuer une identification sûre à partir des caractéristiques phénotypiques ou du profil de fermentation des sucres, le séquençage du gène de l'ARN 16S est nécessaire pour cette espèce. Les résultats obtenus sont parfaitement en accord avec ceux publiés (Vancanneyt et *al.*, 2004). Il est aussi important de souligner que malgré l'identification récente de caractères probiotiques reliés à cette espèce (Santos et *al.*, 2003; Manville et *al.*, 2005), les travaux réalisés par notre groupe sont les premiers à confirmer *in-vivo* des caractéristiques probiotiques. Un brevet fut ainsi déposé pour protéger cette utilisation probiotique (prochain point de ce chapitre).

En conclusion, la description de nouvelles souches de cette espèce permet d'élargir la définition du groupe en y ajoutant des caractéristiques permettant de mieux les discriminer et éventuellement peut-être, d'associer ces caractéristiques à des fonctionnalités technologiques ou biologiques importantes. Par exemple, la résistance à des pH très faibles pourrait être une caractéristique des souches les plus bénéfiques pour la santé intestinale (R2C2). Des travaux supplémentaires seraient nécessaires pour la discrimination des sous-espèces pour les souches R2C2 et ES1. La souche K2 semble appartenir à la sous-espèce *kefirgranum* et la souche INIX à la sous-espèce *kefiranofaciens* étant donné la production du kéfiran.

Pour le profil de fermentation des sucres, il serait nécessaire de vérifier si la forme longue ou courte des cellules peut influencer les résultats et expliquer les différences observées pour l'utilisation du galactose et du mannitol. Ce phénomène de différenciation cellulaire pourrait être relié à une multitude de régulation génétique pouvant aussi influencer les effets santé des souches étudiées. Notons que le type long des bactéries fut observé dans leur milieu naturel lors de l'isolement des souches (figure 18). Pour ce qui est de la formation d'agrégats en culture liquide, une meilleure compréhension de leur composition et de leur formation pourrait permettre des utilisations industrielles particulières tel que proposé par Bergmaier (2002) pour la souche *L. rhamnosus* 9595M. Il est certain que la découverte d'effets bénéfiques pour la santé de ce groupe bactérien permettra d'effectuer des associations entre les caractéristiques phénotypiques et génétiques des souches et les effets santé bénéfiques recherchés.

5.2 Brevet en dépôt international.

Simard, E., Précourt, L.P., Lemieux, P. 2005. Use Lactobacillus kefiranofaciens as a probiotic. US60/651 657. 59 pages.

Ce brevet fut déposé le 11 février 2005 en dépôt provisoire au Etats-Unis et le 11 février 2006 pour son dépôt international.

J'ai participé de façon majeure à la rédaction du brevet, à la réalisation complète de la revue des brevets existants et à la revue de littérature nécessaire à la prise du brevet. J'ai été en charge de la révision du brevet pour son dépôt international.

USE OF *LACTOBACILLUS KEFIRANOFACIENS* AS A PROBIOTIC AND A SYNBIOTIC

ABSTRACT

In accordance with the present invention, there is provided a probiotic composition comprising an effective amount of *Lactobacillus kefiranofaciens* in association with a suitable carrier. The probiotic composition has many probiotic effects such as intestinal adherence, intestinal persistence, positive modulation of the intestinal microflora, protection against intestinal pathogens, immunomodulation, protection against systemic inflammation, protection against intestinal inflammation, protection against allergies, protection against diarrhea, protection against diabetes, protection against hyperlipidemia and protection against colon cancer. In accordance with the present invention, there is also provided a method of treatment and/or prevention of a number of diseases for which the composition has a beneficial effect. For example, the composition is useful for fermenting a product which in turn finds utility in the treatment of hypertension, the treatment of weight disorder, the treatment of hyperlipidemia or the treatment of triglyceride disorder.

USE OF *LACTOBACILLUS KEFIRANOFACIENS* AS A PROBIOTIC AND A SYNBIOTIC

BACKGROUND OF THE INVENTION

(a) Field of the Invention

[0001] This invention relates to the use of *Lactobacillus kefiranofaciens* as a probiotic having effects on intestinal health, modulation of immunity, obesity-associated problem such as control of blood lipid levels, hypertension and body weight and protection against tumors.

(b) Description of Prior Art

[0002] The traditional definition of a probiotic microorganism requires that the bacteria (and its components) be non-toxic, survive through gastric/intestinal environments, adhere/persist in the gastro-intestinal tract, exist as part of the normal human microflora and exert health benefits. However, recent evidence has shown that some probiotic effects can be obtained from lactobacilli of non-human origin and that probiotic effects can be obtained with *lactobacillus* that were heat or radiation inactivated, and in some case from bacterial lysates (U.S. Pat. No 4,347,240). The health benefits observed from probiotic lactobacilli are often strain specific and can vary greatly. Some of the effects described for probiotic lactobacilli include: modulation of intestinal microflora, competition with and elimination of pathogenic microorganisms, modulation of immune function, control of allergies, promotion of gastrointestinal health, regulation of blood lipid levels, control of diabetes (regulation of glucose and insulin in blood), protection against colon cancer and control of body weight.

[0003] Kefir has been used to ferment milk for centuries. Kefir grains are composed of Gram-positive hetero- and homofermentive lactic acid bacteria. Gram-negative acetic acid bacteria, and lactose fermenting and non-fermenting yeasts, held together by kefiran, a biopolymer of the exopolysaccharide family secreted by *Lactobacillus kefiranofaciens* sub-specie (subsp.) *kefiranofaciens* bacteria. Lactobacilli of the *L. kefiranofaciens* species are homofermentive lactobacilli and represent the major bacterial population of kefir grains. While originally classified as 2

independent species, *Lactobacillus kefiranofaciens* and *Lactobacillus kefirgranum* were recently re-classified as sub-species of the *L. kefiranofaciens* species based on their identical 16S rRNA sequences (Vancanneyt et *al.*, *Int J Syst Evol Microbiol.* 54(Pt 2):551-556, 2004). The classification to the two subspecies is done on the basis of morphology on agar plates and in liquid culture, on acid production from different sugars, and on protein profiling from PAGE. Strains from the subspecies *kefiranofaciens* are usually high producers of kefiran, which is essential in the composition and formation of kefir grains. Kefiran production from *kefiranofaciens* strains is recognizable from the colony morphology (showing glossy or slimy appearance) on agar plates, while strains from the subspecies *kefirgranum* (showing dry and compact colonies) do not produce significant levels of kefiran. However, kefiran production from *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* has been shown to be very sensitive to subculturing. *Kefirgranum* strains can produce acid from threalose, while *kefiranofaciens* strains cannot. In addition, strains from the *kefirgranum* sub-species are flocculent and sediment in liquid cultures.

[0004] The fact that no toxicity has been associated with kefir over the years is a strong argument to the safety and non-toxicity of lactobacillus strains isolated from it. The species *Lactobacillus kefiranofaciens* has been classified in the *L. acidophilus* group phylogenically close to *L. crispatus* and *L. acidophilus* species, which contain several well described probiotic strains.

[0005] Santos et al. (*System. Appl. Microbiol.*, 26:434-437, 2003) describe four strains of *Lactobacillus kefiranofaciens* which show resistance to acid and bile, and different adhesion and antimicrobial properties.

[0006] U.S. Patent No 4,347,240 describes the isolation of a novel strain of *lactobacillus* KPB-176 of undefined strain classification (*Lactobacillus kefiranofaciens* subsp. *kefiranofaciens*) from kefir grains, which produces large quantities of kefiran, does not possess strict selectivity for specific media and involves no reduction in the productivity of polysaccharides even during subculture.

SUMMARY OF THE INVENTION

[0007] In accordance with the present invention there is provided a probiotic composition comprising an effective amount of *Lactobacillus kefiranofaciens* in association with a suitable carrier. The *Lactobacillus kefiranofaciens* may be for example selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. kefiranofaciens and *Lactobacillus kefiranofaciens* subsp. *kefirgranum*.

[0008] In one embodiment of the invention, the *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2 (IDAC accession number 041202-3), INIX (IDAC accession number 041202-4), K2 (IDAC accession number 041202-1); ES1 (IDAC accession number 041202-2) and BioSp strain from Technologie Biolactis inc.

[0009] The probiotic effect may be for example selected from the group consisting of intestinal adherence, intestinal persistence, positive modulation of the intestinal microflora, protection against intestinal pathogens, immunomodulation, protection against systemic inflammation, protection against intestinal inflammation, protection against allergies, protection against diarrhea, protection against diabetes, protection against hyperlipidemia and protection against colon cancer. The composition may be suitably formulated for oral, rectal or vaginal administration.

[0010] Still in accordance with the present invention, there is also provided a method for providing positive modulation of the intestinal microflora in a subject comprising the step of administering to said subject an effective amount of such *Lactobacillus kefiranofaciens*.

[0011] Also in accordance with the present invention, there is provided a method for protecting a subject against intestinal inflammation comprising the step of administering to said subject an effective amount of *Lactobacillus kefiranofaciens*. Such intestinal inflammation can be caused for example by an inflammatory bowel disease (IBD), Crohn's disease (CD), ulcerative colitis (UC) or by an irritable bowel syndrome (IBS).

[0012] Further in accordance with the present invention, there is provided a method for protecting a subject against allergies and/or

autoimmune diseases comprising the step of administering to said subject an effective amount of such *Lactobacillus kefiranofaciens*.

[0013] Also in accordance with the present invention, there is also provided a method for protecting a subject against diarrhea comprising the step of administering to said subject an effective amount of such *Lactobacillus kefiranofaciens*.

[0014] According to the present invention, there is still provided a method for protecting a subject against diabetes comprising the step of administering to said subject an effective amount of such *Lactobacillus kefiranofaciens*.

[0015] There is also provided in accordance to the present invention a method for protecting a subject against hyperlipidemia comprising the step of administering to said subject an effective amount of such *Lactobacillus kefiranofaciens*.

[0016] Still in accordance with the present invention, there is also provided a method for protecting a subject against colon cancer comprising the step of administering to said subject an effective amount of such *Lactobacillus kefiranofaciens*.

[0017] Also in accordance with the present invention, there is provided a method for treating and/or preventing against intestinal inflammation in a subject comprising the step of administering to said subject an effective amount of *Lactobacillus kefiranofaciens*. Such intestinal inflammation can be caused for example by an inflammatory bowel disease (IBD), Crohn's disease (CD), ulcerative colitis (UC) or by an irritable bowel syndrome (IBS).

[0018] Further in accordance with the present invention, there is provided a method for treating and/or preventing against allergies and/or autoimmune diseases in a subject comprising the step of administering to said subject an effective amount of such *Lactobacillus kefiranofaciens*.

[0019] Also in accordance with the present invention, there is also provided a method treating and/or preventing against diarrhea in a subject comprising the step of administering to said subject an effective amount of such *Lactobacillus kefiranofaciens*.

[0020] According to the present invention, there is still provided a method treating and/or preventing against diabetes in a subject comprising the step of administering to said subject an effective amount of such *Lactobacillus kefiranofaciens*.

[0021] There is also provided in accordance to the present invention a method for treating and/or preventing against hyperlipidemia in a subject comprising the step of administering to said subject an effective amount of such *Lactobacillus kefiranofaciens*.

[0022] Still in accordance with the present invention, there is also provided a method for protecting a subject against colon cancer comprising the step of administering to said subject an effective amount of such *Lactobacillus kefiranofaciens*.

[0023] The present invention further provides for the use of such *Lactobacillus kefiranofaciens* as a probiotic compound. The probiotic compound may have a probiotic effect selected from the group consisting of intestinal adherence, intestinal persistence, positive modulation of the intestinal microflora, protection against systemic inflammation, protection against intestinal pathogens, immunomodulation, protection against intestinal inflammation, protection against allergies, protection against diarrhea, protection against diabetes, protection against hyperlipidemia and protection against colon cancer. The use is suitable for oral administration.

[0024] In one embodiment, the *Lactobacillus kefiranofaciens* is administered in a form selected from the group consisting of a live bacterial population, a lyophilized bacterial population, as a fermented dairy product and as a non-viable bacterial sample, such as a heat-killed bacteria, an irradiated bacteria or a lysed bacteria.

[0025] One embodiment of the present invention further provides for the use of such *Lactobacillus kefiranofaciens* as a probiotic compound having an anti-inflammatory effect.

[0026] One embodiment provides for the use of such *Lactobacillus kefiranofaciens* as a probiotic compound for treating psoriasis.

[0027] In accordance with the present invention, there is also provided the use of *Lactobacillus kefiranofaciens* in association with an anti-

inflammatory compound, wherein the inflammatory compound is 5-ASA or a corticosteroid.

[0028] The present invention further provides for the use of *Lactobacillus kefiranofaciens* as a probiotic compound for the manufacture of a medicament for treating psoriasis.

[0029] In one embodiment, the *Lactobacillus kefiranofaciens* is used to ferment whey, wherein said whey is cheese whey.

[0030] In accordance with the present invention, there is also provided the use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* for the treatment of a cardiovascular disease.

[0031] In accordance with the present invention, there is also provided the use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* for the treatment of hypertension.

[0032] The present invention further provides for the use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* for the treatment of weight disorder.

[0033] In accordance with the present invention, there is also provided the use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* for the treatment of hyperlipidemia

[0034] The present invention further provides for the use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* for the treatment of triglyceride disorder.

[0035] In accordance with the present invention, there is also provided the use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* in the manufacture of a medicament for the treatment of cardiovascular disease.

[0036] In accordance with the present invention, there is also provided the use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* in the manufacture of a medicament for the treatment of hypertension.

[0037] The present invention further provides for the use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* in the manufacture of a medicament for the treatment of weight disorder.

[0038] The present invention further provides for the use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* in the manufacture of a medicament for the treatment of hyperlipidemia.

[0039] In accordance with the present invention, there is also provided the use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* in the manufacture of a medicament for the treatment of triglyceride disorder.

[0040] The present invention further provides for the use of such *Lactobacillus kefiranofaciens* as a probiotic compound. The probiotic compound may be used for treating metabolic syndrome which is associated with 5 problems which are hypertension, low HDL, fat in belly, insulin resistance and high level of triglyceride. Thus, the probiotic compound of the present invention can be used for treating and/or preventing the problems associated with metabolic syndrome.

[0041] The present invention further provides for the use of such *Lactobacillus kefiranofaciens* as a probiotic compound for controlling weight management.

DETAILED DESCRIPTION OF THE INVENTION

[0042] In accordance with the present invention, there is provided probiotic lactobacilli with various applications in foodstuffs and in medicine. More specifically, the invention relates to a probiotic species *Lactobacillus kefiranofaciens* (including *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp, *Kefirgranum*), defined by the 4 strains described below, which have shown significant probiotic potential following *per os* treatment in animal models. Among these probiotic effects it was observed intestinal adherence, positive modulation of the intestinal microflora, immunomodulation, allergies, diarrhea, weight management, hypertension and hyperlipidemia. The different strains isolated from this species have shown both common and strain specific beneficial health effects. They can be administered orally

either as live or lyophilized bacterial population, as a fermented dairy product (milk or whey based) or as non-viable bacterial samples (heat-killed, irradiated or lysed bacteria).

[0043] The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

Example 1

Identification of five probiotic strains of *lactobacillus* from kefir grains

[0044] Three of the strains described in the present application, R2C2 (IDAC accession number 041202-3), K2 (IDAC accession number 041202-1) and ES1 (IDAC accession number 041202-2) were isolated from kefir grain previously adapted for growth in whey.

[0045] One strain, INIX (IDAC accession number 041202-4) was isolated from a kefiran-overproducing colony on agar plates of the ATCC *kefiranofaciens* reference strain (ATCC accession number 43761).

[0046] BioSp was a *Lactobacillus kefiranofaciens* subsp, *kefirgranum* strain strongly flocculent in liquid cultures from Technologie Biolactis inc.

[0047] Based on their 16S rRNA gene sequences, the strains described in the present application have been assigned to the species *Lactobacillus kefiranofaciens*. Morphological properties in liquid culture and on agar plates and carbohydrate fermentation profile have also permitted a more specific assignment of the strains to either *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* (R2C2, ES1 and INIX) and to *Lactobacillus kefiranofaciens* subsp. *kefirgranum* (K2 and BioSp).

Example 2

In vitro characterization of the *lactobacillus* strains

[0048] R2C2: generally short bacilli, gram positive, single or in chains of 2 to 4, not producing or producing few exopolysaccharides. The length and the width of the bacilli vary much according to the culture medium used and the medium of conservation. On RCW plates, it forms dry and smooth, crystalline looking colonies, white with beige center, convex, with embossed top. This strain has a good growth in poor mediums (for

example in whey not supplemented) and produces agglomerates of small sizes in liquid culture (approximately 10 bacteria and less).

[0049] INIX: elongated, generally longer bacilli than R2C2, gram positive, single or in chains of 2 to 4 bacilli. On RCW plate, it forms slimy or sticky looking colonies, which fuse to neighboring colonies. Kefiran production rates in liquid RCW cultures are high and stable upon subculturing. This strain has a weak growth in poor mediums; typical to *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* strains and produce agglomerates of small sizes in liquid culture (approximately 10 to 50 bacteria).

[0050] ES1: Similar to R2C2 strain, but presents a worse growth in poor mediums.

[0051] K2: Bacilli of average length slightly wither than R2C2, single or in chains of 2 to 4 bacilli. Colonies similar to R2C2. Does not produce or few exopolysaccharides. Growth in poor medium, but reached the stationary phase at level lower than the other strains for all the culture media tested (RCW: 5 to 7 X 10^8 bacteria compared to 1,5 to 3 X 10^9 for R2C2 and ES1). Presents agglomerates of average size in liquid culture (approximately 50 to 100 bacteria).

[0052] BioSP: Bacilli of average length, with irregular cells surface, wither than all other strains, single or in chains of 2 to 4 bacilli, not producing or few exopolysaccharides. Gram positive, formation of large size aggregates of lactobacilli in liquid culture also containing precipitated proteins (more than 100 bacteria). On RCW plates, it forms white colonies, convex, with the more uniform top, but presenting fine white points on the surface. Growth in poor mediums, but strongly reduced in the presence of strong calcium concentration (1% CaCl2 p/v).

Fermentation profiles

[0053] Table 1 provides data about the fermentation profiles of the strains described above.

Table 1

Fermentation results for carbohydrates using API 50CH

Substrates	R2C2	INIX	K2	ES1	TCC 43761
Nomber of tests	4	2	3	3	3
Glycérol					
Erythritol					
D-Arabinose					
L-Arabinose					
Ribose					
D-Xylose					
L-Xylose					
Adonitol					
β-Methyl glycoside					
Galactose					
D-Glucose					
D-Fructose					
D-Mannose					
L-Sorbose					
Rhamnose					
Dulcitol					
Inositol					
Mannitol					
Sorbitol					
α-Methyl-D-Mannoside					
α-Methyl-D-Glucoside					
N-acetyl glucosamnie					
Amygdaline					
Arbutine					
Esculine					
Salicine					
Cellobiose					
Maltose					
Lactose					
Melibiose					
Saccharose					
Trehalose					
Inuline					
Melezitose					
D-Raffinose					
Amidon					
Glycogene					
Xylitol					
β-Gentibiose					
D-Turanose					
D-Lyxose					
D-Tagarose					
D-Fucose					
L-Fucose					
D-Arabitol					
L-Arabitol					
Gluconate					
2-ceto-gluconate					
5-ceto-gluconate					

Strong Moderate Weak

Metabolite fermentation profiles

[0054] Tables 2 to 4 provide data about metabolite fermentation (scale of 1 to 5 defined as 1 being weak to 5 being strong, + = 5 and – means no reaction.

Table 2

Metabolite fermentation for the strain R2C2

	JM 3				JM 19				2 JM 7				JJFT			
Incubation time (h)	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
Glycerol																
Erythritol																
D-Arabinose																
L-Arabinose																
Ribose																
D-Xylose																
L-Xylose																
Adonitol																
β-Methyl glycoside																
Galactose	5	5	5	5												
D-Glucose	5	5	5	5	5	5	5	5	+	+	+	+	4	5	5	
D-Fructose	5	5	5	5	5	5	5	5	+	+	+	+	4	5	5	
D-Mannose	3	5	5	5	-	3	3	3	+	+	+	+	-	-	1	
L-Sorbose																
Rhamnose																
Dulcitol																
Inositol																
Mannitol	4	4	5	5	2	4	5	5	4	4	+	+	1	4	5	
Sorbitol																
α-Methyl-D-Mannoside																
α-Methyl-D-Glucoside														1	-	-
N-acetyl glucosamine	4	5	5	5					+	+	+	+	0	4	5	
Amygdaline																
Arbutine					-	-	1	2	1	3	4	+				
Esculine	1	4	4	4	3	4	4	4	3	4	+	+	4	5	5	
Salicine	1	1	1	2	-	3	3	3	1	4	+	+	-	-	2	
Cellobiose																
Maltose	3	4	5	5	2	4	5	5	+	+	+	+	1	3	3	
Lactose	5	5	5	5	5	5	5	5	+	+	+	+	2	5	5	
Melbiose									-	-	1	3	-	-	2	
Saccharose	4	5	5	5	-	1	2	1	3	+	+	+	1	5	5	
Trehalose	5	5	5	5	-	3	5	5	4	+	+	+	-	5	5	
Inuline																
Melezitose																
D-Raffinose	3	5	5	5	-	1	2	1	-	3	+	+	-	2	2	
Amidon																
Glycogene																
Xylitol																
β-																

	JM 3				JM 19				2 JM 7				JJFT			
Incubation time (h)	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
Gentibiose																
D-Turanose																
D-Lyxose																
D-Tagarose																
D-Fucose																
D-Arabitol																
L-Arabitol																
Gluconate																
2-ceto-gluconate																
5-ceto-gluconate																

Table 3

Metabolite formation for the strain INIX

	JM 35				JM 18			
Incubation time (h)	24	48	72	96	24	48	72	96
Glycerol								
Erythritol								
D-Arabinose								
L-Arabinose								
Ribose								
D-Xylose								
L-Xylose								
Adonitol								
β-Methyl glycoside								
Galactose								
D-Glucose	5	5	5	5	5	5	5	5
D-Fructose	5	5	5	5	5	5	5	5
D-Mannose	4	4	5	5	2	5	5	5
L-Sorbose								
Rhamnose								
Dulcitol								
Inositol								
Mannitol	4	4	5	5	1	3	5	5
Sorbitol								
α-Methyl-D-Mannoside								
α-Methyl-D-Glucoside								
N-acetyl glucosamine	1	3	5	5	-	1	1	1
Amygdaline								
Arbutine					1	1	2	2
Esculine	2	4	4	4	4	4	4	5
Salicine	1	3	3	3	1	2	3	4
Cellobiose								
Maltose	3	4	5	5	2	4	5	5
Lactose	5	5	5	5	5	5	5	5
Melbiose								
Saccharose	3	4	5	5	-	1	3	3

	JM 35				JM 18			
Incubation time (h)	24	48	72	96	24	48	72	96
Trehalose	5	5	5	5	1-	5	5	5
Inuline								
Melezitose								
D-Raffinose	1	3	5	5	-	1	2	2
Amidon								
Glycogene								
Xylitol								
β-Gentibiose								
D-Turanose								
D-Lyxose								
D-Tagarose								
D-Fucose								
D-Arabitol								
L-Arabitol								
Gluconate								
2-ceto-gluconate								
5-ceto-gluconate								

Table 4

Metabolite fermentation for the strain K2

	JM 34				JM 21				2 JM 6			
Incubation time (h)	24	48	72	96	24	48	72	96	24	48	72	96
Glycerol												
Erythritol												
D-Arabinose												
L-Arabinose												
Ribose												
D-Xylose												
L-Xylose												
Adonitol												
β-Methyl glycoside												
Galactose												
D-Glucose	5	5	5	5	5	5	5	5	+	+	+	+
D-Fructose	5	5	5	5	5	5	5	5	+	+	+	+
D-Mannose	4	5	5	5	4	5	5	5	+	+	+	+
L-Sorbose												
Rhamnose												
Dulcitol												
Inositol												
Mannitol												
Sorbitol												
α-Methyl-D-Mannoside												
α-Methyl-D-Glucoside												
N-acetyl glucosamine					3	5	5	5	+	+	+	+
Amygdaline	1	1	-	-	-	-	3	5	1	4	+	+

	JM 34				JM 21				2 JM 6			
Incubation time (h)	24	48	72	96	24	48	72	96	24	48	72	96
Arbutine									1	4	+	+
Esculine	5	5	5	5	5	5	5	5	+	+	+	+
Salicine	4	5	5	5	3	4	5	5	4	+	+	+
Cellobiose	5	5	5	5	5	5	5	5	+	+	+	+
Maltose					-	3	5	5				
Lactose	5	5	5	5	5	5	5	5	4	+	+	+
Melbiose												
Saccharose												
Trehalose												
Inuline												
Melezitose												
D-Raffinose	1	0	-	-	2	4	5	5	-	3	+	+
Amidon												
Glycogene												
Xylitol												
β-Gentibiose	4	5	5	5	4	4	4	4	+	+	+	+
D-Turanose												
D-Lyxose												
D-Tagarose												
D-Fucose												
D-Arabitol												
L-Arabitol												
Gluconate												
2-ceto-gluconate												
5-ceto-gluconate												

Strain classification to *Lactobacillus kefiranofaciens* species:

[0055] Comparison to 16S rRNA sequences is a widely accepted means for classification of *lactobacillus* strains. The 16S gene of the five different lactobacilli strains were amplified by PCR (using forward primer SEQ ID NO:1 and reverse primer SEQ ID NO:2 sequences) and sequenced. The different strains were all phylogenically classified to the species *Lactobacillus kefiranofaciens* through an alignment of the obtained sequences with that of 80 *lactobacillus* 16S sequences available on NCBI and of the reference strain ATCC 43761.

[0056] The corresponding complementary sequences of 16S rRNA sequences of strains R2C2 (SEQ ID NO:3), INIX (SEQ ID NO:4) , BioSP (SEQ ID NO:5), K2 (SEQ ID NO:6) and ES1 (SEQ ID NO:7) are listed in the sequence listing.

Bacterial counting procedures

[0057] The FACS method for evaluating bacterial numbers and survival was used as follows. Before performing a bacterial count using a cytometer, all aggregates have to be dissociated. Lactobacilli strains R2C2 and INIX presented herein have a tendency to form small and unstable aggregates. Dissociation can be easily achieved by resuspending the strains in PBS pyrophosphate (15 mM). Strains BioSP and K2 (particularly BioSP) form large size aggregates that cannot be dissociated using PBS pyrophosphate only. Following resuspension, these two strains must be heated at 55°C for a period of 30 minutes. Then, the strains are exposed for 15 minutes at room temperature to ethidium bromide, which binds to DNA so that each bacteria becomes detectable by cytometry. Series of dilutions (1/10, 1/100, 1/1000) are then performed to make a more precise counting.

[0058] Other methods such as plate counting method and Most Probable Number (series of inoculations with different dilutions that allow counting) can also be used.

Growth characteristics in MRS, RCW and whey

[0059] A preculture of the different strains was prepared in RCW medium at 37°C for 12-24 hours. Cells from fresh exponentially growing cultures were counted and inoculated at a concentration of 10^6 cfu/ml in the different media. Cultures were incubated at 37°C and cells were counted at intervals of 2, 4, 6, 8, 12, 24, 32 and 48 hours using the FACS count methods.

Development of a direct PCR detection method

[0060] A species specific PCR amplification method was developed to allow the detection of *L. kefiranofaciens* in different tissues. The PCR detection test consists in an amplification of the 16S rRNA gene. These primers were designed from unique *L. kefiranofaciens* DNA sequences identified through the results of the alignment of *lactobacillus* 16S sequences with that of our strains and of the reference strain ATCC accession number 43761. The specificity of the primers was tested experimentally against the DNA of 5 different lactobacillus strains. This test

has also been used successfully to detect *L. kefiranofaciens* DNA in experimental samples isolated from feces, colonic content, mucosa and whole colon. These results demonstrated that the primers are highly specific, detecting the presence of *L. kefiranofaciens* DNA throughout the important diversity of bacterial DNA in the intestinal flora.

[0061] *L. kefiranofaciens* specific PCR primer sequences are R2C2-16SF (SEQ ID NO:8) and R2C2-16SR (SEQ ID NO:9).

[0062] The PCR amplification cycling parameters are as follows: 94 degrees from 10 minutes followed by 30 repetitions of 94 degrees for 30 seconds, 69 degrees for 30 seconds, and 72 degrees for 1 minute, and then finishing with a single step of 72 degrees for 10 minutes. PCR products are analyzed by electrophoresis on a 2% agarose gel.

Survival in gastric/intestinal solutions

[0063] The survival and growth of these different *Lactobacillus kefiranofaciens* strains in a low pH and gastric solutions was examined. For the experiments, the different strains were pre-cultured in RCW medium and whey at 37°C for 24 hours, before being submitted to sterile gastric solution. Survival of the different strains under acidic conditions was tested as follows.

a) Survival of strains of lactobacillus in acid solution

[0064] Cells from fresh cultures are counted and then harvested by centrifugation, washed twice in PBS and resuspended to a concentration of 10^7 cfu/ml in MRS broth or whey adjusted to pH 3.5, 3.0, 2.5 and 2.0. Cells are incubated at 37°C and survival measured at intervals of 15, 30, 60 and 120 min. using the FACS and plate count methods.

b) Survival of strains of lactobacillus in human gastric solution

[0065] Cells from fresh cultures in RCW were counted and then harvested by centrifugation, washed twice in PBS and resuspended in human gastric solution to a final concentration of 10^8 cfu/ml. After 30 minutes, 1 hour or 2 hours incubating at 37°C in human gastric solution, cultures were washed twice in PBS and resuspended in RCW broth. Survival was monitored after 24 hours of incubation at 37°C in RCW by spectrometry (640 nm) and compared with the O.D. values of non-treated

cultures. The gastric solution is prepared as follows: 3.2 g/L of pepsine, 2.0 g/L of NaCl and was prepared for the purpose of this experiment at pH 2.0. Survival of the strains was excellent after 30 minutes of incubation in gastric solutions. All the strains seemed to reach the equivalent culture level as the non-treated strains as shown in Table 5.

Table 5

O.D. values for cultures incubated 30 minutes in gastric solution compared with non-treated cultures

		O.D. (640 nm)
R2C2	non-treated	4.63
	30 min.	6.96
INIX	non-treated	3.29
	30 min.	4.13
BioSP	non-treated	2.91
	30 min.	4.94
K2	non-treated	3.30
	30 min	3.45

Bile resistance

[0066] Cells from fresh cultures in RCW were counted and then harvested by centrifugation, washed twice in PBS and resuspended in sterile human intestinal solution to a final concentration of 10^8 cfu/ml. After 30 minutes, 1 hour or 2 hours incubating at 37°C in intestinal solution, cultures were washed twice in PBS and resuspended in RCW broth. Survival was monitored after 24 hours of incubation at 37°C in RCW by spectrometry (640 nm) and compared with the O.D. values of non-treated cultures. The intestinal solution is prepared as follows: 10 g/L of pancreatine, 6.8 g/L of KH_2PO_4, 0.15% of bile salts and was prepared, for the purpose of this experiment, at pH 8.0. Survival of the strains was excellent after 30 minutes of incubation in intestinal solution. All the strains

seemed to reach the equivalent culture level as the non-treated strains as shown in Table 6.

Table 6

O.D. values for cultures incubated 30 minutes in intestinal solution compared with non-treated cultures

		O.D. (640 nm)
R2C2	non-treated	4.63
	30 min.	6.99
INIX	non-treated	3.29
	30 min.	2.87
BioSP	non-treated	2.91
	30 min.	3.99
K2	non-treated	3.30
	30 min.	3.15

In vitro adhesion to CaCo-2/HT-29

[0067] The capacity of the different strains to adhere to intestinal epithelial cells was evaluated. Monolayers of CaCo-2 and HT-29 cells were allowed to grow to confluence and to differentiate for 14 days in 24-well plates. 108 cfu/ml of the different bacteria strains, previously marked with Syto9 dye (BacLight kit), were added to each well (in triplicate) in DMEM (without antibiotics) and incubated for 1 hour at 37°C. Following this, the cells were washed 4 times with PBS, the entire well content harvested by trypsin treatment for 10 minutes, and the number of bacteria per well evaluated by FACS. The strain R2C2 showed good adhesion properties on CaCo-2 cells since 15% of the bacteria were still in the well after the washes. Moreover, adhesion of R2C2 on cells was clearly higher than that of *L.* GG, a probiotic well-known for its good adhesion properties.

Table 7

***In vitro* adhesion of bacteria on human intestinal CaCo-2 cells**

	Percentage of adherent bacteria
R2C2	15%
L. GG	3%

In vitro immunomodulatory potential in co-cultures

[0068] A co-culture system of human intestinal epithelial cells (HT-29) and human PBMC is used as an *in vitro* model to evaluate the immunomodulatory effects of the bacterial strains at the intestinal level. Briefly, human HT-29 epithelial cells are seeded in the upper chamber of a transwell system (at 10^6 cells per ml) and cultured until differentiation occurred (4 weeks in RPMI 1640 changed daily). Human peripheral blood mononuclear cells (PBMCs) are isolated by density gradient centrifugation from fresh human blood and 10^6 cells are added to the lower chamber. The immunomodulatory effects of the different strains are evaluated by addition of 10^6 bacteria in triplicate to either to lower or upper chamber followed by 48 hours incubation. Controls contained media alone. Following this, cell culture supernatants are removed and evaluated for extracellular cytokine levels using standard ELISA kits (R&D systems, Minneapolis, MN USA). Evaluation of cytokine expression levels is determined by RT-PCR of total RNA obtained from pooled triplicate samples of each group.

In vitro immunomodulatory potential of whey fermented with R2C2 on human intestinal cells

[0069] A culture system of human intestinal epithelial cells (HT-29) is used as an *in vitro* model to evaluate the immunomodulatory effects of whey fermented with R2C2. Human HT-29 epithelial cells (at 10^6 cells per ml) are cultured until differentiation occurred (4 weeks in RPMI 1640 changed daily). The immunomodulatory effect of the different strains is evaluated by addition of MPM (malleable protein matrice) in various concentrations followed by 48 hours incubation. Controls contained media alone. For determination of the anti-inflammatory potential of MPM on human intestinal cells,

lipopolysaccharides (LPS) were added in the culture media to induce production of inflammatory cytokines. Following these different protocols, cell culture supernatants were removed and evaluated for extracellular cytokine levels using standard ELISA kits (R&D systems). Evaluation of cytokine expression levels was determined by RT-PCR of total RNA obtained from pooled triplicate samples of each group. Cells exposed to LPS and various concentrations of MPM showed a clear reduction of TNFα expression. Results are shown in Table 8.

Table 8

***In vitro* immunomodulation of MPM on HT-29 cells exposed to LPS**

	Relative expression of TNFα
Controls	1,0
Controls+LPS	4,2
MPM 1/100+LPS	1,3
MPM 1/1000+LPS	2,1
MPM 1/10000+LPS	2,3

Example 3

In vivo characterization of the *lactobacillus* strains and probiotic potential

L. kefiranofaciens adhesion *in vivo*

[0070] *In vivo* adhesion and persistence of the different strains was examined. In this model, C57BL/6 mice were treated by gavage (p.o.) with 10^8 cfu/ml of the different strains for 7 days. Colon samples were then collected from 3 mice of each treatment group on day 1, day 3 and day 10 following the end of gavages to evaluate the persistence of these bacteria in the colonic tissue. The samples were opened longitudinally, colonic content collected by rinsing with saline and mucosal layer collected by scraping. Feces were also collected on the last day of gavages to evaluate the presence of the strains in treated animals. The presence of the different bacteria was evaluated by PCR, using the species specific primers

developed and described above, in the feces, colonic content and mucosal samples.

Modulation of intestinal microflora in mice

[0071] The capacity of the different strains to modulate the intestinal microflora was evaluated. C57BL/6 mice were treated by gavage (p.o.) with 10^8 cfu/ml of the different strains for 7 days. Fecal samples were then collected from each group to evaluate the levels of coliforms, lactic acid bacteria (LAB) and fecal pH was also measured. The feces are mechanically disrupted in saline and the presence of coliforms is evaluated with Perifilm Coliform Count plates™ (3M). Levels of LAB were evaluated using Petrifilm Total Aerobic Count plates, incubated anaerobically with MRS broth. All four strains tested showed a 3 to 4-fold reduction of coliforms levels in fecal samples. The strains showing the most important and constant effect are R2C2 and BioSP as shown in Table 9. Moreover, strain R2C2 showed the capacity to slightly increase LAB counts while reducing fecal pH. These effects suggest that R2C2 can possibly adhere in the intestinal tract of the animals.

Table 9

Modulation of intestinal microflora in mice

	Coliforms/mg of fece	LAB/mg of feces	Fecal pH
Controls	625.24	$9.5x10^6$	7.32
R2C2	193.17	$1.4x10^7$	7.18
INIX	258.64	n/d	n/d
BioSP	160.71	n/d	n/d
K2	244.19	n/d	n/d

Immunomodulatory potential: Modulation of leukocytes populations

[0072] The effect of the strains to modify leukocytes cell populations was tested. C57BL/6 mice were treated by gavage (p.o.) with 10^8 cfu/ml of the different strains for 7 days. At the beginning and end of the treatment

period, blood samples were collected and analyzed by flow cytometry for evaluation of leukocyte populations. The different strains showed variable effects on the leukocyte populations. The four strains seemed to modulate the mice immune system, boosting total lymphocytes and leukocytes numbers. However, only the strain R2C2 has the capacity to increase the number of polymorphonuclear (PMN) cells and only BioSP can slightly boost monocytes as shown in Table 10.

Table 10

Immunomodulation: Mice leukocyte populations following a 7-day treatment (# of cells per 20 ul of blood)

	Lymphocytes	Monocytes	PMN	Total leukocytes
Controls	1553.25	205.25	209.00	1967.50
R2C2	2175.00	236.20	244.60	2655.80
INIX	2512.80	245.40	227.20	2985.40
BioSP	2520.75	281.50	230.75	3033.00
K2	2083.75	194.25	205.50	2483.50

L. kefiranofaciens strains in the prevention/treatment of intestinal inflammation

[0073] The potential of *L. kefiranofaciens* strains to prevent and reduce symptoms of intestinal inflammation was evaluated in the DSS-induced mouse model.

a) Prevention of intestinal inflammation

[0074] C57BL/6 mice were treated by gavage (p.o.) with 10^8 cfu/ml of the different stains once per day for 7 days, before the induction of inflammation with DSS, and then until the end of the experiment. Intestinal inflammation is induced, on day 8, by the addition of DSS (2.5%) in the drinking water for 7 days. The level and progression of inflammation was evaluated through measurements of weight loss, diarrhea, occult blood, hematocrits, and colon lengths (post-mortem). The different treatment

groups showed varying degrees of effects on the different parameters followed. Strains R2C2, BioSP and K2 showed a strong preventive effect against the development of inflammation, while strain INIX had a more moderate effect. In addition, strain *L.* GG showed no beneficial effect in this model, except for combined scores of occult blood and diarrhea and hematocrit. Results are shown in Tables 11-14.

[0075] Experimental groups:

Group 1: 5 mice, water + saline p.o.

Group 2: 5 mice, water-DSS + saline p.o.

Group 3: 5 mice, water-DSS + R2C2 ($1x10^8$ cfu/ml) p.o.

Group 4: 5 mice, water-DSS + INIX ($1x10^8$ cfu/ml) p.o.

Group 5: 5 mice, water-DSS + BioSP ($1x10^8$ cfu/ml) p.o.

Group 6: 5 mice, water-DSS + K2 ($1x10^8$ cfu/ml) p.o.

Group 7: 5 mice, water-DSS + *L.* GG ($1x10^8$ cfu/ml) p.o.

Table 11

Weight loss (%) associated with a certain number of days consuming DSS

Group	Day 4	Day 5	Day 6	Day 7
2	-0.54	-1.87	-5.92	-6.50
3	0.10	-0.52	-4.00	-4.69
4	0.13	-0.88	-4.83	-6.41
5	0.44	0.62	-3.39	-2.99
6	2.28	1.95	-2.97	-3.46
7	-1.10	-1.30	-6.09	-6.67

Table 12

Hematocrit levels following 8 days of DSS exposure

Group	Hematocrit level (%)
1	46.00
2	37.33
3	40.50
4	37.20
5	39.83
6	38.83
7	38.60

Table 13

Combined occult blood and diarrhea scores (evaluated on a scale of 8) following a certain number of days consuming DSS. (More specifically the score is done as follows: Hemoccult II (Beckman Coulter, Mississauga, Ontario, Canada) serial test slides of routine screening for fecal occult blood were used to evaluate rectal bleedings on a scale of 0-4, defined as follows : 0- No blood, 4- Feces like blood. Feces consistency was also evaluated on a scale of 0-4, defined as follows: 0- Normal consistency, 4- Liquid feces. Feces consistency and rectal bleedings values were combined and defined as the "clinical score". Scores were given by a blinded evaluator**).**

Group	Day 6	Day 7
2	4.50	5.17
3	2.67	3.40
4	3.00	4.67
5	2.20	3.00
6	3.00	3.60
7	4.00	4.67

Table 14

Colon length (post-mortem) after 7 days consuming DSS

Group	Colon length (cm)
1	7.43
2	5.70
3	6.30
4	5.50
5	6.03
6	5.85
7	5.68

b) treatment of intestinal inflammation

[0076] C57BL/6 mice were treated by gavage (p.o.) with 10^8 cfu/ml of the different strains once per day for the duration of the experiment. Intestinal inflammation was induced, on day 0, by the addition of DSS (2.5%) to the drinking water for 8 days and then replaced by fresh water for 8 days to evaluate the recovery from inflammation of the different treatment groups. The level and progression of inflammation was evaluated through measurements of weight loss, diarrhea, occult blood, hematocrits, and colon lengths (post-mortem). All *L. kefiranofaciens* strains showed positive effects, although varying in strength for the different strains, in the post-inflammatory recovery period. The strains R2C2 and BioSP showed the best potential in helping the animals recovering from DSS-induced injury. In fact, the mice receiving strain BioSP started gaining weight back 3 days before every other group. Strains R2C2 and BioSP showed better improvement in colon integrity. In addition, strain *L.* GG showed no beneficial effect in this model. These results are shown in Tables 15 and 16.

[0077] Experimental groups:

Group 1: 5 mice, water + saline p.o.

Group 2: 5 mice, water-DSS + saline p.o.

Group 3: 5 mice, water-DSS + R2C2 p.o.

Group 4: 5 mice, water-DSS + INIX p.o.

Group 5: 5 mice, water-DSS + BioSP p.o.

Group 6: 5 mice, water-DSS + K2 p.o.

Group 7: 5 mice, water-DSS + *L.* GG p.o.

Table 15

Weight variation (%) during the recovery period, after 8 days of DSS consumption

Group	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
2	-15.37	-16.27	-17.09	-16.99	-15.75	-14.39	-14.48	-13.51
3	-17.01	-18.79	-18.39	-17.56	-14.60	-13.29	-13.55	-9.75
4	-17.13	-17.37	-16.90	-14.21	-13.06	-11.80	-10.83	-7.30
5	-14.99	-15.72	-13.78	-10.36	-8.54	-5.77	-6.01	-3.58
6	-16.95	-16.95	-16.41	-16.74	-14.16	-13.59	-12.21	-9.74
7	-20.97	-23.21	-25.18	-24.69	-22.36	-19.98	-17.82	-14.24

Table 16

Colon length (post-mortem) after 8 days of DSS consumption and 8 days of recuperation

	Colon length (cm)
1	7.44
2	6.13
3	6.55
4	6.32
5	6.70
6	6.42
7	6.15

Effect of pasteurized or irradiated bacteria on intestinal inflammation

[0078] C57BL/6 mice were treated by gavage (p.o.) with 10^8 cfu/ml of different *L. kefiranofaciens* strains once per day for the duration of the experiment. Animals received the strains R2C2 or BioSP as live, pasteurized or irradiated bacteria. Intestinal inflammation was induced by the addition of DSS (2.5%) to the drinking water for 8 days and then replaced with fresh water for 8 days to evaluate the rapidity of recovery process from inflammation for the different treatment groups. The level and progression of inflammation was evaluated through measurements of weight variation and combined scores of diarrhea and occult blood. Colon length and myeloperoxidase (MPO) activity in the colon were also evaluated at the end of the experiment. The strains R2C2 and BioSP showed positive effects, reducing weight loss during exposure to DSS, reducing combined scores of diarrhea and occult blood. Weight gain also started earlier during the post-inflammatory recovery period. Colon integrity was also better for these groups, as indicated by closer to normal length

and MPO activity. No major difference was observed between groups treated with live, pasteurized or irradiated bacteria. For all these groups, a similar protective effect was observed. 5-ASA was used to compare efficacy in that experiment because of its well-known anti-inflammatory effects. For all parameters tested, 5-ASA, R2C2 and BioSP showed comparable protective effects. Results are shown in Tables 17-20.

Experimental groups :

Group 1 : 5 mice, normal water + saline p.o.

Group 2 : 5 mice, water-DSS + saline p.o.

Group 3 : 5 mice, water-DSS + Live R2C2 p.o.

Group 4 : 5 mice, water-DSS + Pasteurized R2C2 p.o.

Group 5 : 5 mice, water-DSS + Irradiated R2C2 p.o.

Group 6 : 5 mice, water-DSS + Live BioSP p.o

Group 7 : 5 mice, water-DSS + Pasteurized BioSP p.o.

Group 8 : 5 mice, water-DSS + Irradiated BioSP p.o.

Group 9 : 5 mice, water -DSS + 5-ASA p.o

Table 17

Weight loss (%) associated with 8 days of DSS exposure

Group	Day 5	Day 6	Day 7	Day 8
1	-0.20	2.26	0.35	1.26
2	-1.83	-5.23	-8.79	-13.5
3	-1.59	-3.74	-6.03	-8.65
4	-1.48	-2.23	-5.18	-7.02
5	-1.83	-3.78	-8.47	-10.4
6	-1.60	-2.69	-5.33	-8.51
7	-1.80	-3.70	-7.04	-9.94
8	-2.50	-4.73	-7.61	-9.18
9	-3.21	-3.64	-5.90	-8.07

Table 18

Weight variation (%) during a recovery period, following 8 days of DSS exposure

Group	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
1	4.23	3.56	2.29	1.12	3.26	5.21
2	-25.65	-27.36	-29.33	-33.27	-32.98	-34.0
3	-19.05	-18.13	-15.03	-14.49	-12.63	-11.7
4	-18.71	-16.31	-11.67	-10.72	-9.49	-7.22
5	-24.57	-27.39	-25.57	-23.87	-21.71	-20.7
6	-23.15	-22.54	-20.00	-21.06	-17.76	-14.1
7	-21.33	-21.25	-19.61	-21.30	-20.01	-13.0
8	-18.78	-17.05	-15.32	-15.80	-14.03	-11.1
9	-21.64	-20.97	-17.66	-17.11	-16.96	-11.6

Table 19

Combined occult blood and diarrhea scores (evaluated on a scale of 8) following a certain number of days consuming DSS

Group	Day 5	Day 6	Day 7	Day 8
1	0	0	0	0
2	2.25	4.50	6.50	6.50
3	2.00	1.50	4.75	5.25
4	2.00	2.80	2.75	5.25
5	2.00	2.25	4.00	5.25
6	1.50	3.75	4.75	6.00
7	2.00	3.00	5.20	5.40
8	1.40	3.00	4.25	6.00
9	1.75	3.75	4.25	5.50

Table 20

Colon length and MPO activity in the colon (post-mortem) following 8 days consuming DSS and 8 days of recovery

Group	Colon length (cm)	MPO activity (U/g of tissue)
1	7.17	0.056
2	4.79	0.226
3	5.86	0.081
4	6.06	0.066
5	5.84	n/d
6	6.38	n/d
7	6.20	n/d
8	6.06	n/d
9	5.81	0.043

Protection against allergies

[0079] The goal of this assay is to verify the capacity of the strains to modulate an allergic response in a mouse model. BALB/c mice are immunized by i.p.(intraperitoneal) injection of OVA (ovalbumin) in Alum ($Al(OH)_3$ gel) on day 0 and day 14, and serum is collected on days 21, 35, and 42 to detect total IgG and OVA specific antibody response. This immunization schedule is sufficient to induce a strong allergic reaction to OVA in the control mice. The anti-allergenic effect of the *lactobacillus* strains (compared with *L. casei*) are evaluated in groups of mice treated orally with the different strains for the duration of the immunization protocol. Development of an allergic reaction is evaluated through the detection of total IgG production and OVA specific antibodies in the serum by ELISA. Cultured spleenocytes, from the different treatment groups are also exposed to OVA *in vitro* to evaluate the allergic reaction through cytokine and antibody production.

Treatment of hyperlipidemia

[0080] The animal models selected to evaluate the effects of the strains on hyperlipidemia have several phenotypic parameters in common (such

as hyperlipidemia, obesity and diabetes) and have been used to confirm independently the beneficial effects of the strains on these.

[0081] The different bacterial strains were tested for their capacity to regulate blood lipid levels in a rat model of hyperlipidemia. This protocol described the comparative evaluation of the different strains to niacin (vitB3), a potent hypo-lipidemic agent, in regulating artificially induced hyperlipidemia in rats. Wistar rats injected i.p. with poloxamer 407 rapidly develop severe but transient hyperlipidemia. Serum levels of glucose are also increased by this treatment. The hypo-lipidemic effect of *L. kefiranofaciens* strains is evaluated in groups of rats pre-treated orally for 7 days before injection of poloxamer. Blood lipids were measured before injection and at 24 and 72 hours following induction of hyperlipidemia. Plasma levels of triglycerides, and cholesterol (LDL) were evaluated. After a pre-treatment of 7 days, plasma triglycerides were reduced in the niacin-treated group, while a less pronounced reduction was observed in the R2C2-treated group. A reduction of plasma triglycerides was also observed in all treatment groups 72 hours after the induction of hyperlipidemia. The most important reduction was observed in the group receiving niacin (Table 21).

Group 1: 4 rats, gavage saline

Group 2: 4 rats, gavage niacin (100 mg/kg)

Group 3: 4 rats, gavage R2C2 ($1x10^8$ cfu/ml)

Group 4: 4 rats, gavage INIX ($1x10^8$ cfu/ml)

Group 5: 4 rats, gavage BioSP ($1x10^8$ cfu/ml)

Group 6: 4 rats, gavage K2 ($1x10^8$ cfu/ml)

Table 21

Plasma triglycerides (mmol/L) in animals treated for 7 days and 72 hours after induction of hyperlipidemia

Group	Triglycerides (mmol/L) after 7 days of treatment	Triglycerides (mmol/L) 72h post-induction of hyperlipidemia
1	1.55	95.42
2	0.97	69.96
3	1.31	84.24
4	1.52	75.70
5	1.43	82.75
6	1.89	85.90

Protection against colon cancer

The goal of the present assay was to verify the capacity of the strains to protect mice against tumor formation in the genetic model C57BL/6J-ApcMin. In this model, 100% of ApcMin heterozygous mice develop at least 30 spontaneous intestinal adenomas when exposed to a fat-rich diet. The anti-tumorigenic effect of the strains was evaluated in groups of ApcMin heterozygous mice treated orally 3 times per week with the different strains during the tumor formation period.

Example 4

Digestion of protein found in whey

[0082] *L. kefiranofaciens R2C2* was evaluated for its capacity to hydrolyze a protein substrate during fermentation. Whey was used as a substrate to show that the bacteria has a capacity to digest common protein found in whey such Bovine Serum Albumine (BSA), Alpha-lactalbumine (α-LAC), Beta-lactoglobilin (bLG) over time. The analysis was done by HPLC (Column RP C-4 300 Å (Phenomenex, Torrance, CA, USA) with an elution gradient (Table 22).**Table 22**

HPLC analysis of degradation (%) of protein

Time (hours)	*% of degradation α-LAC*	*% of degradation BSA*	*% of degradation bLG*
0	100	100	100
24	90	96	25
48	81	93	4
72	45	79	0
96	24	64	0

Example 5

Anti-inflammatory potential of a whey product fermented with *L. kefiranofaciens* R2C2 in atopic contact dermatitis

[0083] A murine model of atopic contact dermatitis induced with oxazolone in mice was used to determine the anti-inflammatory effect of the whey fermented with *Lactobacillus kefiranofaciens* R2C2. This model of inflammation has proven to be a sensitive and useful tool to determine efficacy and potency of several anti-inflammatory and immunosuppressive drugs used in dermatological disorders like psoriasis for example. Drugs like glucocorticoids are commonly used to relieve skin and joint inflammation. Whey fermented with R2C2 administered orally either in a prophylactic (Table 24) or therapeutic fashion (Table 23) reduced the inflammation as shown with a reduction of around 30% of ear and thickness in both cases. In more details, the murine model of atopic contact dermatitis was based on those firstly described by Garrigue et *al.* (*Contact Dermatitis.*, 30(4):231-273, 1994) and modified as follows: the CD-1 mice's abdomen were removed of hair and the sensitization phase was done with the application of 100 microliters of oxazolone 5 % in acetone on the abdomen (Sigma-Aldrich, Oakville, On). After 4 days, the elicitation phase (first challenge) was done with application of 50 microliters of oxazolone 5% in acetone on the right ear (25 microliters each side of the ear). The second challenge was done 7 days after the first challenge with

the same procedure. The ear thickness of the mice was measured every day.

Prophylactic protocol (MPM) - Mouse atopic contact dermatitis

[0084] The prophylactic anti-inflammatory potential of MPM (patent PCT/CA2002/01988) was evaluated firstly by the administration of MPM, 7 days prior to sensitization. Three groups of 10 CD-1 mice received by gavages, each day, 100 microliters of reconstituted lyophilized MPM, water and 1 mg of water-soluble hydrocortisone (10 mg/mL). Dermatitis was induced as described previously and ear thickness was measured every day.The therapeutic anti-inflammatory potential of MPM was evaluated by the administration of MPM only after the first challenge. Three groups of 10 CD-1 mice received by gavages, each day, 100 microliters of reconstituted lyophilized MPM, water and 1 mg of water-soluble hydrocortisone (10 mg/mL). The mouse atopic contact dermatitis was done as described previously and ear thickness was measured every day. The mice's weight was measured twice a week.

Therapeutic protocol (R2C2) - Mouse model of atopic contact dermatitis

[0085] The therapeutic anti-inflammatory potential of R2C2 was evaluated in an animal model of atopic contact dermatitis by feeding the bacterial suspension after the first challenge. The protective effect of R2C2 was compared to that of hydrocortisone, because of its well-known anti-inflammatory effects on dermatitis. R2C2 showed a good reduction of inflammation, demonstrated by reduced ear thickness. Efficacy was comparable to that of hydrocortisone. Results are shown in Table 23-25.

Table 23

Treatment of atopic contact dermatitis (day 17)

Treatment	Ear thickness (mm)
Controls	0,55
Whey fermented with R2C2	0,38
Hydrocortisone	0,29

Table 24

Protection against atopic contact dermatitis (day 9)

Treatment	Ear thickness (mm)
Controls	0,45
Whey fermented with R2C2	0,32
Hydrocortisone	0,30

Table 25

Measurements of ear thickness (mm) during atopic contact dermatitis in laboratory animals

	Day 15	Day 17	Day 22
Controls	0.26	0.39	0.34
R2C2	0.19	0.28	0.17
Hydrocortisone	0.18	0.27	0.15

EXAMPLE 6

Anti-triglyceridemia potential of a whey product fermented with *L. kefiranofaciens* R2C2

Animals

[0086] For the experiment, female Wistar rats, 7 weeks old, weighing 125-150g. were purchased from Charles River Canada. Rats were

randomized into 4 different groups, each composed of at least 6 animals. Rats received a 1 mL dose of MPM, 1 mL of a saline suspension containing 10^9 bacteria/mL of *Lactobacillus* R2C2 (the *Lactobacillus* strain used to ferment whey), 1 mL PBS (Invitrogen, Burlington, Ontario, Canada) and 100 mg/kg of niacin (Sigma) as controls. They were housed under specific pathogen-free conditions and maintained in a 24h light/dark cycle. All animals consumed standard diet and received water *ad libitum*. MPM was in a lyophilized form and prepared daily by adding 80% of water and mixed to create back a yoghurt-like product. The bacterial strain *Lactobacillus* R2C2 was routinely cultured in MRS broth (BD Biosciences, Mississauga, ON, Canada) at 37°C for a period of 24h. Bacteria were then pelleted by centrifugation at 4000 rpm for 8 minutes and re-suspended at a concentration of 10^9 cells/mL in sterile PBS (Invitrogen). Niacin was dissolved in water in order for the rats to receive a dose of 100 mg of treatment per kg. Niacin did not solubilize perfectly so it had to be sonicated 20 minutes. The animals received the treatments for a 7-day period prior to the poloxamer 407 pluronic F-127 injection (BASF corporation, Mississauga, ON, Canada). The poloxamer 407 solution for intraperitoneal (i.p.) injection was prepared by combining the agent with sterile water and refrigerating overnight to facilitate dissolution of the polymer by the cold method of incorporation.

Induction of hyperlipidemia

[0087] Following the 7-day treatment with the different products, all animals were made hyperlipidemic by an i.p. injection of a 300 mg dose of poloxamer 407. All syringes were placed on ice prior to poloxamer 407 administration to maintain the polymer in a mobile viscous state in order to facilitate injection, since poloxamer 407 solutions at concentrations greater than about 23 % w/w exhibit reverse thermal gelatin properties.

Collection of blood

[0088] Approximately 1 mL of blood was collected from the jugular vein in 3 mL syringes and immediately transferred in lithium-heparinized plastic tubes (Sarstedt, Montréal, QC, Canada). Tubes were lightly shaken for 10 seconds, and then centrifuged to allow separation of the plasma. Plasma samples were collected in clean 1,5 mL eppendorf and immediately frozen

at –80°C until the time of analysis. For blood collection, each animal was anesthetized using isoflurane (AErrane, Baxter Corporation, Deerfield, IL, USA). Blood collection was performed after the 7-day treatment with the different products, before poloxamer 407 injection (t=0, comparison of post-treatment lipid levels), 24 hours after the injection (t=24h, comparison of induced lipid levels) and 72h after the injection (t=72h, comparison of recovery towards normal levels). Animals were sacrificed after the last blood collection by the CO_2 asphyxia method. All procedures for the feeding of the different treatments, for the poloxamer 407 administration and subsequent blood collections were in accordance with the institution's guide for the care and use of laboratory animals and accepted by the Ethic Committee.

Lipid analysis

[0089] Samples from all experimental groups were analyzed for total cholesterol and triglycerides. All analysis were performed in an independent laboratory (Laboratoire médical Biron, 4105-F Matte Blvd, Brossard, Québec, Canada, J4Y 2P4), ISO 9002 certified, offering a reliable plasma lipids quantification service.

[0090] R2C2 showed a slight capacity to regulate basal triglyceride levels (mmol/L) after a 7-day treatment, as shown in Table 15. The best effect was obtained with whey fermented with that bacteria. The bacteria R2C2 reduced basal triglyceride levels by close to 30% and whey fermented with R2C2 reduced basal triglyceride levels by close to 40%, similar to niacin, after 7 days of treatments. Triglyceride levels were also reduced 72h after the induction of hyperlipidemia in the R2C2-treated group, but were particularly modulated in niacin or MPM treated groups.

Table 26

Plasma triglycerides (mmol/L) in animals treated for 7 days and 72 hours after induction of hyperlipidemia

Treatment	Triglyceride levels (7-day treatment)	Triglyceride levels (72 hours post induction of hyperlipidemia)
Controls	1,83	95.42
R2C2	1,30	84.24
Whey fermented with R2C2	1,12	61.99
Niacin	1,00	66.96

Table 27

Cholesterol levels (%) in animals treated for 7 days and 72 hours after the induction of hyperlipidemia

Treatment	CH levels (72 hours post induction of hyperlipidemia) (%)
Controls	100
R2C2	95
Whey fermented with R2C2	29
Niacin	33

EXAMPLE 7

Anti-hypertensive potential of a whey product fermented with *L. kefiranofaciens* R2C2

[0091] Anti-hypertensive potential of whey fermented with R2C2 (MPM) was evaluated by using SHR female rats (6 weeks old). 12 rats were randomized according to their weight in each treatment group. They were housed under specific pathogen-free conditions and maintained in a 12 hour- light/dark cycle. All animals consumed standard diet and received water *ad libitum*. Groups were forced-fed daily either 1 mL of water (placebo group), MPM (5 mL/kg) or Enalapril-malate (10 mg/kg), because of its well-known hypotensive effects. Systolic blood pressure (SBP) was measured weekly by the tail-cuff method with the automated RTBP2000 Tail Blood Pressure system (Kent Scientific, Torrington, CT, USA). Data collections were made weekly and an average of 3 measurements was taken as initial mean SBP. Data was acquired and analysed with Biopac Student Lab Pro® software version 3.6.1 (Biopac System, Goleta, CA, USA). After 2 weeks of treatments, Enalapril-treated group had a normalized SBP going from 184 mm Hg to 156 mm Hg. A steady decrease of the SBP was observed every week with a maximum change of at least 25 % at week 4 for the animals forced-fed with MPM. Results are shown in Table 28.

Table 28

Systolic blood pressure (mm Hg) of SHR rats receiving various treatments

	Week 0	Week 1	Week 2	Week 3	Week 4
Controls	185	181	184	183	187
Whey fermented with R2C2	186	175	174	172	162
Enalapril	184	165	156	154	154
Enalapril + Whey fermented with R2C2	185	155	150	145	148

EXAMPLE 8

Treatment of hyperlipidemia, obesity and diabetes

[0092] The animal models selected to evaluate the effects of the strains on hyperlipidemia, obesity and diabetes have several phenotypic parameters in common (such as hyperlipidemia, obesity and diabetes) and have been used to confirm independently the beneficial effects of whey fermented with L. kefiranofaciens R2C2.

a) Model of hyperlipidemia

[0093] The whey fermented with *L. kefiranofaciens* R2C2 was tested for their capacity to regulate blood lipid levels in a rat model of hyperlipidemia. This protocol described the comparative evaluation to niacin (vitB3), a potent hypo-lipidemic agent, in regulating artificially induced hyperlipidemia in rats. Wistar rats injected i.p. with poloxamer 407 rapidly develop severe but transient hyperlipidemia. Serum levels of glucose are also increased by this treatment. The hypo-lipidemic effect of *L. kefiranofaciens* strains is evaluated in groups of rats pre-treated orally for 7 days before injection of poloxamer. Blood lipids were measured before injection and at 24 and 72 hours following induction of hyperlipidemia. Plasma levels of triglycerides, cholesterol, HDL, LDL and glucose were evaluated. After a pre-treatment of 7 days, plasma triglycerides were reduced in the niacin-treated group. A reduction of plasma triglycerides was also observed in the whey fermented with *L. kefiranofaciens* R2C2 group 72 hours after the induction of hyperlipidemia.

Group 1: 4 rats, gavage saline

Group 2: 4 rats, gavage niacin (100 mg/kg)

Group 3: 4 rats, gavage whey fermented with *L. kefiranofaciens* R2C2 (1 ml per gavage)

Table 29

Plasma triglycerides (mmol/L) in animals treated for 7 days and 72 hours after induction of hyperlipidemia

Group	Triglycerides (mmol/L) after 7 days of treatment	Triglycerides (mmol/L) 72h post-induction of hyperlipidemia
1	1.55	97.72
2	0.97	69.96
3	1.31	68.76

b) Model of hypertriglyceridemia and obesity

[0094] The whey fermented with *L. kefiranofaciens* R2C2 was tested for its capacity to regulate weight gain and blood lipid levels in a diet-induced rat model of hyperlipidemia. This protocol allows a comparison to niacin (vitB3) in regulating diet-induced hyperlipidemia in rats. Wistar rats exposed to fructose (at a concentration of 10%) in their drinking water show a gradual weight gain and develop hyperlipidemia over a four week period. The hypo-lipidemic effects of the whey fermented with *L. kefiranofaciens* R2C2 during the fructose treatment period were evaluated. Blood samples are collected before the start of fructose treatment, and once a week for 4 weeks during the induction of hyperlipidemia. Serum levels of triglycerides were measured.

Group 1: gavage saline

Group 2: gavage niacine (100 mg/kg)

Group 3: gavage whey fermented with L. kefiranofaciens R2C2 (1 ml per gavage twice a day)

Group 4: gavage of 1% Exopolysaccharide

Table 30:

Levels of triglyceride

Group	% relative to saline on day 0 Day 0	% over saline group Day 7	% over saline group Day 21
1	100	260	270
2	100	150	160
3	100	170	150
4	100	220	290

Table 31

Weight gain on fructose

Group	% relative to day 0 Day 7	% relative to day 0 Day 16	% relative to day 0 Day 25	% relative to day 0 Day 33
1	7	12	17	21
3	2	6	11	14
4	3	9	17	22

c) Models of weight and fat management

[0095] The whey fermented with *L. kefiranofaciens* R2C2 was tested for its capacity to regulate fat distribution as well as weight gain in the Spontaneous Hypertensive Rat model and in ovariectomized rats. The SHR model was used as previously described but used to monitor the levels of visceral fat accumulating in the belly following. The animals were fed with 1 ml of whey fermented with *L. kefiranofaciens* R2C2 (20% solid) once a day for 56 days (P.O. q1x56)

Group 1: 12 rats, gavage saline

Group 2: 12 rats, gavage niacine (100 mg/kg)

Group 3: 12 rats, gavage whey fermented with *L. kefiranofaciens* R2C2 (1 ml per gavage)

Table 32

SHR model

Group	% of animals with visceral fat
1	100
2	70
3	20

Table 33

Ovariectomized rats

Forty-five 12-month-old female Wistar rats were used and randomly assigned into 2 sham-operated groups and 2 ovariectomy (OVX) groups, i.e. OVX with saline (OVX group), an OVX with whey fermented with *L. kefiranofaciens* R2C2 (1 ml per gavage). Daily oral administration starting on day 4 after OVX for 12 weeks. The difference is about 8% of weight gain in favor of animals fed with whey fermented with *L. kefiranofaciens* R2C2.

Group	weight gain compared to sham-operated fed with either saline of whey fermented with *L. kefiranofaciens* R2C2 (1 ml per gavage)
1 (OVX saline)	1.32 X
2 (OVX with whey fermented with *L. kefiranofaciens* R2C2	1.24 X

EXAMPLE 9

Effect of whey fermented with L. kefiranofaciens R2C2 on human skin

[0096] Topical activity of whey fermented with *L. kefiranofaciens* R2C2 was monitored by means of sensitive and meaningful biomarkers of skin integrity such as prostaglandin E2 (PGE2) and cyclooxygenase 2 (Cox-2), both guardians of the degree of epithelial homeostasis (8). Whey fermented with *L. kefiranofaciens* R2C2 was compared to a nonsteroidal anti-inflammatory drug (Ibuprofen), a non selective inhibitor of Cox-2 and to an expensive commercial product with a popular brand name, Regenerist Olay®. The goal was to monitor the effect of whey fermented with *L. kefiranofaciens* R2C2 on Cox-2 expression and also basal and induced levels of PGE2 following a solar and environmental ultraviolet (UVB)-induced insult. In these experiments whey fermented with *L. kefiranofaciens* R2C2 was used prophylactically or therapeutically on human skin. In all experimental conditions tested, whey fermented with *L. kefiranofaciens* R2C2 showed a significant inhibitory effect on both biomarkers of integrity. The expression of Cox-2 was reduced following whey fermented with *L. kefiranofaciens* R2C2 exposure as shown by RT-PCR. Following this observation, we sought to explain the inhibition of Cox-2 by exploring the genes that could differentiate and explain this activity and rule out a potential negative affect. We found that, in addition to reducing expression of Cox-2, that the expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), a prostaglandin-degrading enzyme that physiologically and naturally antagonizes Cox-2 was enhanced. To push further this observation, we measured the consequence of Cox-2 reduction on the biosynthesis of PGE2 in human keratinocytes and human skin exposed to whey fermented with *L. kefiranofaciens* R2C2. We found that whey fermented with *L. kefiranofaciens* R2C2 was reducing basal levels of PGE2 by about 75% after a 24-hour exposure in absence of external insult. In a situation where UVB was used as an environmental insult, whey fermented with *L. kefiranofaciens* R2C2 prevented the induction of PGE2 suggesting a protective role of whey fermented with *L. kefiranofaciens* R2C2. Finally, when used either before or after the UVB exposure, whey fermented with *L. kefiranofaciens* R2C2 exhibited the same protective activity and even a

therapeutic activity as demonstrated when whey fermented with *L. kefiranofaciens* R2C2 was applied after the UVB exposure. It is also important to note that Regenerist Olay® was less efficacious than whey fermented with *L. kefiranofaciens* R2C2 even used non-diluted. Whey fermented with *L. kefiranofaciens* R2C2 could not be tested in a non diluted state in this experimental setting but we believe that its topical activity would even greater be increased if used non-diluted. Taken together, these data suggest that whey fermented with *L. kefiranofaciens* R2C2 exhibit an interesting biological functionality on human skin.

Effect of whey fermented with *L. kefiranofaciens* R2C2

EXAMPLE 10

Effect of whey fermented with L. kefiranofaciens R2C2

[0097] Three volunteers were willing on their own consent to consume the whey fermented with *L. kefiranofaciens* R2C2 product for various period of time. Two individuals had high cholesterol and one had diabetes and high blood pressure translating into occasional numbness in the hands. The first 2 volunteers with high cholesterol took for a period of 10 days the equivalent of 25 grams per day while the volunteer with pregnancy diabetes and high blood pressure took the product (100 ml humid) for a period of 3 weeks. The results were as follows, there was a reduction of 12% and 15% of total cholesterol for the first 2 individuals and a total alleviation of numbness and even a prevention of the pregnancy diabetes. These results are suggestive that some positive beneficial effects are to be tested in a more rigorous and rigid clinical format.

Table 34

Effect of consummation of whey fermented with *L. kefiranofaciens* R2C2 product for various period of time

Analysis	Volunteer 1	Volunteer 2
Cholesterol total (mmol/L)	From to 4.9 to 4.2 (15% reduction)	From 7.68 to 6.77 (12%)
Triglycerides (mmol/L)	From 3 to 3.5	From 1.66 to 1.33
Chol LDL (Calculated)	From 2.7 to 1.9	From 5.57 to 5.04
Chols/HDL ratio	From 6.2 to 6.0	NA

[0098] The *Lactobacillus kefiranofaciens* of the present invention can also be used to ferment substrates like milk products, whey and cheese whey leading to beneficial product having various effects. Fermentations processes of cheese whey are used for production of a ruminant feed supplement rich in protein, in the wine production. Fermented cheese whey have also the ability to act as an antioxidant, antihypertensive, antitumor, hypolipidemic, antiviral, antibacterial, and chelating agent.

[0099] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features herein before set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A probiotic composition comprising an effective amount of *Lactobacillus kefiranofaciens* in association with a suitable carrier.

2. The composition of claim 1, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum*.

3. The composition of claim 1, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSP and ES1.

4. The composition of claim 1, having a probiotic effect selected from the group consisting of intestinal adherence, intestinal persistence, positive modulation of the intestinal microflora, protection against intestinal pathogens, immunomodulation, protection against systemic inflammation, protection against intestinal inflammation, protection against allergies, protection against diarrhea, protection against diabetes, protection against hyperlipidemia and protection against colon cancer.

5. The composition of claim 1, wherein said composition is for oral, rectal or vaginal administration.

6. A method for providing positive modulation of the intestinal microflora in a subject comprising the step of administering to said subject an effective amount of *Lactobacillus kefiranofaciens*.

7. The method of claim 6, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum*.

8. The method of claim 6, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

9. A method for protecting a subject against intestinal inflammation comprising the step of administering to said subject an effective amount of *Lactobacillus kefiranofaciens*.

10. The method of claim 9, wherein said intestinal inflammation is caused by an inflammatory bowel disease (IBD), Crohn's disease (CD), ulcerative colitis (UC) or by an irritable bowel syndrome (IBS).

11. The method of claim 9, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum*.

12. The method of claim 9, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

13. A method for protecting a subject against allergies and/or autoimmune diseases comprising the step of administering to said subject an effective amount of *Lactobacillus kefiranofaciens*.

14. The method of claim 13, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum*.

15. The method of claim 13, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

16. A method for protecting a subject against diarrhea comprising the step of administering to said subject an effective amount of *Lactobacillus kefiranofaciens*.

17. The method of claim 16, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum*.

18. The method of claim 16, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

19. A method for protecting a subject against diabetes comprising the step of administering to said subject an effective amount of *Lactobacillus kefiranofaciens.*

20. The method of claim 19, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum.*

21. The method of claim 19, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

22. A method for protecting a subject against hyperlipidemia comprising the step of administering to said subject an effective amount of *Lactobacillus kefiranofaciens.*

23. The method of claim 22, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum.*

24. The method of claim 22, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

25. A method for protecting a subject against colon cancer comprising the step of administering to said subject an effective amount of *Lactobacillus kefiranofaciens.*

26. The method of claim 25, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum.*

27. The method of claim 25, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

28. A method for treating and/or preventing intestinal inflammation in a subject comprising the step of administering to said subject an effective amount of *Lactobacillus kefiranofaciens*.

29. The method of claim 28, wherein said intestinal inflammation is caused by an inflammatory bowel disease (IBD), Crohn's disease (CD), ulcerative colitis (UC) or by an irritable bowel syndrome (IBS).

30. The method of claim 28, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum*.

31. The method of claim 28, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

32. A method for treating and/or preventing allergies and/or autoimmune diseases in a subject comprising the step of administering to said subject an effective amount of *Lactobacillus kefiranofaciens*.

33. The method of claim 32, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum*.

34. The method of claim 32, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

35. A method for treating and/or preventing diarrhea in a subject comprising the step of administering to said subject an effective amount of *Lactobacillus kefiranofaciens*.

36. The method of claim 35, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus*

kefiranofaciens subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum.*

37. The method of claim 35, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

38. A method for treating and/or preventing diabetes in a subject comprising the step of administering to said subject an effective amount of *Lactobacillus kefiranofaciens.*

39. The method of claim 38, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum.*

40. The method of claim 38, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

41. A method for treating and/or preventing hyperlipidemia in a subject comprising the step of administering to said subject an effective amount of *Lactobacillus kefiranofaciens.*

42. The method of claim 41, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum.*

43. The method of claim 41, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

44. A method for treating and/or preventing colon cancer in a subject comprising the step of administering to said subject an effective amount of *Lactobacillus kefiranofaciens.*

45. The method of claim 44, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum.*

46. The method of claim 44, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

47. Use of *Lactobacillus kefiranofaciens* as a probiotic compound.

48. The use as claimed in claim 47, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum*.

49. The use as claimed in claim 47, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

50. The use as claimed in claim 47, having a probiotic effect selected from the group consisting of intestinal adherence, intestinal persistence, positive modulation of the intestinal microflora, protection against intestinal pathogens, immunomodulation, protection against systemic inflammation, protection against intestinal inflammation, protection against allergies, protection against diarrhea, protection against diabetes, protection against hyperlipidemia and protection against colon cancer.

51. The use as claimed in claim 47, wherein said *Lactobacillus kefiranofaciens* is for oral administration.

52. The use as claimed in claim 51, wherein said *Lactobacillus kefiranofaciens* is administered is a form selected from the group consisting of a live bacterial population, a lyophilized bacterial population, as a fermented dairy product and as a non-viable bacterial sample.

53. The use as claimed in claim 51, wherein said non-viable bacterial sample is selected from the group consisting of a heat-killed bacteria, an irradiated bacteria and a lysed bacteria.

54. The use as claimed in claim 47, wherein the probiotic compound has an anti-inflammatory effect.

55. The use of claim 54 for treating psoriasis.

56. The use of any one of claims 47-55, in association with an anti-inflammatory compound.

57. The use of claim 57 wherein the anti-inflammatory compound is 5-ASA or a corticosteroid.

58. The used of claim 47, for the manufacture of a medicament for treating psoriasis.

59. Use of *Lactobacillus kefiranofaciens* to ferment whey.

60. The use as claimed in claim 60, wherein said whey is cheese whey.

61. The use as claimed in any one of claims 60 and 61, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum*.

62. The use as claimed in any one of claims 60 and 61, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

63. Use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* for the treatment of a cardiovascular disease.

64. Use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* for the treatment of hypertension.

65. Use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* for the treatment of weight disorder.

66. Use a of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* for the treatment of hyperlipidemia

67. Use a of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* for the treatment of triglyceride disorder.

68. The use as claimed in anyone of claim 64-68, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum*.

69. The use as claimed in anyone of claim 64-68, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

70. Use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* in the manufacture of a medicament for the treatment of cardiovascular disease.

71. Use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* in the manufacture of a medicament for the treatment of hypertension.

72. Use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* in the manufacture of a medicament for the treatment of weight disorder.

73. Use of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* in the manufacture of a medicament for the treatment of hyperlipidemia.

74. of a product obtained from the fermentation of whey by *Lactobacillus kefiranofaciens* in the manufacture of a medicament for the treatment of triglyceride disorder.

75. The use as claimed in anyone of claims 71-75, wherein said *Lactobacillus kefiranofaciens* is selected from the group consisting of *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum*.

76. The use as claimed in anyone of claim 71-75, wherein said *Lactobacillus kefiranofaciens* is a strain selected from the group consisting of R2C2, INIX, K2 , BioSp and ES1.

SEQUENCE LISTING

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LEMIEUX, Pierre

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tacaaagttc cttcggggac gctaagacag gtggtgcatg gctgtcgtca
gctcgtgtcg 1020
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tgacgtcaag 1140

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ttgacggtaa tcaaccagaa agtcacggct aactacgtgc cagcagccgc ggtaatacgt 480
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Chapitre 6 : Production d'enzymes protéolytiques naturelles à partir des streptomycètes.

Contenu du chapitre: résultats partiels ne faisant pas partit d'un manuscrit et manuscrit soumis en révision.

Résultats partiels

Certains résultats sont présentés en regard des objectifs initiaux de modification d'une enzyme par mutagénèse dirigée. Suite à la modification de la stratégie pour l'obtention des enzymes protéolytiques, cette approche fut abandonnée.

Manuscrit déposé en révision.

Simard, E., Shareck, F., Dupont, C. 2005. New enzyme and analysis of S2 peptidase family: MULTI-WAY EVOLUTION BY DUPLICATION, RECOMBINATION AND LATERAL TRANSFER. 41 pages.

Contributions : J'ai participé à l'élaboration du projet avec Claude Dupont et François Shareck. J'ai sélectionné les gènes du génome de *Streptomyces coelicolor*, j'ai procédé à l'isolement de l'ADN génomique de *Streptomyces lividans*, pour par la suite amplifier les séquences, produire les transformants et les sélectionner à l'aide de nouvelles méthodes de criblage que j'ai développées. J'ai effectué la production des enzymes et l'ensemble des travaux de purification et caractérisation. J'ai effectué l'analyse phylogénétique du groupe d'enzyme pour finalement rédiger le manuscrit avec la participation de Claude Dupont et François Shareck.

6.1 Résultats partiels

6.1.1 Introduction et choix des gènes

Deux groupes d'enzymes sont connus pour réaliser une hydrolyse efficace de la β-lactoglobuline (β-lg) et provoquer la formation d'agglomérats : les subtilisines et les protéases spécifiques à l'acide glutamique. Pour ces enzymes, les représentants bien connus ayant été utilisés pour la sélection des gènes sont l'enzyme Carlsberg de *Bacillus subtilis* (subtilisine : PDB (Protein Data Bank) 1SBC) et l'enzyme spécifique à l'acide glutamique de *Streptomyces griseus* (Swissprot : Q07006). Par comparaison des gènes connus avec le génome de *Streptomyces coelicolor* (www.sanger.ac.uk), dix gènes codant pour des subtilisines (Prot 1 à 10) et cinq (Prot 21 à 25) pour des protéases spécifiques à l'acide glutamique ont été sélectionnées.

En étendant la recherche, il fut possible de sélectionner aussi cinq gènes codant pour des protéases similaires à la thermolysine de *Geobacillus stearothermophylus* (une métalloprotéase : Swissprot AAB02774) et trois gènes codant pour un autre type de protéase spécifique à l'acide glutamique par homologie avec la protéase V8 de *Staphylococcus aureus* (Swissprot : P04188). Ces gènes sont présentés au tableau 18.

Initialement, l'axe exploratoire de production d'enzymes protéolytiques devait porter sur la modification de la spécificité d'une subtilisine par mutagenèse dirigée. À cette fin, les neufs premières protéines encodées par le groupe de gènes des subtilisines (Prot 1 à 9) furent modélisées pour développer les outils nécessaires à l'amélioration de leur spécificité. Malgré l'abandon de cette approche, due à la difficulté qu'aurait représentée l'utilisation d'enzymes génétiquement modifiées en alimentation, un exemple des résultats produits est fourni pour une des protéases modélisées afin de démontrer la faisabilité de cette approche. Ces résultats sont donc partiels et présentés comme une note en relation avec les objectifs initiaux.

Tableau 18 : Identification des gènes sélectionnés à partir du génome de *S. coelicolor*.

Nom	Cosmide	Gène	a.a. S.C.*	a.a. clonés	Cloné ? **	Taille du gène (bp)
Prot1	8A11	8A11.04C	1253	517	oui	1551
Prot2	8A11	8A11.16C	1234	516	oui	1548
Prot3	C24	C24.17C	1220	492	oui	1659
Prot4	F51A	51A10	1245	512	oui	1536
Prot5	I35	I35.29	393	393	oui	1182
Prot6	I8	SSP (I8.09)	512	512	oui	1539
Prot7	3C3	3C3.08	413	413	oui	1242
Prot8	3C3	3C3.17	450	450	oui	1353
Prot9	2G61	2G61I.37C	537	423	oui	1263
Prot10	SC8E4A	SC8E4A.07	1098	1098	non	3296
Prot21	SCI11	I11.30C	360	360	oui	1083
Prot22	SCI11	I11.35C	300	351	oui	1056
Prot23	SCF81	F81.11C	437	372	oui	1116
Prot24	3SC5B7	35B7.10	454	363	oui	1089
Prot24B	3SC5B7	35B7.10	454	454	oui	1365
Prot25	SC10G8	10G8.13C	390	390	oui	1173
Prot31	SC3D11	3D11.03	549	549	non	1650
Prot32	SC3D11	3D11.04	684	684	oui	2055
Prot33	SC7A8	7A8.13	547	547	oui	1644
Prot34	SCC117	C117.02	356	356	oui	1071
Prot35	2SCG58	2SCG58.26	713	713	non	2142
Prot41	SC5F7	5F7.30	362	362	oui	1089
Prot42	SCP8	P8.12	542	542	oui	1629
Prot43	SCBAC25E3	BAC25E3.14	519	519	non	1560

*Sanger Center : selon la séquence du gène.

** Le gène fut incéré dans un vecteur multicopie et transformé dans la souche *S. lividans* 10-164 (Hurtubise et *al.*, 1995).

6.1.2 Modélisation des subtilisines

Les subtilisines furent modélisées à partir du logiciel MOE, par comparaison avec la structure connue de la subtilisine Carlsberg de *B. licheniformis* (PDB : 1CSE) en utilisant les paramètres par défaut du logiciel. Le choix de cette structure fut effectué en fonction de son haut niveau de définition (1,2 Å). Les enzymes de ce groupe présentent une forte homologie entre elles ce qui rend la modélisation et l'identification des résidus impliqués dans la spécificité plus facile. Le tableau 19 présente l'homologie réelle et l'homologie conservatrice (considérant les positions conservées comme équivalentes : un acide glutamique pour un acide aspartique par exemple) pour trois enzymes bien connues et pour le groupe des subtilisines modélisées de *S. coelicolor*.

Afin de valider cette approche, un modèle de l'enzyme BPN fut produit à partir de l'information de la structure de l'enzyme Calsberg et il fut superposé à la structure réelle de l'enzyme (figure 24). On peut y voir que le modèle produit respecte très bien la structure connue de l'enzyme. La figure 25 présente les positions des résidus impliqués dans la spécificité de l'enzyme toujours pour cette modélisation. Les mêmes figures sont présentées pour la modélisation de la protéase 3 (Prot 3) des gènes sélectionnés du génome de *S. coelicolor* (figures 26 et 27).

La modélisation et la superposition à des structures connues permettent de bien définir les résidus impliqués dans la spécificité. L'évaluation de ces résidus responsables de la spécificité fut effectuée par comparaison avec des séquences connues d'enzymes pour lesquelles une grande quantité d'informations est disponible. Les séquences retenues à cette fin sont la subtilisine Carslberg et la protéase alcaline de *Bacillus licheniformis* et la subtilisine BPN de *Bacillus amyloliquefaciens*. Le tableau 20 présente les résidus potentiellement impliqués dans la spécificité de l'enzyme Prot 3 en comparaison avec ces mêmes résidus pour les enzymes connues. Il s'agit donc d'une approche intéressante pour la réalisation de travaux de mutagenèse dirigée.

Tableau 19 : Homologie de séquence des enzymes matures probables de *S. coelicolor* par rapport à trois subtilisines connues; la subtilisine Carslberg de *Bacillus licheniformis*, la subtilisine BPN de *Bacillus amyloliquefaciens* et la subtilisine E de *Bacillus subtilis*.

Séquences	Nb. de résidus	Homologie réelle			Homologie conservée		
		Carls.	BPN	E	Carls.	BPN	E
Carlsberg	274	100	69	69	100	79	80
BPN	275	70	100	86	79	100	92
E	275	70	86	100	80	92	100
Prot1	293	43	42	43	56	58	58
Prot2	298	43	43	43	57	60	58
Prot3	292	40	39	38	57	58	56
Prot4	298	43	46	46	59	57	57
Prot5	307	42	39	38	54	53	52
Prot6	337	38	38	39	50	48	48
Prot7	286	36	37	36	52	53	52
Prot8	292	30	33	37	46	47	48
Prot9	279	38	37	37	53	52	53

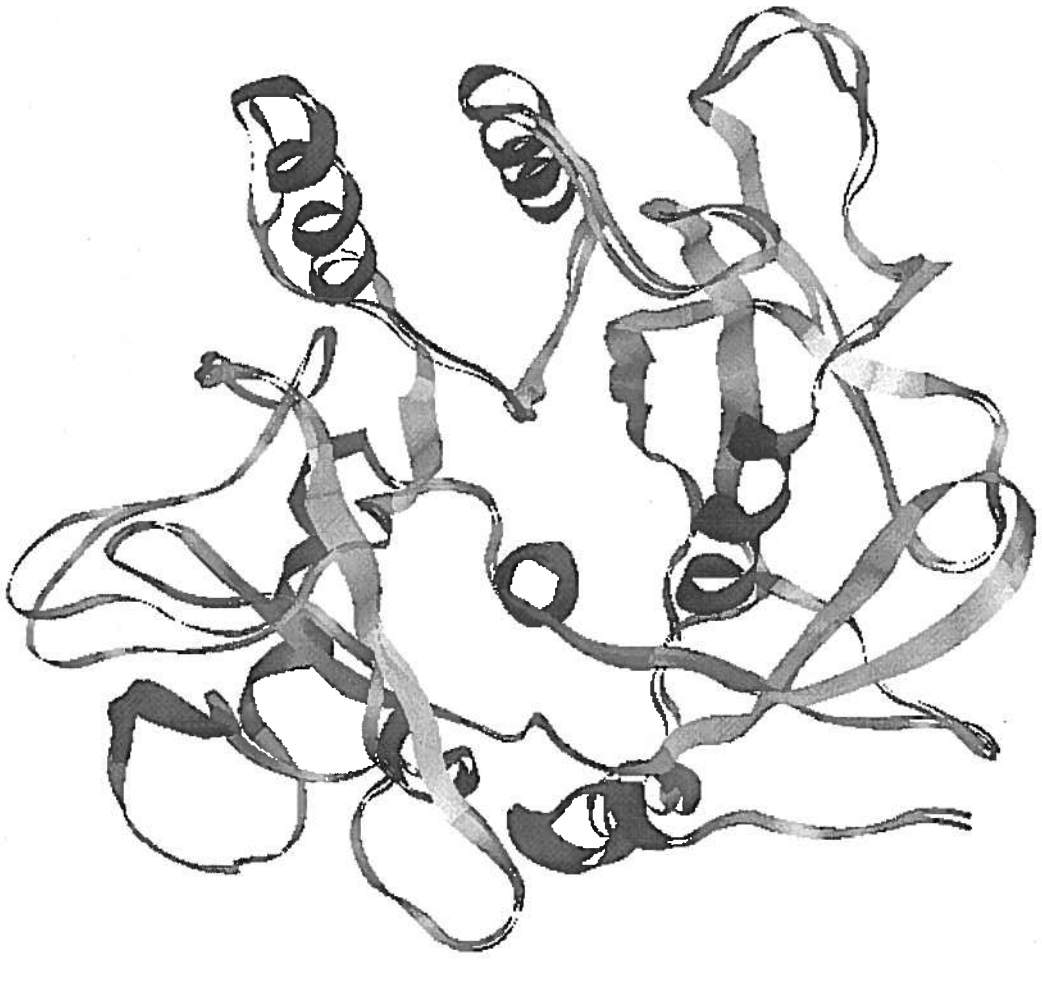

Figure 24 : Superposition du modèle réalisé à l'aide de la séquence protéique de la subtilisine BPN de *Bacillus amyloliquefaciens* avec la structure réelle de l'enzyme (PDB : 2SNI : 2,10 Å de résolution).

La structure utilisée pour la modélisation est celle de l'enzyme Carlsberg (PDB : 1CSE ; 1,2 Å de résolution). RMSD : 0, 582.

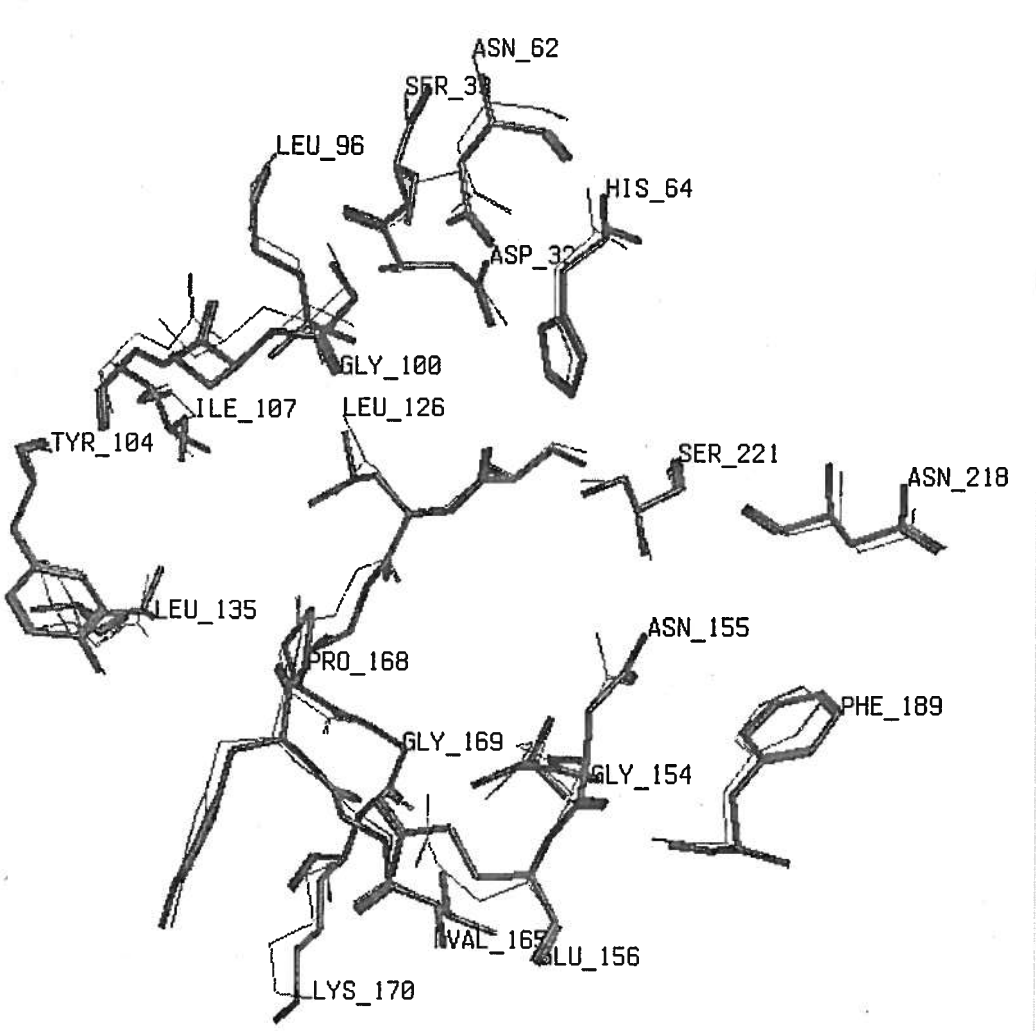

Figure 25 : Résidus entrant en interaction (selon Barrett et *al.,* 1998) avec un substrat éventuel, pour le modèle de l'enzyme BPN réalisé à partir de la séquence connue de l'enzyme BPN et de la structure de l'enzyme Carlsberg (PDB : 1CSE).

Le modèle est superposé à la structure réelle de l'enzyme. Les résidus sous forme de ligne sont ceux de l'enzyme BPN, ceux en bâtonnet sont ceux du modèle.

Figure 26 : Superposition du modèle réalisé à partir de la séquence protéique Prot3 de *Streptomyces coelicolor*, avec la structure de l'enzyme Carlsberg (PDB : 1CSE ; 1,2 Å de résolution).

La structure utilisée pour la modélisation est celle de l'enzyme Carlsberg (PDB : 1CSE ; 1,2 Å de résolution). RMSD : 0,814.

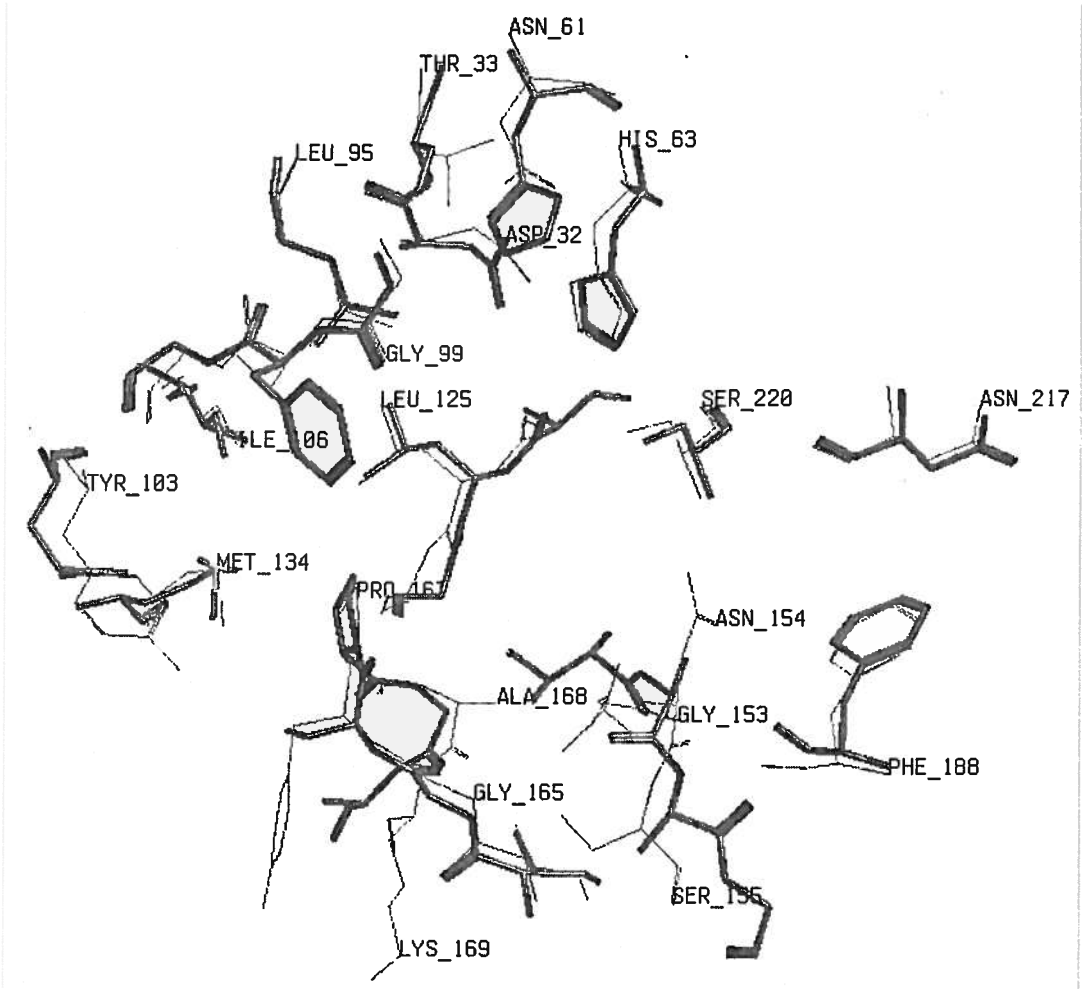

Figure 27 : Résidus entrant en interaction (selon Barrett et *al.*, 1998) avec un substrat éventuel, pour le modèle Prot3 réalisé à partir de la structure connue de la subtilisine Carlsberg (PDB : 1CSE) et superposé à celle-ci.

Les résidus sous forme de ligne sont ceux de l'enzyme Carlsberg, ceux en bâtonnet sont ceux de l'enzyme.

Tableau 20 : Homologie de séquence nous permettant d'étudier la spécificité théorique de la protéase probable Prot3 de *S. coelicolor*.

Les enzymes connues utilisées pour la comparaison sont la subtilisine Carslberg et la protéase alcaline de *Bacillus licheniformis*, la subtilisine BPN de *Bacillus amyloliquefaciens*.

Site catalytique (résidus)	Prot 3	Protéase alcaline	Carlsberg	BPN
P1 (152-155)	AAGN	AAGN	AAGN	AAGN
P1 (166-169)	GSPG	GYPG	GYPA	GYPG
156	T	S	S	E
166	G	G	G	G
P1-P4 (125-128)	SLGS	SLGG	SLGG	SLGG
P4 (101-104)	FGSE	SGSY	SGTY	SGQY
96	L	L	L	L
104	E	Y	Y	Y
107	I	I	I	I
126	L	L	L	L
P1'-P3' (217-219)	MDG	LNG	LNG	YNG

6.1.3 Criblage des transformants

Suite à l'abandon de l'approche de mutagenèse dirigée, la presque totalité des gènes sélectionnés furent clonés (voir tableau 18) pour tenter de produire des enzymes naturelles des streptomycètes à partir de *Streptomyces lividans*. Les transformants furent sélectionnés sur la base de leur activité protéolytique.

Un premier criblage a été réalisé sur gélose à la caséine (milieu Stewart additionné de 2,5 % de lait écrémé stérile : décrit dans le manuscrit présenté) où les transformants sont jugés actifs s'il y a apparition d'une zone claire entourant la colonie. Un témoin négatif (*Stretomyces lividans* 10-164 avec le vecteur pIAF C-109 ou pIAF 550 selon le cas) est toujours utilisé pour fin de comparaison et est totalement négatif. Des cultures sont ensuite préparées pour évaluer l'activité protéolytique des surnageants (fraction extracellulaire), des membranes cellulaires (fraction membranaire) et de la fraction intracellulaire par le test de l'azocaséine. Par comparaison avec le témoin négatif, il est ainsi possible de déduire si l'activité est principalement retrouvée dans une de ces fractions. Le tableau 21 présente un résumé de l'évaluation qualitative (gélose à la caséine) et quantitative (test à l'azocaséine) de l'activité de ces transformants.

L'activité des trois fractions de chacun des transformants est exprimée de façon relative à l'activité de ces fractions pour le témoin négatif. Le test à l'azocaséine est suffisamment sensible pour évaluer l'activité de base de la souche 10-164 et ainsi y comparer les transformants. La valeur 2,5X signifie donc, que cette fraction du transformant exprime une activité 2,5 fois plus élevée que la fraction correspondante du témoin. Ces travaux ont mené à la purification et à la caractérisation sommaire de l'enzyme SLP25 dont les résultats sont présentés par le manuscrit en préparation de ce chapitre.

Tableau 21 : Activité protéolytique des différents transformants

Nom	Gélose	Azocaséine		
		Extra	Intra	Memb
Prot1	+++		2,5X	
Prot2	+	1,75X	4X	1,8X
Prot3	+			
Prot4	+	2X		2,25
Prot5	+*	47x	5X	7X
Prot6	++	2,25X	2,25X	
Prot7	++			
Prot8	++			
Prot9	+++	12x		
Prot21	+++			
Prot22	+		18X	2X
Prot23	++		11X	3X
Prot24	+++	3,5X		
Prot25	+++	9X		

* Croissance très faible.

6.1.4 Discussion et conclusion

La grande quantité d'information structurale de certains groupes enzymatiques permet maintenant de modéliser avec fidélité de nouvelles enzymes probables afin d'orienter le choix des enzymes à produire ou encore, de modifier leur activité par mutagénèse dirigée. Il devient ainsi possible d'utiliser l'information génétique afin d'orienter des choix avant même que la fonction de certaines enzymes ne soit connue.

De plus, la possibilité de surproduire des enzymes protéolytiques naturelles de *S. lividans* fut démontrée avec succès. Cependant, pour la majorité des clonages effectués, il demeure très difficile de purifier et de caractériser les enzymes produites. Les réactions d'hydrolyse peuvent subvenir à toutes les étapes des travaux et leur forte activité enzymatique en permet la détection pour des quantités infimes de protéine difficilement analysables.

6.2 Manuscrit en révision

Simard, E., Shareck, F., Dupont, C. 2005. New enzyme and analysis of S2 peptidase family: MULTI-WAY EVOLUTION BY DUPLICATION, RECOMBINATION AND LATERAL TRANSFER. 41 pages.

Soumis à Journal of Biological Chemistry et ensuite à Microbiology (MS27468). Réponse de Microbiology le 30 août 2004 avec les commentaires des examinateurs que les travaux sont d'intérêt, mais que certains compléments devraient être apportés et que la publication devrait être effectuée dans un journal traitant plus d'évolution ou de phylogénie. Les modifications ont été apportées, mais quelques analyses statistiques sont à complétées ainsi que les figures de phylogénie.

J'ai effectué la production des enzymes et l'ensemble des travaux de purification et caractérisation. J'ai effectué l'analyse phylogénétique du groupe d'enzyme pour finalement rédiger le manuscrit avec la participation de Claude Dupont et François Shareck.

TITLE: New enzyme and analysis of S2 peptidase family: MULTI-WAY EVOLUTION BY DUPLICATION, RECOMBINATION AND LATERAL TRANSFER.

RUNNING HEADER: SLP25 enzyme and philogenetic analysis of S2 family peptidase

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ABSTRACT

Peptidases are ubiquitous enzymes found in all living organisms that have evolved in order to hydrolyze the various substrates they encounter. Their strong relationships to the ecological adaptation of the organism make them a group with a great interest for the appreciation of the evolutionary processes. Within bacteria, *Streptomyces* proteases are well known and with the determination of *S. coelicolor* genome, it was possible to putatively identify specific peptidases and clone them. Here we report the construction of a homologous expression vector for *Streptomyces* (pIAF550) and the cloning, production and purification of a new S2 family peptidase (Slp25). Amino acid sequence analysis of the Slp25 peptidase showed that this enzyme belongs to a subfamily of S2 peptidase, the "α-lytic like enzyme" group. The peptidases from that subfamily differentiate themselves from the S2 family members by harboring a third disulfide bond and a rather long prodomain. Phylogenetic analysis of S2 family peptidases indicated lateral gene transfer from an actinomycete to a eukaryote in the case of the Slp25 peptidase. The evolution of this enzyme family has progressed from a continuous evolutionary divergence with duplication, recombination and lateral transfer.

INTRODUCTION

Streptomyces, Gram-positive bacteria with a complex life cycle, are known to secrete a multitude of proteins (Gilbert *et al.*, 1995), many of which are hydrolytic enzymes converting soil residues, carbohydrates and polypeptides into nutriments for growth. In *Streptomyces,* the putative secreted hydrolytic enzymes are over-represented when compared with those of five other species (*Bacillus subtilis*, *Escherichia coli*, *Corynebacterium diphteriae*, *Mesorhizobium loti* and *Mycobacterium tuberculosis*) for which the complete genomes are known (Bentley *et al.*, 2002). These enzymes are the keys of soil regeneration, especially for the degradation of insoluble substrates such as keratin (Bockle *et al.*, 1995; Bressollier *et al.*, 1999). The contribution of these gene families diversification and the nature of the evolutionary forces acting during this process, are linked to the ecological traits associates to their life strategies (Hu & Leger, 2004). This paper reports about a group of enzymes of great interest for the appreciation of the evolutionary processes.

Many species of Streptomycetes have been identified for peptidase production with a wide variety of activities: including subtilisin-like, glutamic-acid-specific, metalloprotease, fibrinolytic enzyme, streptogrisin, etc... (Chitte & Dey, 2000; Dammann & Wohlleben, 1992; Sidhu *et al.*, 1993; Sidhu *et al.*, 1995; Suzuki *et al.*, 1997). Indeed, *Streptomyces griseus* is the source of a commercial crude enzyme preparation, known as pronase, which is the best-known group of streptomyces proteases (Sidhu *et al.*, 1995). Obviously, the varieties of peptidases that can be produced are at the level of the microbial degrading role of streptomyces strains. For efficient degradation of proteins, peptides and glycoproteins, the *S. coelicolor* genome contains numerous putative genes

(www.sanger.ac.uk/Projects/S_coelicolor/scheme.shtml) which includes over 150 putative peptidases of different families. The completion of the total genome sequence of *S. coelicolor* A3 has led to the possible amplification of many new genes for enzyme production in the closely related strain such as *S. lividans* 1326 (Bentley *et al.*, 2002).

The best-represented family of peptidases in streptomyces is the S2 family of clan SA of serine proteases (Barrett *et al.*, 1998). This group encompasses the streptogrisin (*S. griseus*), the glutamyl endopeptidase II (*S. fradiae* and *S. griseus*), the SAM-P20 (*S. albogriseolus*) and the Sfase (*S. fradiae*). The homology between these peptidase gene sequences allowed Taguchi *et al*, (Taguchi *et al.*, 1995a) and Binnie *et al*, (Binnie *et al.*, 1996) to use oligonucleotide probes to clone the encoding genes from *S. lividans* (SALO and SAL respectively). In this paper, we describe a new enzyme of this family isolated on the basis of *S. coelicolor* genome information. We report the following: (i) the cloning and sequencing of a novel serine peptidase gene of *S. lividans*; (ii) the phylogenetic analysis of the S2 peptidase family and (iii) the purification and initial characterization of the enzyme.

The peptidase Slp25 is a new enzyme of the S2 family peptidase with strong relationship to a fungal peptidase. Phylogenetic analyses of Slp25 and other S2 peptidases provide a better understanding of this family and allow us to propose two peptidase subfamilies: the stretogrisin and α-lytic like peptidases. We conclude that Slp25 is an evolutionary example of lateral gene transfer from bacteria to fungi. The comparison of the proteins sequences, selected amino acid of the active site-related residues and the 16S based species phylogeny within the S2 peptidase family, reveal that the evolution of this family has progressed from a continuous evolutionary divergence with duplication, recombination and lateral transfer.

METHODS

DNA amplification and cloning

The gene coding for Slp25 was amplified by PCR from chromosomal DNA of *S. lividans* 1326 using the following oligonucleotide primers, designed from *S. coelicolor* SC10G8.13C gene sequence (2) : CD1601 : 5'-GGGGCATGCGACGCAGCAGACTCAGGC-3' and CD1602: 5'-CCCGAGCTCTCAGGCCGTGTGCAGGG-3'. All amplifications were performed using a GeneATAQ controller (LKB-Pharmacia) in 50 µL reaction with 1 ng of template, 50 pmoles of each primer, 4% propionamide, 0.05% Tween20, 20 nmoles of each deoxynucleotide, 2.5 U of *Pwo* DNA polymerase (Roche) and the reaction buffer supplied by the manufacturer. The first PCR cycle consisted of a denaturation step at 95°C for 5 min., an annealing step at 55°C for 5 min. and a polymerization step at 72°C for 3 min. Then, DNA was amplified by 30 cycles: 94°C for 1 min., 55°C for 1 min. and 72°C for 3 min. followed by an extension of the unfinished products at 72°C for 7 min. (Sulzenbacher *et al.*, 1997). A sample (10 µL) was analyzed by agarose gel electrophoresis and the amplicons were recovered using the High GFX PCR DNA and Gel band purification kit (GE Healthcare). The amplification product and plasmid pIAF550 were digested with *Sph*I and *Sac*I endonucleases and ligated using established procedures. Protoplasts of *S. lividans* 10-164 (*msi*K$^-$) (Hurtubise *et al.*, 1995) were then transformed and regenerated on R5 medium according to Hopwood *et al.*,(Hopwood *et al.*, 1985). Transformants were grown for 5 days at 34 °C on Stewart solid medium containing 2.5 % (w/v) skim milk. The proteolytic activity was monitored for the presence of clearing zone formation and was identical for all transformants.

DNA sequencing and analysis

DNA sequence was determined using pIAF2025 plasmid isolated from positive transformants as described previously by Hopwood *et al* (Hopwood *et al.*, 1985). The plasmid was digested with *Sph*I and *Sac*I endonucleases for 2 h at 37 °C. The insert was purified and ligated into pTZ19U *E. coli* vector at 16 °C overnight. Competent *E. coli* DH11S cells were transformed with the ligation mixture and plated on 2xTY ampicillin agar plates containing X-gal and IPTG. Transformants were selected and the plasmid isolated using FlexiPrep purification kit (GE Healthcare) accordingly to the manufacturer's instructions. The insert was sequenced in both directions from the plasmid using universal and reverse primers and the DNA sequences were assembled, aligned and analyzed using the Vector NTI 7.0 software. Homology search of NCBI databanks was carried out using the BLAST software (Altschul *et al.*, 1997).

Phylogenetic data analysis

Amino acid sequences were first aligned using Vector NTI (Align) and phylogenetic analysis performed using PHYLIP Version 3.6 (Department of Genome Sciences, University of Washington). The pairwise sequence distances were calculated with PRODIST and the confidence values were assessed from 1000 bootstrap replicates of the original sequence data using SEQBOOT. Distance trees were constructed using FITCH and the consensus tree was obtained using CONSENSE. The final tree was manually edited using PRODIST original distances and Windows PAINT software.

Enzymes production and purification.

Spore suspensions of *S. lividans* IAF2025 obtained from 7-day old Bennett agar plates, were used to inoculate Erlenmeyer flasks containing trypticase soy broth (Difco) to which 5 µg/mL of thiostrepton had been added as a selective marker. Abundant growth

was achieved after 48h incubation at 34°C on a rotary shaker (240 rpm). Thirty-five mL of this pre-culture served to inoculate 2 L Erlenmeyer flask containing 500 mL of M14 medium (Mondou *et al.*, 1986) supplemented with 1% (w/v) mannitol as carbon source. Bacteria were removed by filtration on a 0.2 µ membrane and secreted proteins were first filtered by ultrafiltration on a 100 kDa cut-off membrane (Omega) to remove high molecular weight material and the filtrate containing the Slp25 protein concentrated on a 3 kDa cut-off membrane (Omega). The concentrated proteins (12.5 mL) were loaded on a 5 mL Hi-Trap™ Benzamidine column (GE Healthcare). Unbound material was removed with successive washes of 25 mL of 50 mM Tris-HCl containing 1 M NaCl at pH 7.5 and then 25 mL of 50 mM Tris-HCl pH 7.5. Bound proteins were eluted with 50 mM glycine-HCl buffer pH 3.0 at a flow rate of 1.6 mL/min. To prevent acidic denaturation of the protein, collection tubes contained 16 to 75 µL of 1 M Tris-HCl pH 9.0.

Protein analysis.

Protein concentration was determined with the Folin-Lowry reagent (Lowry *et al.*, 1951) using bovine serum albumin as standard (BioRad) and purity was assessed by SDS-PAGE (Laemmli, 1970). The N-terminal sequence of the purified mature Slp25 was determined at the Sheldon Biotechnology Center of McGill University (Montreal, Canada) by automated Edman degradation performed with a gas-phase/pulsed-liquid sequencer (model 492, Applied Biosystems). Proteins in gels were transferred onto PVDF membrane using a Mini TransBlot apparatus (BioRad).

Zymogram

Protein samples were prepared in 5X zymogram sample buffer (62 mM Tris, pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol and 0,05 % (w/v) bromphenol blue) and were loaded into 1 mm SDS-PAGE containing 0,2% (w/v) of gelatin. The electrophoresis was

carried out at 200 V until the dye marker ran off the gel. Gels were soaked for 1h in 2.5% (v/v) Triton X-100 solution on a rotary shaker at room temperature. The Triton X-100 solution was decanted and the gel subjected to two brief washes in 100 mL of assay buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$ and 0.2% (v/v) Brij-35) and then incubated for 18 h at 37°C in the assay buffer followed by staining and destaining on a rotary shaker at room temperature. Staining consisted of a 60 min soak in 0.1% (w/v) Coomassie blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid followed by destaining using 40% (v/v) methanol and 10% (v/v) acetic acid solution until clear bands were detected.

Assays for enzyme activity

The activity of SLP25 protease in each purification step was measured by monitoring the increase of absorbancy at 412 nm resulting from the release of p-nitroaniline due to the enzymatic hydrolysis of N-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroaniline (AAPF) at 30°C using a Cary 100 Bio UV–visible spectrophotometer (Varian, Victoria, Australia) equipped with a 6X6 multicell holder (Varian) and thermostatic water bath Lauda K-2/R (Brink Mann Instruments). The reaction was initiated by adding 50 µL of enzyme solution to 0.1 mM of substrate in 50 mM Tris-HCl , pH 8.5 containing 5% (v/v) methanol in a final volume of 1.8 mL. One unit of activity is defined as the amount of enzyme required to produce 1 µmol of *p*-nitroaniline in 1 min under the above conditions.

Tryptic digest and mass spectroscopy analysis

(i) In gel digestion.

The proteins were in-gel digested using Sequencing grade modified trypsin (Promega) according to a protocol adapted from Blum *et al.* (Blum *et al.*, 1987) Briefly, after Coomassie staining, the protein band was cut out from the gel and incubated in 500 µL of 100 mM NH_4HCO_3 for 60 min. Protein was reduced in 150 µL of the same buffer containing 3 mM DTT for 30 min. at 60 °C, then alkylated for 30 min. at room temperature by adding 10 µL of 100 mM iodoacetamide. The gel piece volume was dehydrated by successive incubations in 50% CH_3CN/100 mM NH_4HCO_3, then in 100% CH_3CN and finally dried in a Savant speed-vac. Trypsin solution (0.1 µg/µL) was added to reswell the gel piece and the digestion was performed at 37 °C for 16 hours. The gel piece was centrifuged and the supernatant was collected, remaining peptides were extracted by further washes of the gel with 60% CH_3CN in 0.1% TFA (3x). All supernatants were pooled and the peptides dried by Speed-Vac then resuspended in 1% TFA.

(ii) Nano-ESI-MS/MS analysis and interpretation of the ion spectra from tryptic peptides.

The resulting peptide mixture was desalted and concentrated using ZipTip C18 (Millipore, Boston, MA, USA). Peptides were eluted in 9µL of a solution consisting of 50% (v/v) methanol, and 0.1% acetic acid and sonicated for 5 min before MS analysis. The solution containing tryptic peptides was sprayed into a tandem mass spectrometer (Quattro II, Micromass, Pointe-Claire, Canada) using nanospray capillary tips (D6028627, Micromass, Canada). The mass spectra were acquired in full scan mode. Nitrogen was the drying gas and the capillary and cone voltages were 0.7-1.0 kvolt and 25 volts respectively. Low-energy collision-induced dissociation (CID) experiments

were performed at 1.8x 10^{-3} (units) using argon as the collision gas. The interpretation strategy of product ion mass spectra was built on the characteristics of tandem mass spectra tryptic peptides and determined using the peptide sequencing application of Masslynx software (3.5 version, Micromass Canada).

(iii) Computer search for protein identification

The best amino-acid sequence candidates from the product ion interpretation were used to start database searching. The *S. coelicolor* protein motif search from the Sanger Center server (www.sanger.ac.uk) or peptide search (Peptide Mass fingerprint tool from EMBL Heidelberg, ExPASy server) were used for protein identification. The peptide mapping experiments or the coverage determination was performed using Peptidemass of proteomic tools (ExPASy server).

RESULTS

Construction of pIAF550, amplification and cloning of slp25

The expression vector pIAF550 was constructed as following: the replication origin (2558 bp) was derived from a stable deletion of plasmid pIJ702. The thiostrepton resistance gene was amplified using pIJ702 as template by PCR with the primer TSR5 5'TTTGGTACCTGCAGTTTGCATGCCCGGCCGAGCTCTGATCAAGGCGAATA CTTCA3' which incorporated unique KpnI, PstI, SphI and SacI restriction sites at the 5'end and primer TSR3 5'TTTGGTACCTGATCACGTACGGAATCGAGGTCGAGGA3' to add a KpnI site at the 3' end. The 1052 bp amplicon was cloned into the unique Kpn1 site of the deleted plasmid. Then a DNA fragment of 383 bp containing a mutated promoter of the xlnC gene of Streptomyces lividans and a ribosome binding site located at – 9 nt from the ATG start codon present in the unique Sph1 restriction site was cloned into restriction

sites PstI and SphI which were added into the PCR primer at the 5' end of the tsr gene. The final plasmid is a high copy number expression vector of 4042 bp.

The gene encoding the S. lividans peptidase Slp25 was amplified using primers CD1601, CD1602 and *S. lividans* chomosomal DNA as template. The primers CD1601 allowed the incorporation of a *Sph*I restriction site at the 5' end, while CD1602 incorporated a unique *Sac*I restriction site at the 3' end following the TGA stop codon. The incorporation of unique restriction sites at both ends of the PCR product allowed directional cloning of the DNA fragment into the vector pIAF550 downstream of a strong promoter and a consensus RBS leading to plasmid pIAF2025.

DNA and predicted amino acid sequences analysis

Sequencing of the cloned DNA fragment showed 99.4% identity with the *S. coelicolor* CAB76287 open reading frame from which the putative peptidase encoding gene was originally deduced (Figure 1). This gene encodes a 390 amino acids polypeptide, preproSlp25 of a calculated molecular mass of 40329 Da from which the first 30 amino acids showed a hydrophilic N-terminal core rich in arginine residues (R), followed by a hydrophobic core (A, L and V) and a typical peptidase cleavage site (PAXA) corresponding to a classical *Streptomyces* signal peptide. BLAST analysis (16) of the predicted polypeptide proSlp25 360 amino acid sequence showed high similarity with other members of the clan SA of serine peptidase, more particularly, peptidases that would belong to the S2 family of that clan (Table I). The maturation site was confirmed by the N-terminal sequencing of the mature enzyme to be AAGTVGGDPY. The presence of two residues Ala-190 and Thr-213 (bovine chymotrypsin numbering),

which determine S1 specificity for hydrophobic substrates like *S.griseus* proteases A, B and C (*Sidhu et al., 1993*) are consistent with the phenylalanine N-terminal residue of the premature junction for a self-processing enzyme. Mature SLP25 constitute of the 189 final amino acids and its predicted molecular mass is similar to that of the bacterial chymotrypsin (19,441). Tryptic digest analysis have shown 3 peptides of the mature enzyme and two of these were sequenced by collision-induced dissociation (CID) and are consistent with peptides corresponding to amino acids 1 to 13 and 36 to 41 of the mature enzyme.

Phylogenetic analysis was performed using a variety of approaches from complete prepro-mature sequence presented in Table 1 and mature sequence with and without C-terminal chitin binding domain (like SGPC; data not shown). Results were in agreement with the utilization of complete prepro-mature sequence of the peptidases. The alignment of these proteins was used for pairwise distance calculation and this distance was conserved in the final consensus tree. Bootstrap analysis was used to place the confidence limits on the phylogenetic tree presented in Fig. 2 and 3. The non-significant branch (< 500/1000) was revoked.

The *S. fradiae* trypsin (SfTRY) was used as outgroup for Figure 2. The first analysis of 38 most homologue peptidases of Slp25 groups this secreted S2 enzyme into one distinct cluster with a strong junction (979/1000) to the S1 family peptidase represented by *S. griseus* trypsin (SgTRY) (resultats show for 27 sequences).

In this S2 group, 14 peptidases form a cluster (namely Streptogrisin subfamily) with a high evolution distance to the common ancestor of 13 other proteases. This first cluster forms three groups statistically different and one enzyme stand alone. Other peptidases, including Slp25, share a particular feature: six cysteine residues which contribute to the formation of three disulphide bonds typical of the α-lytic-like proteases (namely α-lytic-like subfamily: Figure 2). In these α-lytic-like proteases, only Slp25 and its *S. coelicolor* homologue (SC10G8.13c), were not grouped in a unique streptomyces cluster. However, this cluster shows a weak statistical support (570/1000) and the other nine peptidases remain ungrouped. To improve the assessment of phylogenic distance evaluation and grouping, a second analysis was processed using only the α-lytic-like protease members and two other peptidases of the first cluster as outgroup and as representative of this cluster respectively (Sfase-1 and CAC14934,). The resulting phylogenetic tree is presented in Figure 3.

This α-lytic-like cluster groups contain peptidases of five different bacterial genera and one eukaryotic genus. All bacterial features of this enzymatic tree are consistent with the 16S rRNA tree (figure 4: gene listed in Table 2). Evolution of this bacterial enzyme group was in accord to the genetic lineage. However, the bootstrap analysis provides a strong statistical support (952/1000) to indicate that the enzymes Slp25 and MaCHY had diverged furthest from the common ancestor than the other streptomyces enzymes. The streptomyces cluster was already distinguishable with a robust junction (960/1000). This α-lytic-like cluster has also a strong junction (994/1000) to the SC5B7.10 peptidase, in agreement with the initial analysis.

On the other hand, Krem and Di Cera (2001), have outlined the use of active site-related residues (ASRR) for the identification of common ancestries. Similarly like them, selections of ASRR (amino acid markers) are processed in phylogenetic tree (figure 2 and 3). The amino acid neighboring the catalytic triad (H32, D60 and S143: mature bovine chymotrypsin numbering) and the nine residues 114 to 122, between the two cysteine residues involved of the third disulphide bond of the α-lytic-like proteases, were used. This method allowed the comparison of incomplete sequences like that of Sfase-2 for which the prepro region is unknown.

These markers tree were generally consistent with the enzymatic tree and the homology between all peptidases revealed a comparable homology between the streptomyces ones and a protease from *M. anisopliae* (67%) with a poorest homology for one cluster formed by the glutamic specific peptidases ((SGPE and SFase-1), *Nocardia* (2) and *Rarobacter* proteases (50 %, 52 %, 35%, 50% and 50% respectively). It is also important to mention that this cluster is not in agreement with the genetic lineage by gathering three various bacterial genus.

Enzyme screening production, purification and characterization

Secretion of active Slp25 was observed on Stewart skim-milk agar by visualizing clearing zones around thiostrepton-resistant colonies. All showed similar clearing zones, therefore, four randomly selected isolates were cultured in liquid media (25 mL) for up to 72 hours and peptidase secretion was monitored using a casein hydrolysis assay. Maximum activity was found after 69 hours of growth (results not shown). One strain was selected, cultured in 2 L flasks and Slp25 recovered from the supernatant by

ultrafiltration followed by affinity chromatography as described above. Monitoring of active fractions was performed by zymogram analysis, which showed a single hydrolysis band on gelatin-containing polyacrylamide gels (result not shown). The production yield was calculated at 2.2 mg of pure enzyme per liter of culture.

SDS-PAGE showed a single band at 20 kDa corresponding to the expected molecular weight of the mature Slp25 (data not shown). This protein was transferred to a PVDF membrane and the N-terminal amino acid sequence was determined by Edman degradation. The sequence AAGTVGGDPY was obtained and therefore identifying the maturation site of the proSlp25. This site corresponded to the amino acid 202 of the preproSlp25 (Figure 1). Tryptic digest of the 20 kDa protein extracted from the SDS-PAGE followed by mass spectroscopy analysis identified ions corresponding to three peptides of the mature Slp25. Collision induced dissociation experiments were performed and sequences of two of the peptides were obtained that confirmed the identity of the mature Slp25 .

Activity parameters were determined using N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroaniline. The enzyme is active over a broad pH range (6 to 9) with 90% of its relative activity found between pH 7.5 and 8.5 (figure 6).

DISCUSSION

Sequence analysis

Serine peptidases, which possess a catalytic triad of serine, histidine and aspartic acid, are classified into seven clans (Barrett et al., 1998). Streptomyces serine peptidases are reported for four of theses clans (SA, SB, SC, and SE) with predominance within clan SA where the S2 family is well represented by the Streptomyces peptidases. These enzymes are translated in the form of pre-pro-mature precursors, with only the mature parts of the polypeptides appearing in culture supernatants (Baardsnes et al., 1998). The high degree of homology between S. coelicolor and S. lividans made possible the isolation of a new gene from S. lividans. The slp25 gene is almost identical to its homologue in S. coelicolor conveying the close relationship of these two species. We can assert that the seven nucleotide differences which result in three amino acid changes in the pro-domain of the predicted protein will not have any impact on the activity of the mature enzyme. The changes E97D and P178R are not in the conserved section of the sequence and V124A is a conserved hydrophobic change (Figure 1).

The Slp25 protein possesses a large propeptide region (171 residues), almost identical to the proregion of the S. griseus protease C (162 residues) and Streptomyces sp. chymotrypsin 1 and II (171 and 168 residues estimated by homology, respectively). Many other streptomyces peptidases have shorter propeptide regions like SGPA and SGPB (78 and 76 amino acid residues respectively), or very short like S. griseus trypsin (4 residues (Kim et al., 1991)). The microbial trypsins and related mammalian enzymes, with very short propeptide regions, are classified in the S1 peptidase family.

Like other bacterial peptidases, the processing of proSlp25 is probably autocatalytic, because the amino acid in Pi position (phenylalanine) is the preferred residue for the chemotrypsin-like peptidases. The presence of this residue in Pi position of Slp25 pro-mature junction underlines its preference of large aromatic or aliphatic residues like SGPA, SGPB, SGPC and SGPD, that contain leucine residues at the equivalent position (Chitte & Dey, 2000; Dammann & Wohlleben, 1992; Sidhu et al., 1993; Sidhu et al., 1995; Suzuki et al., 1997). Assays with substrate bearing leucine or alanine at Pi showed reduced activity in the same proportion of its fungal homologue MaCHY (Screen & St Leger, 2000)(data not shown). The activity of Slp25 in function of the pH and the temperature are also similar with that of MaCHY (Screen & St Leger, 2000).

Amino acid sequence comparison of the Slp25 (BLASTP, NCBI) revealed that it belongs to the α-lytic like endopeptidase of the S2 peptidase family. This group is characterized by three disulphide bonds and by relatively long propeptide regions. If the secreted S1 and S2 serine proteases were diverged early from an endocellular form (Screen et al., 2000), the longer of propeptide region was a strong molecular evolutionary marker of this secreted enzymes group. Moreover, this propeptide region, in the same way of the subtilisin, has co-evolued with the enzyme to finally carry out a folding function (Silen & Agard, 1989; Kitadokoro, K, et al., 1994) and such functional properties seem to be connected with the lifetime of these enzymes in this highly proteolytic extracellular environment (Jaswal et al, 2005).

Finally, the *slp25* gene is physically located downstream of the AUD1 (amplifiable unit of DNA 1) chromosomal region in *S. coelicolor*, we could assume that the encoded

enzyme is not essential for the bacteria survival. The AUD1 chromosomal region being known for its instability, the bacteria could easily lose or rearrange the genes located in that sector of the chromosome (Volff & Altenbuchner, 1998).

Gene duplication

Thus, the phylogenic analysis of S2 family suggests the formation of two phylogenic subfamilies in agreement with the evolutionary importance of the propeptide region. In this S2 group, 15 peptidases form a cluster (namely Streptogrisin subfamily) with a high evolutive distance to the common ancestor of the 13 other proteases. This cluster forms three groups statistically different, including one for the glutamic-acid specific endopeptidase (SGPE and Sfase-1). The bootstrap analysis provides strong support to indicate the validity of this sub-grouping. However, all enzymes arose from a common ancestor within the α-lytic-like cluster.

The grouping of all peptidases presenting two disulphides bridges, with a single ancestor, allowed prediction of duplication of the initial gene associated to the loss of the third pairs of cysteines and one can suppose that this ancestor had three disulphide bonds. However, this raises also the following question: which evolutionary advantage was conferred by the loss of one of the disulphide bridge?

Truhlar and its collaborator (2004), showed that the proregion of *L. enzymogenes* α-lytic protease accelerates folding by a factor of 2 $X10^9$ and the much smaller proregion of SGPB accelerates folding only by a factor of 400. It seems to exist a strong connection between proregion size and folding efficiency like the one reported for

subtilisin proregion (Bryan, 2002). It also seems there is a close link between the number of disulphide bonds and the size of the prodomain. If the size of the prodomain is linked to its folding effectiveness and that having enzymes maintained a structure with three disulphides bonds have also the largest prodomains, we can put forth the assumption that the third bridge disulphide play a role in the establishment of the unfolding free energy barriers as in the case of the α-lytic proteases (Truhlar et al, 2004; Jaswal et al, 2005). This evolutionary adaptation would have conferred diversity in kinetic of folding allowing a faster folding up, possibly with disadvantage of a poorer stability.

The presences of the *S. hygroscopiccus enzyme* with three bonds among the streptogrisin with two bonds support this assumption. This enzyme would come from an ancestor common to the group which would have presented two disulphides bonds, probably by duplication even, would have evolved to a structure with three bonds to meet its stability need of its thermophilic environment. A study on folding and unfolding kinetic of this enzyme could allow to evaluate if there were a co-evolution of its proregion as folding catalysis was consistent with the L. enzymogenes α-lytic protease.

Recombination even

Our phylogenetic analysis allowed us to link the C-terminal extension of *S. griseus* protease C to six homologues, which also carry this binding domain. For SGPC, this binding domain was associated with two chitinase binding domains of *Bacillus circulans*, suggesting that the enzyme is specialized for the degradation of chitin-linked protein (Sidhu *et al.*, 1994)), and were classified in the family 12 of the carbohydrate-

binding modules (CBM 12: CAZy databank (http://afmb.cnrs-mrs.fr/CAZY). Interestingly, these seven enzymes of the streptomyces α-lytic like subfamily form a subgroup having a common ancestor with *Streptomyces sp.* chymotrypsin I and II (SspCHYI and II). This enables the hypothesis that the acquisition of this domain is a relatively recent event, which occurred after species differentiation. Since chitinases are present in many streptomyces species, it is possible that the origin of a chitin-binding domain linked to a proteolytic domain was a recombination event in a polygenomic mycelial bacteria like streptomyces. We find this CBM12 domain within four different *Streptomyces* strains, one could assume that attachment of peptidase to polysaccharidic substrates will give an evolutionary advantage to the strains harboring this feature. Like Miyamoto and its collaborators (2002), we assume that both chitinases and proteases could be necessary for efficient degradation of chitin structure and the protease with chitin binding domain are a convergent even in multiple protease family like subtilisin and chymotrypsin. In the midst of connect the ecological features to genes; one could name this case of "Ecological convergence of recombination". Similarly, the RFRPI, a yeast-lytic α-lytic like serine protease, have a C-terminal domain with a mannose binding activity. This mannose-binding activity is required for its yeast-lytic activity (Shimoi H. et al, 1992). Then, this binding domain acquisition by a protease can be associated with more than one function and could play a major role in the adaptation of organisms.

Lateral transfert

Similarly to the eukaryotic peptidase of *M. anisopliae* (MaCHY), which was assigned to the prokaryotic S2 enzymes by phylogenic analysis (Screen & St Leger, 2000), Slp25

was the only one of streptomyces α-lytic like protease not included in the same streptomyces cluster. In this group, Slp25 diverged further with MaCHY from the common ancestor. The active site-related residues (ASRR) analysis reinforces the hypothesis of Screen *et al.*,(Screen & St Leger, 2000) for lateral gene transfer from an actinomycete to a eukaryote for the *M.anisopliae* α-lytic like protease. The general homology and phylogenetic analysis of this ASRR marker are also in accord with the phylogenetic 16S rRNA lineage distance between the different species represented. However, this raise also the following question: why the Slp25 peptidase and it's transfer homologue are not in the streptomyces cluster?

One could believe, because of its location downstream of the AUD1 (amplifiable unit of DNA 1) chromosomal region and the absence of Slp25 homologue in the *S.avermitilis* genome, that the *slp25* gene could have an extra-chromosomal origin. The absence of more recent streptomyces counterpart of Slp25, than *S.coelicolor* homologue, lets suppose that it is a gene of another origin, within the Actinobacteria. According to this assumption, the phylogenetic position of SlpP25 would be an indication of the distance, in the genetic lineage, of the donor of this gene at *S .lividans / S. coelicolor.* The analysis of ASRR marker are also in accord with this assumption but the discovery of new proteolytic enzymes will be necessary to elucidate the real origin of Slp25.

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FIGURE LEGENDS.

Figure 1. Nucleotide and predicted amino acid sequence of *slp25* gene in comparison with homologous gene of *S. coelicolor* (*Sc10G8.13C*). The differences, nucleotides and amino acids, are shaded. The putative signal peptide is underline. The first's amino acids of the mature SLP25 and the putative active site residues are in bold. The nucleotide sequence of *slp25* has been deposited in GenBank (accession number A4259141)

Figure 2. Phylogenic trees of SLP25 and other homologous peptidases: S2 family peptidases phylogenic tree.

Figure 3. S2 subfamily α-lyric like peptidases phylogenic tree.

Figure 4. 16S rRNA gene phylogenic trees.

Figure 5. Partial amino acid sequence alignments of S2 peptidase family member showing the conserved cysteine residues: (*) indicated cysteine residues conserved for all member of S2 family. (↓) indicated extra residues conserved in the α-lytic like subgroup. Identification of proteins is listed in table I.

Figure 6. Activity profile of SPL25 as a function of pH (panel A) and as a function of temperature (panel B)

Table I: List of encoding gene and corresponding protein showing homnology with peptidase

Organism	Gene	Protein	Accession number	Reference
S. lividans	***slp25***	**Slp25**		this paper
S. coelicolor	***sc10G8.13c***	**putative**	CAB76287	(Bentley *et al.*, 2002)
S. coelicolor	***scd840A.16c***	**putative**	CAB81861	(Bentley *et al.*, 2002)
S. lividans	***spC***	**putative**	CAD42809	direct submission
S. sp	***sapI***	**SspCHYI**	CAA52206	(Yum *et al.*, 1994)
S. sp	***sapII***	**SspCHYII**	CAA52205	(Yum *et al.*, 1994)
M.anisopliae	***chy1***	**MaCHY**	CAB60729	(Screen & St Leger, 2000)
S. griseus	***sprC***	**SGPC**	AAA26813	(Sidhu *et al.*, 1994)
S. fradiae	***spI***	**SfspI**	CAH04620	direct submission
N. sp.	***NAPase***	**NAPase**	AAO06113	direct submission
S. coelicolor	***scf43A.19***	**putative**	CAB48906	(Bentley *et al.*, 2002)
S. avermitilis	***sprC***	**putative**	BAC68710	(Omura *et al.*, 2001)
S. lividans	***spB***	**putative**	CAD42808	direct submission
T. fusca		**TfCHY**	AAC23545	(Lao & Wilson, 1996)
S. coelicolor	***scI11.30c***	**putative**	CAB50956	(Bentley *et al.*, 2002)
S. avermitilis	***sprB***	**putative**	BAC74264	(Omura *et al.*, 2001)
S. avermitilis	***sprA***	**putative**	BAC74263	(Omura *et al.*, 2001)
L. enzymogenes		**LeCHY**	AAA74111	(Silen *et al.*, 1988; Silen *et al.*, 1989)
S. albogriseolus	***SAM-p20D***	**SaCHY**	BAA21784	(Taguchi *et al.*, 1997)
S. sp.	***sfp***	**SspSFP**	AAM96214	(Gong & Wang, 2001)
S. griseus	***sprD***	**SGPD**	AAA74409	(Sidhu *et al.*, 1995)
S. hygroscopicus	***ORF6***	**putative**	AAO61210	direct submission
R. faecitabidus	***rpI***	**RfRPI**	BAA01585	(Shimoi *et al.*, 1992)
S. coelicolor	***sSc5B7.10***	**putative**	CAC14934	(Bentley *et al.*, 2002)
S. griseus	***sprB***	**SGPB**	AAA26819	(Henderson *et al.*, 1987)
S. fradiae	***sfp2***	**SFP2**	CAH05008	direct submission
S. griseus	***sprE***	**SGPE**	AAA19747	(Sidhu *et al.*, 1993)
S. coelicolor	***scf81.11c***	**putative**	CAB61552	(Bentley *et al.*, 2002)
S. avermitilis		**putative**	BAC75208	(Omura *et al.*, 2001)
S. griseus	***sprA***	**SGPA**	AAA26818	(Henderson *et al.*, 1987)
S. coelicolor	***scI11.35c***	**putative**	CAB50961	(Bentley *et al.*, 2002)
S. albogriseolus	***SAM-p20***	**Sa**	BAA06163	(Taguchi *et al.*, 1995b)
S. lividans	***salo***	**SALO**	BAA08785	(Taguchi *et al.*, 1995a)
S. fradiae	***Sfase-1***	**SfCHY I**	BAA02038	(Kitadokoro *et al*, 1993)
S. fradiae	***Sfase-2***	**SfCHY II**	AAB30080	(Kitadokoro *et al*, 1994)

Table II: 16S rRNA gene used for phylogenetic lineage analysis

Organism	Gene bp	Match	Non Match	% Match	Accession number
L. enzymogenes	***1509***	**1179**	388	75	AAA74111
N.farcinica	***1527***	**1379**	158	89	AP006618
R.faecitabidus	***1503***	**1362**	164	89	AB056128
S. fradiae	***1523***	**1481**	43	97	AF290616
S. albogriseolus	***1519***	**1477**	42	97	AJ494865
S. coelicolor	***1551***	**1480**	71	95	X60514
S. lividans	***1531***	**1484**	47	96	Y00484
S. griseus	***1528***	**1468**	63	95	M76388
S. hygroscopicus	***1483***	**1428**	95	93	AJ391819
S. avermitilis	***1517***	**1467**	52	96	AF145223
T. fusca	***1488***	**1306**	235	84	AB006171
N.metallicus	***1488***	**1306**	236	84	AJ420769

slp25 (1) GCATGCGACGCAGCAGACTCAGGCACCTCGGCCTGGCCGGACTCCTCGTGCTCGGCAGCCTCACCGCGGTC
sc10G8.13c (1) GTGCGACGCAGCAGACTCAGGCACCTCGGCCTGGCCGGACTCCTCGTGCTCGGCAGCCTCACCGCGGTC
M R R S R L R H L G L A G L L V L G S L T A V (23)

slp25 (72) GGCACCCTGCCGGCCTCGGCGGCCGACGCCCCCGCGCACTCCCCCTCCTCCTCCGAGCAGCCGGCCTCCGCC
sc10G8.13c (70) GGCACCCTGCCGGCCTCGGCGGCCGACGCCCCCGCGCACTCCCCCTCCTCCTCGGAGCAGCCGGCCTCCGCC
G T L P A S A A D A P A H S P S S S E Q P A S A (47)

slp25 (144) GGTCTGCTGGACGCGATGCAGCGGGACTTCGGCCTCACCCGGGCCGAGGCCGAGGACCGGCTCGCGGCCGAA
sc10G8.13c (142) GGTCTGCTGGACGCGATGCAACGGGACTTCGGCCTCACCCGGGCCGAGGCCGAGGACCGGCTCGCGGCCGAA
G L L D A M Q R D F G L T R A E A E D R L A A E (71)

slp25 (216) CGCGAAGCCACCGGCATCGAGCCCGCGGCACGCCGGGCGGCCGGAAAGGCCTTCGGCGGTTCGTGGTTCGAC
sc10G8.13c (214) CGCGAAGCCACCGGCATCGAGCCCGCGGCACGCCGGGCGGCCGGAAAGGCCTTCGGCGGTTCGTGGTTCGAC
R E A T G I E P A A R R A A G K A F G G S W F D (95)

slp25 (288) GCGGACGCCGGGCGGCTGACGGTGGCCGTCACCTCGGACGCGACACCCTCGGCCCTGCGGGCGGTCCGCGCC
sc10G8.13c (286) GCGGAAGCCGGGCGGCTGACGGTGGCCGTCACCTCGGACGCGACACCCTCGGCCCTGCGGGCGGTCCGCGCC
A D A G R L T V A V T S D A T P S A L R A V R A (119)

slp25 (360) ACCGGCGCGCAGGCCCGCACCGTGGAGCACAGCGCACAGCAGCTCGACGCGGCCAAGGCGCGCGTCGACCGT
sc10G8.13c (358) ACCGGCGCGCAGGTCCGCACCGTGGAGCACAGCGCACAGCAGCTCGACGCGGCCAAGGCGCGCGTCGACCGT
T G A Q A R T V E H S A Q Q L D A A K A R V D R (143)

slp25 (432) CTCGACGCACCGGCCGGAGTGAGCAGCTGGCGCGCCGACCGCGCCACCAACAAGGTCGTCGTGGACGTGGTC
sc10G8.13c (430) CTCGACGCACCGGCCGGAGTGAGCAGCTGGCGCGCCGACCGGGCCACCAACAAGGTCGTCGTGGACGTGGTC
L D A P A G V S S W R A D R A T N K V V V D V V (167)

slp25 (504) GCGGGCGAGCGCGCTGACAACGATGTCCAGAGCTTCCTGAAGCAGGCCCGCGCGACCGCGCGCGTCACGGTG
sc10G8.13c (502) GCGGGCGAGCGCGCTGACAACGATGTCCAGAGCTTCCTGAAGCAGGCCCGCGCGACCGCGCCGGTCACGGTG
A G E R A D N D V Q S F L K Q A R A T A R V T V (191)

slp25 (576) CGGACGGTGCCGTCGGCCCCGCAGACCTTCGCGGCGGGCACCGTCGGCGGTGACCCCTACTACACCGGCAAC
sc10G8.13c (574) CGGACGGTGCCGTCGGCCCCGCAGACCTTCGCGGCGGGCACCGTCGGCGGTGACCCCTACTACACCGGCAAC
R T V P S A P Q T F **A A G T V G G D P Y** Y T G N (215)

slp25 (648) GTCCGCTGCTCCATCGGCTTCTCGGTGCACGGCGGGTTCGTCACCGCCGGGCACTGCGGCCGGGCCGGCGCC
sc10G8.13c (646) GTCCGCTGCTCCATCGGCTTCTCGGTGCACGGCGGGTTCGTCACCGCCGGGCACTGCGGCCGGGCCGGCGCC
V R C S I G F S V H G G F V T A G **H** C G R A G A (239)

slp25 (720) GGGGTCAGCGGCTGGGACCGCTCCTACATCGGCACCTTCCAGGGCTCGTCCTTCCCCGACAACGACTACGCG
sc10G8.13c (718) GGGGTCAGCGGCTGGGACCGCTCCTACATCGGCACCTTCCAGGGCTCGTCCTTCCCCGACAACGACTACGCG
G V S G W D R S Y I G T F Q G S S F P D N **D** Y A (263)

slp25 (792) TGGGTCAGCGTGGGCAGCGGCTGGTGGACGGTGCCGGTCGTGCTGGGCTGGGGCACCGTCTCCGACCAGCTC
sc10G8.13c (790) TGGGTCAGCGTGGGCAGCGGCTGGTGGACGGTGCCGGTCGTGCTGGGCTGGGGCACCGTCTCCGACCAGCTC
W V S V G S G W W T V P V V L G W G T V S D Q L (287)

slp25 (864) GTGCGCGGCTCGAACGTGGCACCCGTCGGTGCCTCGATCTGCCGCTCCGGGTCGACCACGCACTGGCACTGC
sc10G8.13c (862) GTGCGCGGCTCGAACGTGGCACCCGTCGGTGCCTCGATCTGCCGCTCCGGGTCGACCACGCACTGGCACTGC
V R G S N V A P V G A S I C R S G S T T H W H C (311)

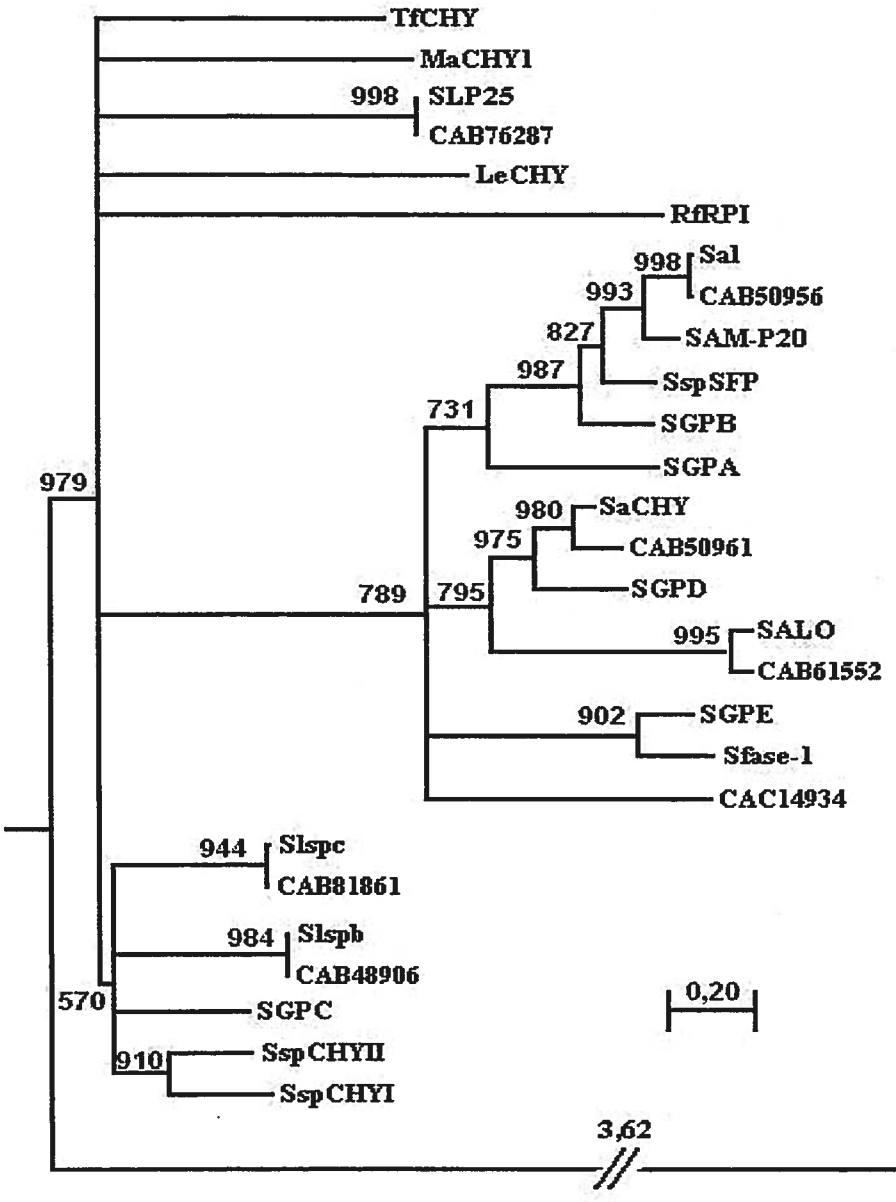
slp25 (936) GGCACCGTGCTCGCCCACAACGAGACGGTCAACTACAGCGACGGCTCGGTGGTGCACCAGCTGACCAAGACC
sc10G8.13c (934) GGCACCGTGCTCGCCCACAACGAGACGGTCAACTACAGCGACGGCTCGGTGGTGCACCAGCTGACCAAGACC
G T V L A H N E T V N Y S D G S V V H Q L T K T (335)

slp25 (1008) AGTGTGTGCGCCGAGGGCGGCGACTCCGGCGGCTCCTTCATCAGCGGTGACCAGGCGCAGGGCGTCACCTCG
sc10G8.13c (1006) AGTGTGTGCGCCGAGGGCGGCGACTCCGGCGGCTCCTTCATCAGCGGTGACCAGGCGCAGGGCGTCACCTCG
S V C A E G G D **S** G G S F I S G D Q A Q G V T S (359)

slp25 (1080) GGCGGCTGGGGCAACTGCTCCAGCGGCGGGGAGACCTGGTTCCAGCCGGTCAACGAGATCCTCAACCGCTAC
sc10G8.13c (1078) GGCGGCTGGGGCAACTGCTCCAGCGGCGGGGAGACCTGGTTCCAGCCGGTCAACGAGATCCTCAACCGCTAC
G G E T W F Q P V N E I L N R Y G G W G N C S S (383)

slp25 (1152) GGCCTGACCCTGCACACGGCCTGAGAGCTCTGAA
sc10G8.13c (1150) GGCCTGACCCTGCACACGGCCTGA
G L T L H T A - (390)

Figure 1. Nucleotide and predicted amino acid sequence of *slp25* gene in comparison with homologous gene of *S. coelicolor* (*Sc10G8.13C*).



Active site-related amino acid residues

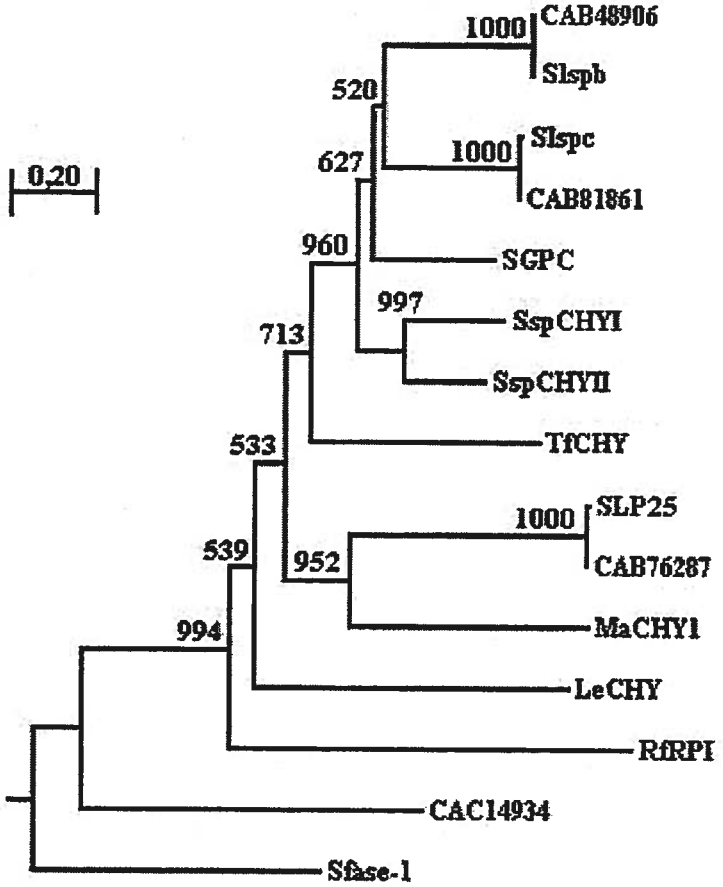
Histidine 32 *	Aspartic.A 60 *	Serine 143 *	114-122
FLTAGHCT	NDYGIVQY	AEPGDSGGS	RDGSTTGTH
FLTAGHCT	NDYGLVKY	AEPGDSGGS	RSGSTTQVH
FLTAGHCT	NDYGIVRY	AEPGDSGGP	RDGSTTGTH
FLTAGHCT	NDYGLVRY	AEPGDSGGP	RRGSTTGTH
FLTAGHCT	NDYGIVRY	AEPGDSGGP	RRGSTTGTH
ALTAGHCT	NDYGIIRH	AEPGDSGGS	RSGSTTGLR
FLTAGHCT	DDYGLVKY	AEPGDSGGS	RSGSTTQVH
FLTAGHCT	NDYGIVQY	AEPGDSGGS	RDGSTTGTH
FLTAGHCT	NDYGLVKY	AEPGDSGGA	RSGSTTQVH
FLTRGHCG	GDFGLVRY	AEPGDSGGS	RMGSTTGLA
FLTAGHCG	GDFALVRY	AEPGDSGGS	RMGSTTGLA
FVTAGHCT	NDYGIVRY	SAGGDSGGA	KSGRTTKTV
FLTAGHCT	NDYGIVRY	SAGGDSGGA	KSGSTTKVT
ILTAGHCG	DDFSLVEY	AEPGDSGGP	RSGSTSGLR

Streptogrisin subfamily (two disulphide bonds and propeptide of 68 to 141 residues)

Figure 2. Phylogenic trees of SLP25 and other homologous peptidases: S2 family peptidases phylogenic tree.

α-Lytic like subfamily

(three disulphide bonds and propeptide of 162 to 171 residues)



Histidine 32 *	Asp. Acid 60 *	Serine 143 *	114-122
FATAGHCG	KDMAWVGV	AEPGDSGGP	RSGSTTGWH
FATAGHCG	KDMAWVGV	AEPGDSGGP	RSGSTTGWH
FATAGHCD	RDMAWVAT	AEPGDSGGS	RSGSTTGWH
FATAGHCG	RDMAWVAT	AEPGDSGGS	RSGSTTGWH
FATAGHCG	RDIAWVAT	AEPGDSGGS	RSGSTTGWH
FVTAGHCG	RDMAWVAV	AQPGDSGGS	RSGSTTGWH
FATAGHCG	DDMAWVSV	AEPGDSGGS	RSGSTTGWR
FATAGHCG	RDMGWVRI	AEGGDSGGP	RSGATTGWR
FVTAGHCG	NDYAWVSV	AEGGDSGGS	RSGSTTHWH
FVTAGHCG	NDYAWVSV	AEGGDSGGS	RSGSTTHWH
FVSAGHCG	ADMAYVRT	AEPGDSGGS	RSGSTTQVH
FVTAGHCG	NDRAWVSL	MGRGDSGGS	RSGRTTGYQ
FLTAGHCA	IDAAWAKN	ALGGDSGGA	KSGRTTKWT
ILTAGHCG	DDFSLVEY	AEPGDSGGP	RSGSTSGLR
FLTAGHCT	NDYGIVRY	SAGGDSGGA	KSGSTTKVT

Streptomyces

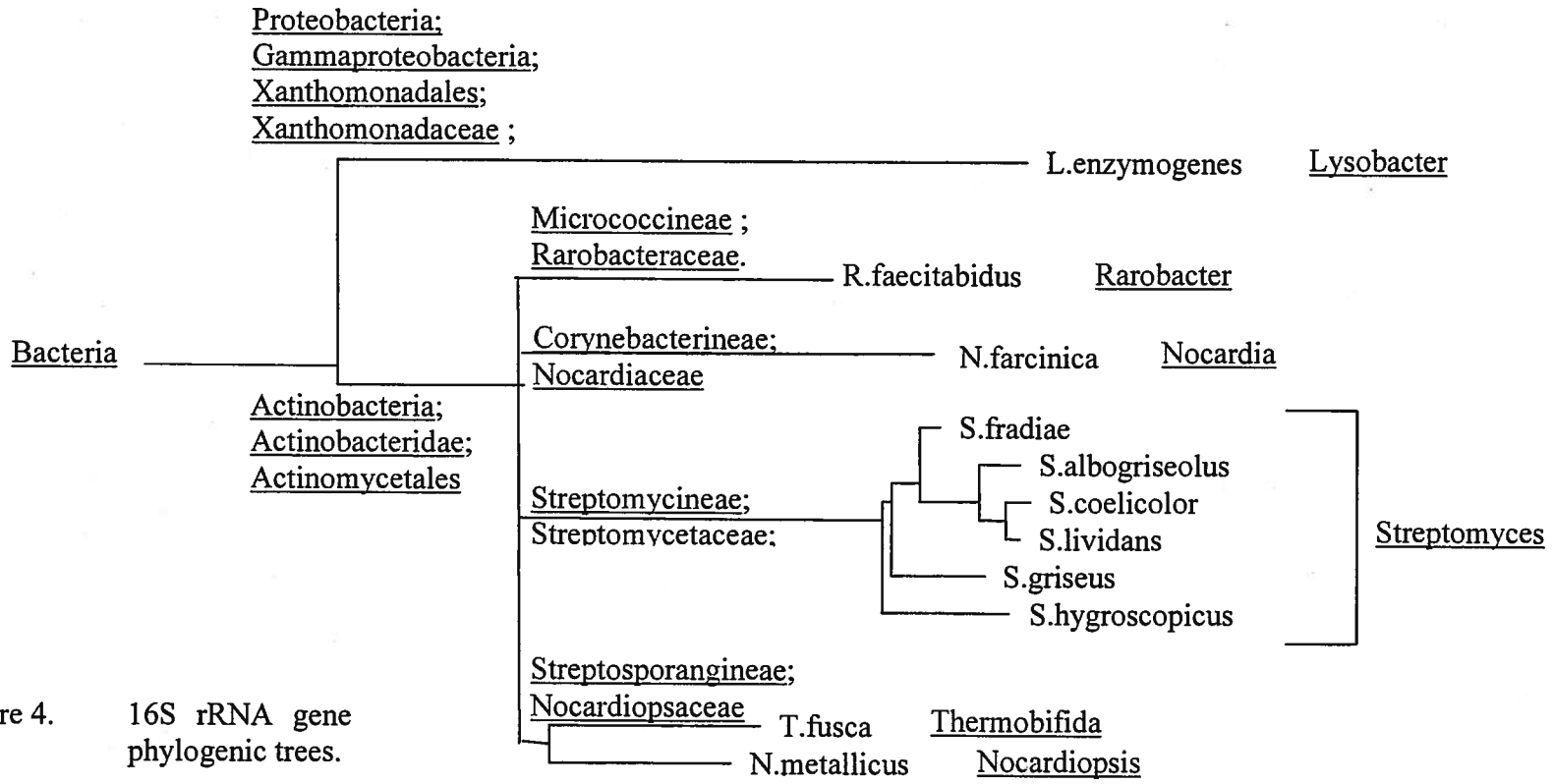


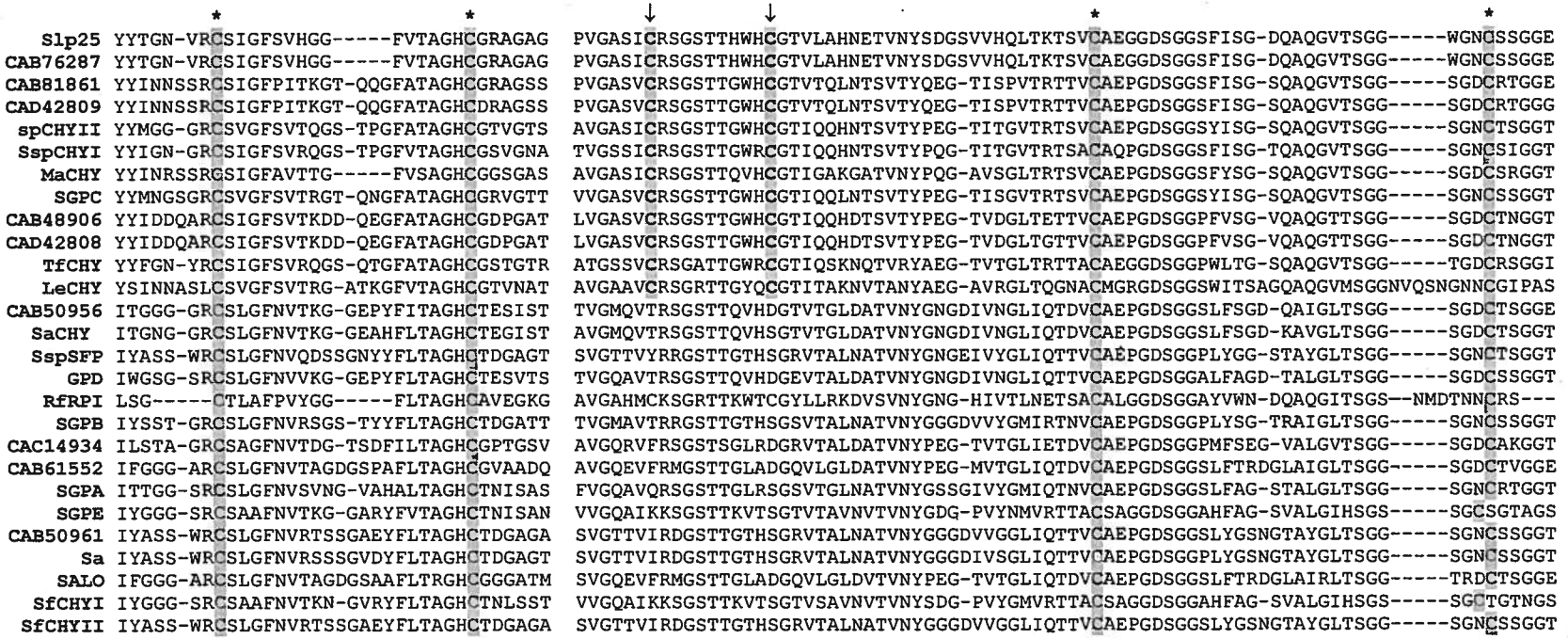
Figure 4. 16S rRNA gene phylogenic trees.

A)

```
                                  *                   *
   Slp25 (174) GTVGGDPYYTGN-VRCSIGFSVHGG-----FVTAGHCGRAGAGVSGWDR-
CAB76287 (174) GTVGGDPYYTGN-VRCSIGFSVHGG-----FVTAGHCGRAGAGVSGWDR-
CAB81861 (164) DLRGGEAYYINNSSRCSIGFPITKGT-QQGFATAGHCGRAGSSTTGANR-
CAD42809 (164) DLRGGEAYYINNSSRCSIGFPITKGT-QQGFATAGHCDRAGSSTTGANR-
 spCHYII (168) DLVGGDAYYMGG-GRCSVGFSVTQGS-TPGFATAGHCGTVGTSTTGYNQ-
 SspCHYI (173) DLVGGDAYYIGN-GRCSIGFSVRQGS-TPGFVTAGHCGSVGNATTGFNR-
   MaCHY (169) TVQGGDVYYINRSSRCSIGFAVTTG-----FVSAGHCGGSGASATTSSG-
    SGPC (164) DIRGGDAYYMNGSGRCSVGFSVTRGT-QNGFATAGHCGRVGTTTNGVNQ-
CAB48906 (177) DLVGGDAYYIDDQARCSIGFSVTKDD-QEGFATAGHGGDPGATTTGYNE-
CAD42808 (164) DLVGGDAYYIDDQARCSIGFSVTKDD-QEGFATAGHCGDPGATTTGYNE-
   TfCHY (153) AIIGGNPYYFGN-YRCSIGFSVRQGS-QTGFATAGHCGSTGTRVS-----
CAB50956 (133) FVAGGDAITGGG-GRCSLGFNVTKG-GEPYFITAGHCTESISTWSDSSG-
   LeCHY (168) NIVGGIEYSINNASLCSVGFSVTRG-ATKGFVTAGHCGTVNATARIGG--
   SaCHY  (68) FIAGGDAITGNG-GRCSLGFNVTKG-GEAHFLTAGHCTEGISTWSDSSG-
  SspSFP  (76) LISGGDAIYASS-WRCSLGFNVQDSSGNYYFLTAGHCTDGAGTWWSNSSH
    GPD (140) LIAGGDAIWGSG-SRCSLGFNVVKG-GEPYFLTAGHCTESVTSWSDTQGG
   RfRPI (181) DYWGGDALSG-----CTLAFPVYGG-----FLTAGHCAVEGKGHILKTEM
    SGPB  (76) LISGGDAIYSST-GRCSLGFNVRSGS-TYYFLTAGHCTDGATTWWANSAR
CAC14934 (132) RLNGAEPILSTA-GRCSAGFNVTDG-TSDFILTAGHCGPTGSVWFGDRPG
CAB61552 (149) FASGGDAIFGGG-ARCSLGFNVTAGDGSPAFLTAGHCGVAADQWSDAQGG
    SGPA  (78) LIAGGEAITTGG-SRCSLGFNVSVNG-VAHALTAGHCTNISASWS-----
    SGPE (139) EVLGGGAIYGGG-SRCSAAFNVTKG-GARYFVTAGHCTNISANWSASSGG
CAB50961  (77) LIQGGDAIYASS-WRCSLGFNVRTSSGAEYFLTAGHCTDGAGAWRASSG-
      Sa  (77) LIQGGDAIYASS-WRCSLGFNVRSSSGVDYFLTAGHCTDGAGTWYSNSAR
    SALO (117) FASGGDAIFGGG-ARCSLGFNVTAGDGSAAFLTRGHCGGGATMWSDAQGG
  SfCHYI (141) EVAGGDAIYGGG-SROSAAFNVTKN-GVRYFLTAGHCTNLSSTWSSTSGG
 SfCHYII  (77) LIQGGDAIYASS-WRCSLGFNVRTSSGAEYFLTAGHCTDGAGAWRASSG-
```

Figure 5 A and B. Partial amino acid sequence alignments of S2 peptidase family member showing the conserved cysteine residues: (*) indicated cysteine residues conserved for all member of S2 family. (↓) indicated extra residues conserved in the α-lytic like subgroup. Identification of proteins is listed in table I.

B)


Slp25 YYTGN-VRCSIGFSVHGG-----FVTAGHCGRAGAG PVGASICRSGSTTHWHCGTVLAHNETVNYSDGSVVHQLTKTSVCAEGGDSGGSFISG-DQAQGVTSGG-----WGNCSSGGE
CAB76287 YYTGN-VRCSIGFSVHGG-----FVTAGHCGRAGAG PVGASICRSGSTTHWHCGTVLAHNETVNYSDGSVVHQLTKTSVCAEGGDSGGSFISG-DQAQGVTSGG-----WGNCSSGGE
CAB81861 YYINNSSRCSIGFPITKGT-QQGFATAGHCGRAGSS PVGASVCRSGSTTGWHCGTVTQLNTSVTYQEG-TISPVTRTTVCAEPGDSGGSFISG-SQAQGVTSGG-----SGDCRTGGE
CAD42809 YYINNSSRCSIGFPITKGT-QQGFATAGHCDRAGSS PVGASVCRSGSTTGWHCGTVTQLNTSVTYQEG-TISPVTRTTVCAEPGDSGGSFISG-SQAQGVTSGG-----SGDCRTGGG
spCHYII YYMGG-GRCSVGFSVTQGS-TPGFATAGHCGTVGTS AVGASICRSGSTTGWHCGTIQQHNTSVTYPEG-TITGVTRTSVCAEPGDSGGSYISG-SQAQGVTSGG-----SGNCTSGGT
SspCHYI YYIGN-GRCSIGFSVRQGS-TPGFVTAGHCGSVGNA TVGSSICRSGSTTGWRCGTIQQHNTSVTYPQG-TITGVTRTSACAQPGDSGGSFISG-TQAQGVTSGG-----SGNCSIGGT
MaCHY YYINRSSRGSIGFAVTTG-----FVSAGHCGGSGAS AVGASICRSGSTTQVHCGTIGAKGATVNYPQG-AVSGLTRTSVCAEPGDSGGSFYSG-SQAQGVTSGG-----SGDCSRGGT
SGPC YYMNGSGRCSVGFSVTRGT-QNGFATAGHCGRVGTT VVGASVCRSGSTTGWHCGTIQQLNTSVTYPEG-TISGVTRTSVCAEPGDSGGSYISG-SQAQGVTSGG-----SGNCSSGGT
CAB48906 YYIDDQARCSIGFSVTKDD-QEGFATAGHCGDPGAT LVGASVCRSGSTTGWHCGTIQQHDTSVTYPEG-TVDGLTETTVCAEPGDSGGPFVSG-VQAQGTTSGG-----SGDCTNGGT
CAD42808 YYIDDQARCSIGFSVTKDD-QEGFATAGHCGDPGAT LVGASVCRSGSTTGWHCGTIQQHDTSVTYPEG-TVDGLTGTTVCAEPGDSGGPFVSG-VQAQGTTSGG-----SGDCTNGGT
TfCHY YYFGN-YRCSIGFSVRQGS-QTGFATAGHCGSTGTR ATGSSVCRSGATTGWRCGTIQSKNQTVRYAEG-TVTGLTRTTACAEGGDSGGPWLTG-SQAQGVTSGG-----TGDCRSGGI
LeCHY YSINNASLCSVGFSVTRG-ATKGFVTAGHCGTVNAT AVGAAVCRSGRTTGYQCGTITAKNVTANYAEG-AVRGLTQGNACMGRGDSGGSWITSAGQAQGVMSGGNVQSNGNNCGIPAS
CAB50956 ITGGG-GRCSLGFNVTKG-GEPYFITAGHCTESIST TVGMQVTRSGSTTQVHDGTVTGLDATVNYGNGDIVNGLIQTDVCAEPGDSGGSLFSGD-QAIGLTSGG-----SGDCTSGGE
SaCHY ITGNG-GRCSLGFNVTKG-GEAHFLTAGHCTEGIST AVGMQVTRSGSTTQVHSGTVTGLDATVNYGNGDIVNGLIQTDVCAEPGDSGGSLFSGD-KAVGLTSGG-----SGDCTSGGT
SspSFP IYASS-WRCSLGFNVQDSSGNYYFLTAGHQTDGAGT SVGTTVYRRGSTTGTHSGRVTALNATVNYGNGEIVYGLIQTTVCAEPGDSGGPLYGG-STAYGLTSGG-----SGNCTSGGT
GPD IWGSG-SRCSLGFNVVKG-GEPYFLTAGHCTESVTS TVGQAVTRSGSTTQVHDGEVTALDATVNYGNGDIVNGLIQTTVCAEPGDSGGALFAGD-TALGLTSGG-----SGDCSSGGT
RfRPI LSG-----CTLAFPVYGG-----FLTAGHCAVEGKG AVGAHMCKSGRTTKWTCGYLLRKDVSVNYGNG-HIVTLNETSACALGGDSGGAYVWN-DQAQGITSGS--NMDTNNCRS---
SGPB IYSST-GRCSLGFNVRSGS-TYYFLTAGHCTDGATT TVGMAVTRRGSTTGTHSGSVTALNATVNYGGGDVVYGMIRTNVCAEPGDSGGPLYSG-TRAIGLTSGG-----SGNCSSGGT
CAC14934 ILSTA-GRCSAGFNVTDG-TSDFILTAGHCGPTGSV AVGQRVFRSGSTSGLRDGRVTALDATVNYPEG-TVTGLIETDVCAEPGDSGGPMFSEG-VALGVTSGG-----SGDCAKGGT
CAB61552 IFGGG-ARCSLGFNVTAGDGSPAFLTAGHCGVAADQ AVGQEVFRMGSTTGLADGQVLGLDATVNYPEG-MVTGLIQTDVCAEPGDSGGSLFTRDGLAIGLTSGG-----SGDCTVGGE
SGPA ITTGG-SRCSLGFNVSVNG-VAHALTAGHCTNISAS FVGQAVQRSGSTTGLRSGSVTGLNATVNYGSSGIVYGMIQTNVCAEPGDSGGSLFAG-STALGLTSGG-----SGNCRTGGT
SGPE IYGGG-SRCSAAFNVTKG-GARYFVTAGHCTNISAN VVGQAIKKSGSTTKVTSGTVTAVNVTVNYGDG-PVYNMVRTTACSAGGDSGGAHFAG-SVALGIHSGS-----SGCSGTAGS
CAB50961 IYASS-WRCSLGFNVRTSSGAEYFLTAGHCTDGAGA SVGTTVIRDGSTTGTHSGRVTALNATVNYGGGDVVGGLIQTTVCAEPGDSGGSLYGSNGTAYGLTSGG-----SGNCSSGGT
Sa IYASS-WRCSLGFNVRSSSGVDYFLTAGHCTDGAGT SVGTTVIRDGSTTGTHSGRVTALNATVNYGGGDIVSGLIQTTVCAEPGDSGGPLYGSNGTAYGLTSGG-----SGNCSSGGT
SALO IFGGG-ARCSLGFNVTAGDGSAAFLTRGHCGGGATM SVGQEVFRMGSTTGLADGQVLGLDVTVNYPEG-TVTGLIQTDVCAEPGDSGGSLFTRDGLAIRLTSGG-----TRDCTSGGE
SfCHYI IYGGG-SRCSAAFNVTKN-GVRYFLTAGHCTNLSST VVGQAIKKSGSTTKVTSGTVSAVNVTVNYSDG-PVYGMVRTTACSAGGDSGGAHFAG-SVALGIHSGS-----SGCTGTNGS
SfCHYII IYASS-WRCSLGFNVRTSSGAEYFLTAGHCTDGAGA SVGTTVIRDGSTTGTHSGRVTALNATVNYGGGDVVGGLIQTTVCAEPGDSGGSLYGSNGTAYGLTSGG-----SGNCSSGGT

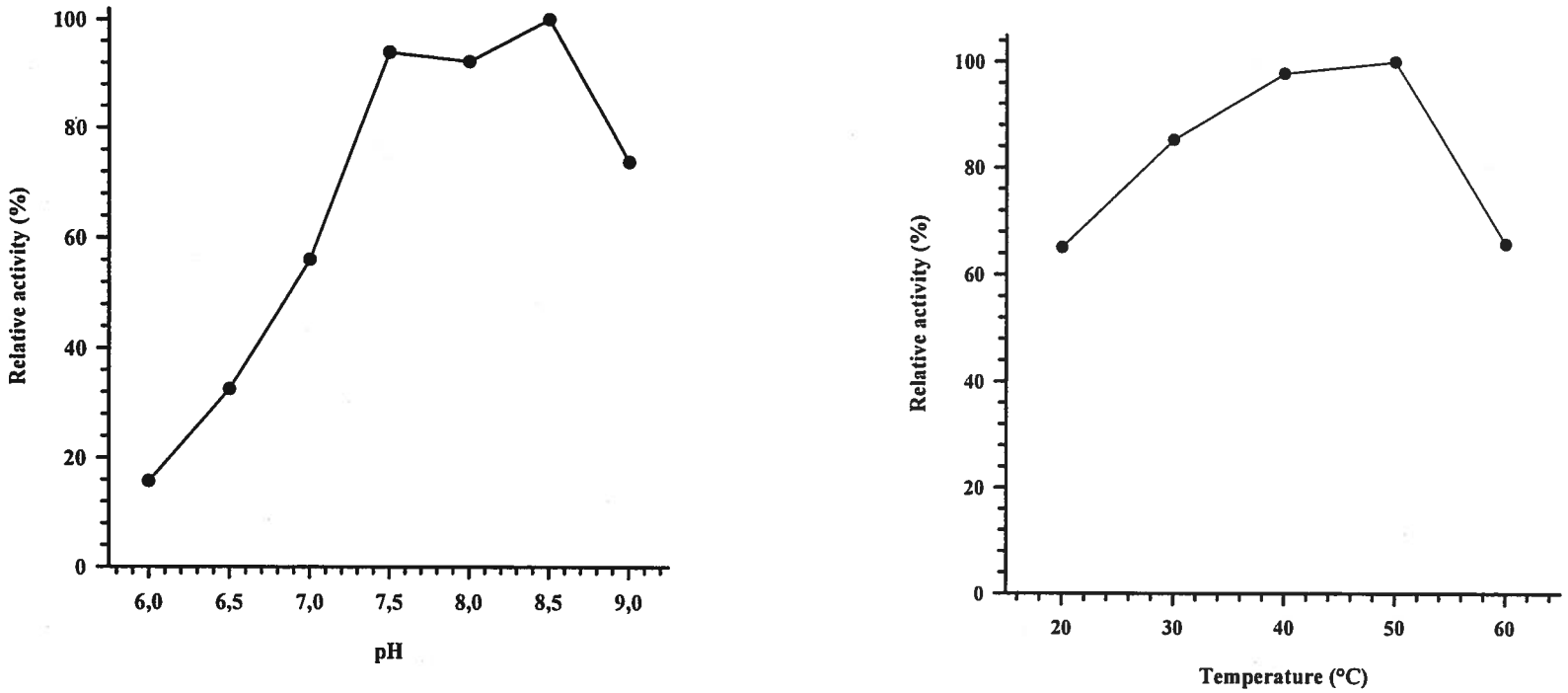


Figure 6. Activity profile of SPL25 as a function of pH (panel A) and as a function of temperature (panel B)

REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). **Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.** Nucleic Acids Res 25, 3389-3402.

Baardsnes, J., Sidhu, S., MacLeod, A., Elliott, J., Morden, D., Watson, J. & Borgford, T. (1998). **Streptomyces griseus protease B: secretion correlates with the length of the propeptide.** J Bacteriol 180, 3241-3244.

Barrett, A. J., Rawlings, N. & Woessner, J. F. (1998). **Handbook of Proteolytic Enzymes.** London: Academic Press.

Bentley, S. D., Chater, K. F., Cerdeno-Tarraga, A. M. & other authors (2002). **Complete genome sequence of the model actinomycete Streptomyces coelicolor A3(2).** Nature 417, 141-147.

Binnie, C., Liao, L., Walczyk, E. & Malek, L. T. (1996). **Isolation and characterization of a gene encoding a chymotrypsin-like serine protease from Streptomyces lividans 66**. Can J Microbiol 42, 284-288.

Blum, H., Chance, B. & Buzby, G. P. (1987). **In vivo noninvasive observation of acute mesenteric ischemia in rats.** Surg Gynecol Obstet 164, 409-414.

Bockle, B., Galunsky, B. & Muller, R. (1995). **Characterization of a keratinolytic serine proteinase from Streptomyces pactum DSM 40530.** Appl Environ Microbiol 61, 3705-3710.

Bressollier, P., Letourneau, F., Urdaci, M. & Verneuil, B. (1999). **Purification and characterization of a keratinolytic serine proteinase from Streptomyces albidoflavus.** Appl Environ Microbiol 65, 2570-2576.

Bryan, P.N. (2002). **Prodomain and protein folding catalysis.** Chem. REV. 102: 4805-4816.

Chitte, R. R. & Dey, S. (2000). **Potent fibrinolytic enzyme from a thermophilic Streptomyces megasporus strain SD5.** Lett Appl Microbiol 31, 405-410.

Dammann, T. & Wohlleben, W. (1992). **A metalloprotease gene from Streptomyces coelicolor 'Muller' and its transcriptional activator, a member of the LysR family.** Mol Microbiol 6, 2267-2278.

Gilbert, M., Morosoli, R., Shareck, F. & Kluepfel, D. (1995). **Production and secretion of proteins by streptomycetes.** Crit Rev Biotechnol 15, 13-39.

Gong, Y. & Wang, Y. (2001). **Purification and gene cloning of a novel fibrinolytic protease from Streptomyces sp. C3662.** Wei Sheng Wu Xue Bao 41, 186-190.

Henderson, G., Krygsman, P., Liu, C. J., Davey, C. C. & Malek, L. T. (1987). **Characterization and structure of genes for proteases A and B from Streptomyces griseus.** J Bacteriol 169, 3778-3784.

Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P. & Ward, J. M. (1985). **Genetic manipulation of streptomyces. A laboratory manual.** Norwich: The John Innes Foundation.

HU, g.& St.Leger, R.J. (2004). **A phylogenomic approach to reconstructing the diversification of serine protease in fungi.** J.Evol.Biol. 17, 1204-1214.

Hurtubise, Y., Shareck, F., Kluepfel, D. & Morosoli, R. (1995). **A cellulase/xylanase-negative mutant of Streptomyces lividans 1326 defective in cellobiose and xylobiose uptake is mutated in a gene encoding a protein homologous to ATP-binding proteins.** Mol Microbiol 17, 367-377.

Krem, M.M., Di cera, E. (2001). **Molecular markers of serine protease evolution.** EMBO journal 12, 3036-3045.

Kim, J. C., Cha, S. H., Jeong, S. T., Oh, S. K. & Byun, S. M. (1991). **Molecular cloning and nucleotide sequence of Streptomyces griseus trypsin gene.** Biochem Biophys Res Commun 181, 707-713.

Kitadokoro, K., Nakamura, E., Tamaki, M. & other authors (1993). **Purification, characterization and molecular cloning of an acidic amino acid-specific proteinase from Streptomyces fradiae ATCC 14544.** Biochim Biophys Acta 1163, 149-157.

Kitadokoro, K., Tsuzuki, H., Nakamura, E., Sato, T. & Teraoka, H. (1994). **Purification, characterization, primary structure, crystallization and preliminary crystallographic study of a serine proteinase from Streptomyces fradiae ATCC 14544.** Eur J Biochem 220, 55-61.

Laemmli, U. K. (1970). **Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4.** Nature 227, 680-685.

Lao, G. & Wilson, D. B. (1996). **Cloning, sequencing, and expression of a Thermomonospora fusca protease gene in Streptomyces lividans.** Appl Environ Microbiol 62, 4256-4259.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). **Protein measurement with the Folin phenol reagent.** J Biol Chem 193, 265-275.

Mondou, F., Shareck, F., Morosoli, R. & Kluepfel, D. (1986). **Cloning of the xylanase gene of Streptomyces lividans.** Gene 49, 323-329.

Screen, S. E. & St Leger, R. J. (2000). **Cloning, expression, and substrate specificity of a fungal chymotrypsin. Evidence for lateral gene transfer from an actinomycete bacterium.** J Biol Chem 275, 6689-6694.

Shimoi, H., Iimura, Y., Obata, T. & Tadenuma, M. (1992). **Molecular structure of Rarobacter faecitabidus protease I. A yeast-lytic serine protease having mannose-binding activity.** J Biol Chem 267, 25189-25195.

Sidhu, S. S., Kalmar, G. B. & Borgford, T. J. (1993). **Characterization of the gene encoding the glutamic-acid-specific protease of Streptomyces griseus.** Biochem Cell Biol 71, 454-461.

Sidhu, S. S., Kalmar, G. B., Willis, L. G. & Borgford, T. J. (1994). **Streptomyces griseus protease C. A novel enzyme of the chymotrypsin superfamily.** J Biol Chem 269, 20167-20171.

Sidhu, S. S., Kalmar, G. B., Willis, L. G. & Borgford, T. J. (1995). **Protease evolution in Streptomyces griseus. Discovery of a novel dimeric enzymes.** J Biol Chem 270, 7594-7600.

Silen, J. L., McGrath, C. N., Smith, K. R. & Agard, D. A. (1988). **Molecular analysis of the gene encoding alpha-lytic protease: evidence for a preproenzyme.** Gene 69, 237-244.

Silen, J. L. & Agard, D. A. (1989). **The alpha-lytic protease pro-region does not require a physical linkage to activate the protease domain in vivo.** Nature 341, 462-464.

Silen, J. L., Frank, D., Fujishige, A., Bone, R. & Agard, D. A. (1989). **Analysis of prepro-alpha-lytic protease expression in Escherichia coli reveals that the pro region is required for activity.** J Bacteriol 171, 1320-1325.

Sulzenbacher, G., Shareck, F., Morosoli, R., Dupont, C. & Davies, G. J. (1997). **The Streptomyces lividans family 12 endoglucanase: construction of the catalytic cre, expression, and X-ray structure at 1.75 A resolution.** Biochemistry 36, 16032-16039.

Suzuki, M., Taguchi, S., Yamada, S., Kojima, S., Miura, K. I. & Momose, H. (1997). **A novel member of the subtilisin-like protease family from Streptomyces albogriseolus.** J Bacteriol 179, 430-438.

Taguchi, S., Endo, T., Naoi, Y. & Momose, H. (1995a). **Molecular cloning and sequence analysis of a gene encoding an extracellular serine protease from Streptomyces lividans 66.** Biosci Biotechnol Biochem 59, 1386-1388.

Taguchi, S., Odaka, A., Watanabe, Y. & Momose, H. (1995b). **Molecular characterization of a gene encoding extracellular serine protease isolated from a subtilisin inhibitor-deficient mutant of Streptomyces albogriseolus S-3253.** Appl Environ Microbiol 61, 180-186.

Taguchi, S., Ogawa, T., Endo, T. & Momose, H. (1997). **A gene homologous to the Streptomyces chymotrypsin-like protease (SAM-p20) gene is tandemly located.** Biosci Biotechnol Biochem 61, 909-913.

Volff, J. N. & Altenbuchner, J. (1998). **Genetic instability of the Streptomyces chromosome.** Mol Microbiol 27, 239-246.

Yum, D. Y., Chung, H. C., Bai, D. H., Oh, D. H. & Yu, J. H. (1994). **Purification and characterization of alkaline serine protease from an alkalophilic Streptomyces sp.** Biosci Biotechnol Biochem 58, 470-474.

6.3 Discussion et conclusion du chapitre

Les travaux réalisés ont permis de démontrer la possibilité d'utiliser *S.lividans* pour la production d'une vaste gamme d'enzymes protéolytiques naturelles. Il fut possible de produire et caractériser une nouvelle enzyme : SLP25. Ces travaux permettent aussi de souligner que la forte relation entre l'activité de ces enzymes et l'adaptation écologique des organismes, en fait un groupe d'un grand intérêt pour l'appréciation des processus évolutifs. L'analyse phylogénétique de cette enzymes et des protéases de la famille S2 a permis de proposer la formation d'une sous-famille de protéases du groupe des chymotrypsines : les protéases α-lytique. De plus, SLP25 présente une forte relation avec une enzyme eucaryote produite par un champignon. Une analyse plus poussée de la phylogénie du groupe d'enzyme permet de démontrer que SLP25 est en fait une preuve qu'un échange latéral d'un gène d'un procaryote à un eucaryote. Ce groupe d'enzyme a utilisé plusieurs voix évolutives dont la duplication, la recombinaison et le transfert latéral.

Discussion générale

Premièrement, les quatre brevets présentés dans cette thèse témoignent du caractère appliqué des travaux. Il est important de rappeler, que la protection de la propriété intellectuelle constitue une étape cruciale de tout projet de recherche à caractère appliqué. Elle demande une grande connaissance de tous les sujets apparentés de façon à être capable de bien faire ressortir le caractère non évident de l'invention tout en évaluant les possibilités de conflit avec les brevets existants. Lorsqu'un brevet existant s'apparente de près avec la découverte à protéger, l'inventeur doit être capable de donner les explications nécessaires justifiant la protection de la nouvelle invention. Une note explicative sur la protection de la propriété intellectuelle peut être consultée à l'annexe 2.

La figure 28, tirée de la présentation industrielle de la plateforme technologique, présente le concept des MPM d'un point de vu technologique et scientifique. Il va de soi que tout concept à caractère industriel est avant tout confronté à la capacité de convaincre les financiers et partenaires industriels d'y investir temps et argent. Les critères de sélection des technologies qui seront supportés financièrement se basent à la fois sur le potentiel de la plateforme technologique et sur l'approche de développement qui assurera la pleine réalisation de ce potentiel. La figure 29, toujours tirée de la présentation de la plateforme technologique, présente l'intégration des capacités de recherche avec les capacités d'innovation en matière de production d'ingrédients à forte valeur ajoutée de cette nouvelle technologie.

Cette figure présente bien l'aboutissement scientifique du concept au niveau des essais cliniques chez l'humain et de la possibilité d'optimiser, à toutes les étapes du développement, l'ingrédient produit en fonction des caractéristiques recherchées. La compréhension des effets santé reliée aux composantes est primordiale afin d'orienter la production en fonction de l'effet santé recherché.

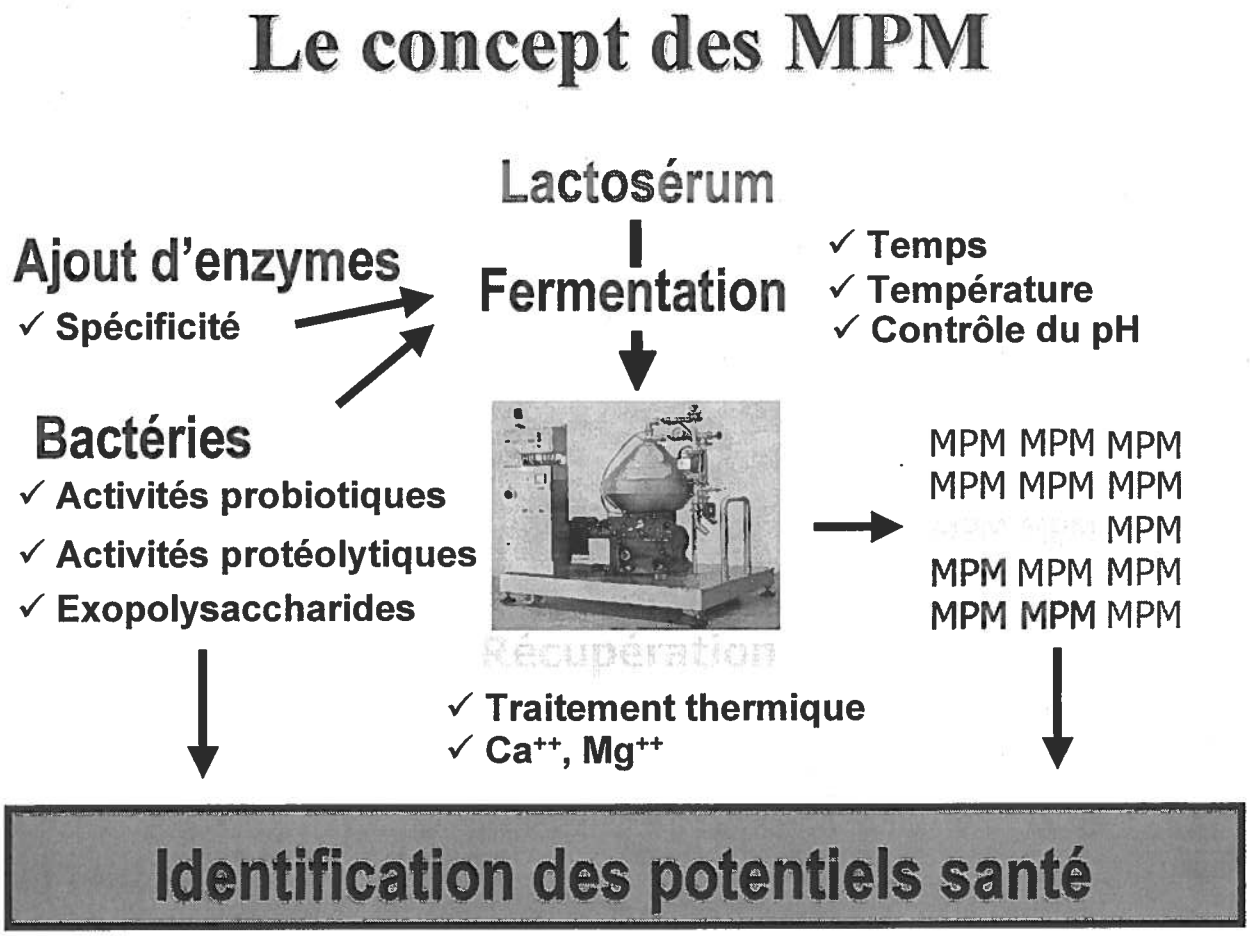


Figure 28 : Présentation de la plateforme technologique des MPM comme nouvelle voie innovatrice de production d'une gamme d'ingrédients bioactifs bénéfiques pour la santé.

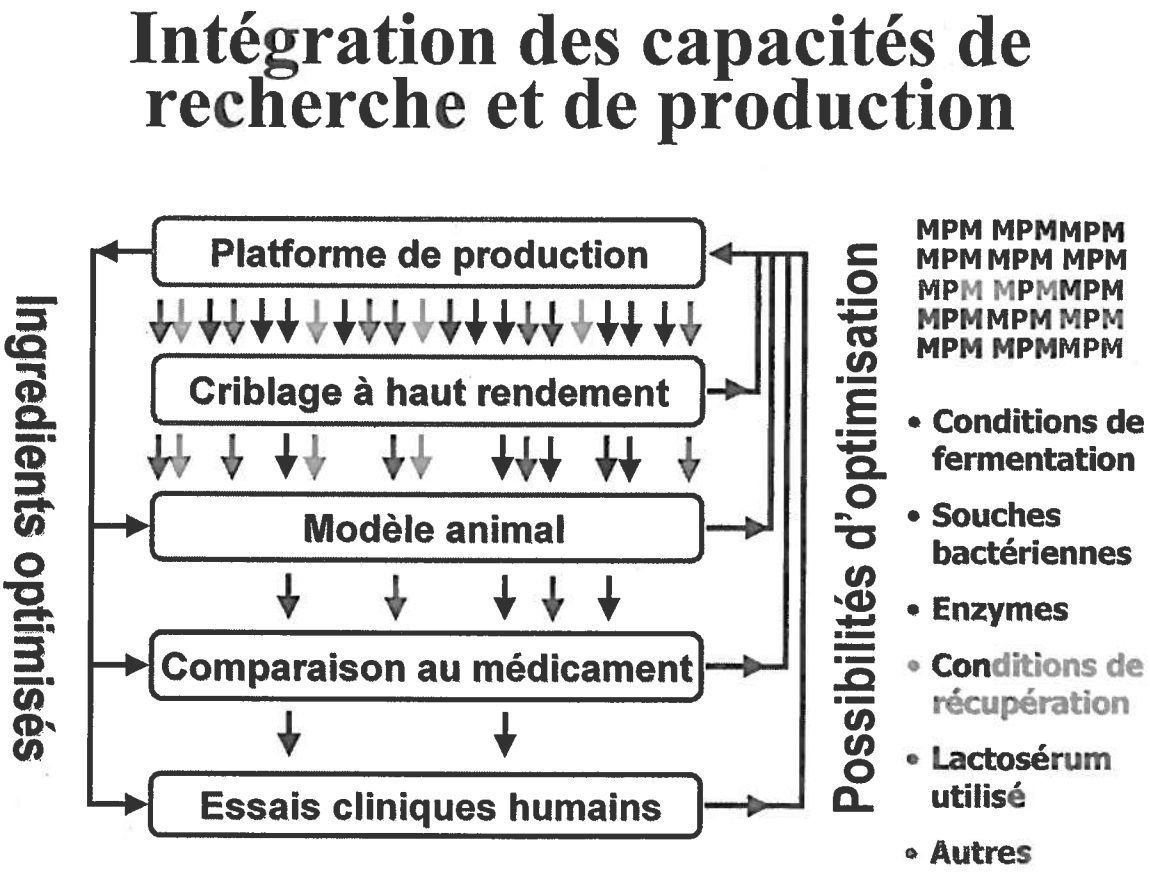


Figure 29 : Intégration des capacités de recherche et des capacités de production technologique dans le but de développer des ingrédients bioactifs aux caractéristiques améliorées.

La caractérisation fine du contenu peptidique et des effets santé bénéfiques des bactéries est une première avenue en cours de réalisation. Par la suite, l'utilisation d'autres souches bactériennes influencera ce contenu en peptides, en exopolysaccharides et en bactéries pour permettre des effets bénéfiques sur la santé différents. Cette description élargie complète la description de la technologie et permet de bien comprendre comment les futurs développements seront effectués.

D'un point de vu commercial et social, l'article présenté à la section 10 discute des impacts possibles des ingrédients santé d'origine laitière sur les coûts de santé et le maintien d'une bonne santé de la population. Les coûts de santé sont fortement en augmentation et les produits d'alimentation fonctionnelle et nutraceutiques peuvent y jouer un rôle important. L'article introduit le terme "lactoceutique" pour désigner tout type de produits contenant des protéines laitières, soient intactes ou modifiées par un traitement léger (comme l'hydrolyse enzymatique), qui possèdent une valeur nutritionnelle supérieure aux produits à base de lait conventionnels et qui présentent des effets santé scientifiquement prouvés. Sur une vision de cinq ans, le rapprochement des secteurs de l'alimentation et de la pharmaceutique devrait s'amplifier et favoriser l'apparition d'aliments fonctionnels et de produits nutraceutiques. Dans ce contexte, les lactoceutiques sont particulièrement bien placés pour jouer un rôle important. Leur origine naturelle et leur effet santé bien documenté viennent s'ajouter à la perception déjà favorable des consommateurs envers les produits laitiers.

La technologie et les produits développés auront bien sûr aussi des impacts environnementaux et commerciaux très importants. D'un point de vue technologique, la production de MPM à partir de lactosérum, couplée à leur utilisation dans la formulation de produits laitiers légers, crée une opportunité de commercialisation technologique de très grande envergure. Les volumes de lactosérum produit annuellement sont gigantesques (145 milliards de litres de lactosérum) et les technologies actuelles valorisent très peu cette ressource à fort potentiel (60% valorisée et principalement séchée).

Conclusion générale

Le lactosérum est une ressource disponible en très grande quantité et ses protéines présentent des avantages nutritifs, technologiques et commerciaux indéniables. Une nouvelles technologie de fermentation fut développée afin d'exploiter les pleins potentiels de cette ressource. Cette plate-forme technologique permet de produire une nouvelle gamme d'actifs biologiques aux applications très variées, sous la forme de matrices protéiques malléables (MPM).

Les MPM sont composés d'environ 80% d'eau, 8% de protéine, 6% de minéraux (dont 1,8 % de calcium), 5 % de glucide, 1% de gras et $6X10^{11}$ bactéries par 100g. Ces matrices permettent d'allier des fonctions technologiques (épaississantes, émulsifiantes et texturisantes), aux fonctions nutritives et santés. Elles permettent la formulation de produits légers (yogourt, mayonnaise, beurre) et de produits santé (modulation immunitaire et amélioration du bilan lipidique). Un brevet, en phase internationale, fut déposé pour la protection du procédé de production, du produit (MPM) et des applications de cette nouvelle technologie.

De nouvelles souches bactériennes furent isolées de grains de kéfir pour le développement de cette technologie. Ces souches appartiennent à l'espèce *Lactobacillus kefiranofaciens*. Parmi celle-ci, la souche R2C2 représente la souche la plus robuste jamais décrite de cette espèce et la souche INIX est surproductrice d'exopolysaccharide. Quatre nouvelles souches de cette espèce furent décrites et comparées aux données actuelles de la littérature. Des amorces d'ADN spécifiques à l'espèce furent développées à partir du gène de l'ARN ribosomiale 16S et l'appartenance de cette espèce au grand groupe des acidophilus souligne son caractère probiotique qui fut démontré *in-vitro* et *in-vivo*. Un brevet, en phase international, fut aussi déposé pour la protection des aspects probiotiques de ces souches.

Outre les composantes et les applications de cette technologie, un axe exploratoire de production d'enzymes naturelles des streptomycètes fut aussi développé dans le but éventuel d'utiliser ces enzymes pour la production de nouveaux MPM. Les travaux réalisés ont mené à la purification et à la caractérisation sommaire d'une nouvelle enzyme (SLP25). Ces travaux ont aussi démontré que la forte relation entre l'activité des protéases et l'adaptation écologique des organismes en fait un groupe d'un grand intérêt pour l'appréciation des processus évolutifs. L'analyse phylogénétique de cette nouvelle enzyme a permis de proposer la formation d'une sous-famille et de démontrer que SLP25 est en fait un exemple d'échange latéral d'un gène d'un procaryote à un eucaryote.

Un article, deux manuscrit déposés, ainsi que deux brevets, sont présenté dans la section « Autres contributions ». Un premier manuscrit présente les effets anti-inflammatoires de *L. kefiranofaciens*. Un article de revue introduit la notion de « Lactoceutique » comme produit d'alimentation fonctionnelle ou nutraceutique d'origine laitier et un autre manuscrit présente les résultats de l'utilisation des MPM comme application cosméceutiques. Les deux brevets portent sur l'utilisation des EPS de *L. kefiranofaciens* pour la formulation de médicament; un décrit son utilisation pour la formulation en générale et un autre décrit une application particulière pour le traitement du cancer. L'invention permet de solubiliser et d'améliorer l'effet de certains médicaments soit par synergie ou par augmentation de l'absorption.

Ces travaux ont démontré avant tout qu'il est toujours possible d'innover en portant un regard nouveau sur un domaine donné. Le premier facteur de réussite à cette innovation est de commencer par croire que c'est possible.

Contributions à l'avancement des connaissances

Les travaux réalisés ont permis le développement et la protection de la propriété intellectuelle d'une nouvelle technologie de valorisation du lactosérum. Malgré le nombre impressionnant de recherches réalisées sur le lactosérum depuis les années 70, il s'agit d'un nouveau concept qui ouvre la voie au développement de toute une gamme de bio-ingrédients actifs. Les brevets déposés démontrent à la fois l'originalité des travaux réalisés et l'intégration d'une grande quantité de connaissances très variées.

Grâce à leur composition et aux qualités nutritives bénéfiques pour la santé de leurs composantes, les MPM ont été qualifiés de "super aliment" par Hélène Bishop-Mc.Donald, une nutritionniste de renommée mondiale. L'importance de cette nouvelle plateforme technologique dans le contexte du développement des aliments fonctionnels et nutraceutiques fut reconnue par la manifestation de l'intérêt de nombreux industriels internationaux. Les effets santé identifiés jusqu'à maintenant sont bénéfiques pour le contrôle du poids corporel, pour l'inflammation, pour le bilan lipidique, pour la tension artérielle, pour la santé intestinale et pour le diabète de type 2.

L'isolement, la description et l'identification de nouvelles souches bactériennes probiotiques sont un apport majeur à l'avancement des connaissances. Parmi les nouvelles souches étudiées, on retrouve la souche la plus robuste de *Lactobacillus kefiranofaciens* jamais décrite. Outre les limites de pH et de température décrites pour la souche R2C2, un grand nombre de caractéristiques de cette espèce sont propres à ces travaux : la sensibilité au calcium, la dissolution des agglomérats cellulaires par la température, la croissance en lactosérum concentré, les nouveaux profils de fermentation des sucres, l'élaboration d'amorces d'ADN spécifiques à l'espèce et la présence pour cette espèce de deux types morphologiques repiquables en culture.

Une des théories proposées dans cette thèse stipule que le phénomène d'élongation cellulaire des bactéries serait relié à une multitude de régulations métaboliques propres au type A du sous-groupe *acidophilus*. Si cette théorie est

confirmée, elle pourrait avoir un impact considérable sur l'identification et la caractérisation des effets bénéfiques sur la santé du plus important groupe de probiotiques. Étant donné que le type court est favorisé pour la production industrielle de probiotiques afin d'améliorer la viabilité des cellules, il est possible que la découverte d'effets santé associés au type long amène l'industrie à reconsidérer cette sélection qui est appliquée depuis plus de 20 ans.

Les travaux sur la production d'enzymes protéolytiques ont permis le clonage de vingt nouveaux gènes, la sélection d'un grand nombre de clones positifs pour l'activité protéolytique et la purification/caractérisation d'une nouvelle enzyme. L'analyse phylogénétique réalisée permet de proposer la formation de deux sous-familles de ce groupe d'enzymes, tout en démontrant plusieurs facettes évolutives importantes pour ces protéases.

Finalement, les brevets sur les applications des EPS décrivent une nouvelle technologie de formulation de médicaments par un produit naturel. Une première application de cette technologie fut protégée pour la préparation et l'utilisation de vaccins anticancéreux. Il s'agit d'une contribution majeure à l'avancement des connaissances.

Références

Abascal D, Lencki RW. 2004. Effect of pH on the ternary solution behavior of beta-lactoglobulin. Biotechnol Prog. Vol. 20, No. 6, pp. 1741-8.

Adachi S., Toba, T., Mukai, T., Watanabe, T., Haruhiko, Y. 1993. Lactobacillus sp. KPB-167 and method of manufacturing viscose polysaccharides employing the same. USPTO 5 204 247.

Adams, M. C., Birdsall, E. L. 1946. New consistometer measures corn consistency. J. Food Industries. Vol. 18, No.6, pp. 78.

Altermann, E., Buck, L.B., Cano, R., Klaenhammer, T.R. 2004. Identification and phenotypic characterization of the cell-division protein CdpA. Gene. Vol. 342, No. 1, pp. 189-197.

Alting AC, Hamer RJ, de Kruif CG, Visschers RW. 2003. Cold-set globular protein gels: interactions, structure and rheology as a function of protein concentration. J Agric Food Chem. Vol. 51, No. 10, pp. 3150-6.

Alting AC, Weijers M, de Hoog EH, van de Pijpekamp AM, Cohen Stuart MA, Hamer RJ, de Kruif CG, Visschers RW. 2004. Acid-induced cold gelation of globular proteins: effects of protein aggregate characteristics and disulfide bonding on rheological properties. J Agric Food Chem. Vol. 52, No. 3, pp. 623-31.

ATCC: American Type Culture Collection P.O. Box 1549, Manassas, VA 20108 USA. (www.atcc.org).

Athanasiadis I, Boskou D, Kanellaki M, Kiosseoglou V, Koutinas AA. 2002. Whey liquid waste of the dairy industry as raw material for potable alcohol production by kefir granules. J Agric Food Chem. Vol. 50, No. 25, pp. 7231-4.

Axelsson, L. 1998. *Lactic acid bacteria : Classification and physiology*, in *Lactic acid bacteria*. S.S.a.A.v. Wright, Editor., Marcel Dekker: New york, pp. 1-72.

Baele M, Vaneechoutte M, Verhelst R, Vancanneyt M, Devriese LA, Haesebrouck F. 2002. Identification of Lactobacillus species using tDNA-PCR. J Microbiol Methods. Vol. 50, No. 3, pp. 263-71.

Barber, F. W., Frazier, W. C. 1945. Dissociants of lactobacilli. Journal of Bacteriology. Vol. 50, pp. 637-649.

Barrett, A.J., Rawling, N.D., Woessner, J.F. 1998. Handbook of proteolytic enzymes. Academic Press. San Diego. USA. 1666 pages.

Barros RM, Malcata FX. 2002. Modeling the kinetics of whey protein hydrolysis brought about by enzymes from Cynara cardunculus. J Agric Food Chem. Vol. 50, No. 15, pp. 4347-56.

Bergmaier, D. 2002. Production d'exopolysaccharides par fermentation avec des cellules immobilisées de *LB rhamnosus* RW-9595M LB. RHAMNOSUS RW-9595M d'un milieu à base de perméat de lactosérum. Thèse de doctorat. Département des sciences des aliments et de la nutrition, Faculté des sciences de l'agriculture et de l'alimentation. Université Laval. 171 pages.

Bertrand-Harb C, Baday A, Dalgalarrondo M, Chobert JM, Haertle T. 2002. Thermal modifications of structure and co-denaturation of alpha-lactalbumin and beta-lactoglobulin induce changes of solubility and susceptibility to proteases. Nahrung. Vol. 46, No. 4, pp. 283-9.

Blenford, D.E. 1996. Whey from waste to gold. IFI NR.1, pp 42-47.

Boyd MA, Antonio MA, Hillier SL. 2005. Comparison of API 50 CH strips to whole-chromosomal DNA probes for identification of Lactobacillus species. J Clin Microbiol. Vol. 43, No. 10, pp. 5309-11.

Boye, J.I., Alli, I., Ismail, A.A., Gibbs, B.F. and Konishi, Y. 1995. Factors affecting molecular characteristics of whey protein gelation. Int. Dairy Journal. Vol. 5. pp. 337-353.

Breddam, K., Meldal., M. 1992. Substrate preferences of glutamic – acid – specific endopeptidases assessed by synthetic peptide substrates based on intramolecular fluorescence quenching. Eur. J. Biochem. Vol. 206, No. 1, pp. 103-107.

Briczinski EP, Roberts RF. 2002. Production of an exopolysaccharide-containing whey protein concentrate by fermentation of whey. J Dairy Sci, Vol. 85, No. 12, pp. 3189-97.

Britten, M. and Giroux, H.J. 1994. Effect of pH during heat processing of partially hydrolyzed whey protein. J. Dairy Sci. Vol. 77. pp. 676-684.

Brode III, P.F., Erwin, C.R., Rauch, D.S., Barnett, B.L., Armpriester, J.M., Wang, E.S.F., Rubingh, D.N. 1996. Subtilisin BPN variants : Increased hydrolytic activity on surface-bound substrates via decresed surface activity. Bichemistry. Vol. 35, No. 10, pp. 3162 – 3169.

Buck, B.L., Altermann, E., Svingerud, T., Klaenhammer, T.R. 2005. Functional Analysis of Putative Adhesion Factors in Lactobacillus acidophilus NCFM. Appl. Environ. Microbiol. Vol. 71, No. 12, pp. 8344-8351.

Burke MD, Ha SY, Pysz MA, Khan SA. 2002. Rheology of protein gels synthesized through a combined enzymatic and heat treatment method. Int J Biol Macromol. Vol. 31, No. 1-3, pp. 37-44.

Byun, R., Nadcarni, M.A., Chhour, K.L., Martin, F.E., Jacques, N.A., Hunter, N. 2004. Quantitative analysis of diverse lactobacillus species present in advanced dental caries. Journal of Clinical Microbiology. Vol. 42. no.7. pp. 3128-3136.

Caessens, P.W.J.R., Daamen, W.F., Gruppen, H., Visser, S., Voragen, A.G.J. 1999a. β-lactoglobuline hydrolysis. 2. Peptide identification, SH/SS exchange, and fonctional properties of hydrolysate fractions formed by the action of plasmin. J. Agric. Food Chem. Vol. 47, No. 8, pp. 2980-2990.

Caessens, P.W.J.R., Visser, S., Gruppen, H., Voragen, A.G.J. 1999b. β-lactoglobuline hydrolysis. 1. Peptide composition and functional properties of hydrolysates obtained by action of plasmin, trypsin, and *Staphylococcus aureus* V8 protease. J. Agric. Food Chem. 47, No. 8, pp. 2973-2979.

Carter, P., Wells, J.A. 1988. Dissecting the catalytic triad of a serine protease. Nature. Vol. 332, No. 6164, 564 – 568.

Cayot, P., Lorient, D. 1998. Structures et technofonction des protéines du lait. Lavoisier. Paris, 363 pages.

Chagnaud, P., Machinis, K., Coutte, L.A., Marecat, A., Mercenier, A. 2001. Rapid PCR-based procedure to identify lactic acid bacteria: application to six common Lactobacillus species. Journal of Microbiological Methods. Vol. 44. no. 2. pp. 139-148.

Champagne, C.P. 1991. The freeze-drying of lactic acid bacteria. A Review. Canadian Institute of Science and Technology Journal. Vol. 24, pp. 118-128.

Chang, S.C., Chang, P.C., Lee, Y.H.W. 1994. The Roles of Propeptide in Maturation and Secretion of Npr Protease from *Streptomyces*. Journal of Biological Chemistry. Vol. 269., pp. 3548-3554.

Cheirsilp, B., Shimizu, H., Shioya, S. 2003B. Enhanced kefiran production by mixed culture of Lactobacillus kefiranofaciens and Saccharomyces cerevisiae. Journal of Biotechnology. Vol. 9, No. 1, pp. 43-53.

Cheirsilp, B., Shimizu, H., Shioya, S. 2005. Modelling and optimization of environmental conditions for kefiran production by Lactobacillus kefiranofaciens. Appl Microbiol Biotechnol. Vol. 57, No. 5-6, pp. 639-646.

Cheirsilp, B., Shoji, H., Shimizu, H., Shioya, S. 2003A. Interactions between Lactobacillus kefiranofaciens and Saccharomyces cerevisiae in mixed culture for kefiran production. J Biosci Bioeng. Vol. 96, No. 3, pp. 279-284.

Chen, S.X., Hardin' C .C., Swaisgood, H.E. 1993. Purification and caracterization of β-strutural domains of β-lactoglobulin liberated by limited proteolysis. Journal of Protein Chemistry. Vol. 12. No. 5. pp 613-625.

Christensen JE, Reynolds CE, Shukla SK, Reed KD. 2004. Rapid molecular diagnosis of lactobacillus bacteremia by terminal restriction fragment length polymorphism analysis of the 16S rRNA gene. Clin Med Res. Vol. 2, No. 1, pp. 37-45.

Couture, R., Gagné, D. Champagne, C.P.1991. Effet de divers additifs sur la survie à la lyophilisation de Lactococcus lactis. Canadian Institute of Science and Technology Journal. Vol. 24, pp. 224-227.

CQVB. 1993. Valorisation des sous-produits de l'industrie de transformation du lait. Dépliant de vulgarisation. CQVB, 18 pages.

Czapinska, H., Otlewski, J. 1999. Structural and energetic determinants of the S_1 –site specificity in serine proteases. Eur. J. Biochem. Vol. 260. pp. 571-595.

de Roissart, H., Luquet, F.M. 1994. *Bactéries Lactiques. Aspects fondamentaux et technologiques.* ed. Lorica, Uriage. Vol. 2, 614 pages.

Demay, S. 1993. Analyse différentielle de protéases à large spécificité. Mise au point d'une méthodologie d'étude sur le modèle sérum albumine bovine / subtilisines. Thèse de doctorat. Université de Limoge. France, 130 pages.

Dong Chen, X., Dong Chen, Z., Nguang, S.K., Anema, S. 1998. Exploring the reaction kinetics of whey protein denaturation / aggregation by assuming the denaturation step is reversible. Biochemical Engineering Journal. Vol. 2, pp. 63-69.

Doucet D, Gauthier SF, Otter DE, Foegeding EA. 2003a. Enzyme-induced gelation of extensively hydrolyzed whey proteins by alcalase: comparison with the plastein reaction and characterization of interactions. J Agric Food Chem. Vol. 51, No. 20, pp. 6036-42.

Doucet D, Otter DE, Gauthier SF, Foegeding EA. 2003b. Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: peptide identification and determination of enzyme specificity. J Agric Food Chem. Vol. 51, No. 21, pp. 6300-8

Dumay, E., Laligant, A., Zasypkin, D., Cheftel, J.C. 1999. Pressure and heat – induced gelation of mixed β-lactoglobulin / polysaccharide solution : scanning electron microscopy of gels. Food Hydrocolloids. Vol.13, pp. 339-351.

Dunn, B.M. 1989. Proteolytic enzymes a pratical approch; chapitre 4 : Determination of protease mechanism. IRL Press at Oxford University Press. Oxford, 255 pages.

Dupont, I., Roy, D., Lapointe, G. 2000. Comparison of exopolysaccharide production by strains of Lactobacillus rhamnosus and Lactobacillus paracasei grown in chemically defined medium and milk. Journal of Industrial Microbiology & Biotechnology. Vol. 24, pp. 251–255.

Dybing, S. T. and Smith, D. E. 1998. The ability of phosphates or κ-carrageenan to coagulate whey proteins and the possible uses of such coagula in chesse manufacture. Journal of Dairy Science. Vol. 81. No. 2. pp 309-317.

Eder, J., Fersht, A.R. 1995. Pro-sequence-assisted protein folding, microreview. Molecular Microbiology. Vol. 16. pp. 609-614.

Eder, J., Rheinnecker, M., Fersht, A.R. 1993a. Folding of Subtilisin BPN : Characterization of a Folding Intermedaite. Biochemistry. Vol. 32., pp. 18-26.

Eder, J., Rheinnecker, M., Fersht, A.R. 1993b. Folding of Subtilisin BPN : Role of the Pro-sequence. Journal of Molecular Biology. Vol. 233., pp. 293-304.

Edwards U, Rogall T, Blocker H, Emde M, Bottger E.C. 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. Vol. 11, No.17, pp. 7843-53.

Eissa AS, Khan SA. 2005. Acid-induced gelation of enzymatically modified, preheated whey proteins. J Agric Food Chem. Vol. 53, No. 12, pp. 5010-7.

Ekici P, Backleh-Sohrt M, Parlar H. 2005. High efficiency enrichment of total and single whey proteins by pH controlled foam fractionation. Int J Food Sci Nutr. Vol. 56, No 3, pp. 223-9.

Ellinger DK, Muller LD, Glantz PJ. 1980. Influence of feeding fermented colostrum and Lactobacillus acidophilus on fecal flora of dairy calves. J Dairy Sci. Vol. 63, No. 3, pp. 478-82.

Ercolini, D., Moschetti, G., Blaiotta, G., Coppola, S. 2001. Behavior of variable V3 region from 16S rDNA of lactic acid bacteria in denaturing gradient gel electrophoresis. Current Microbiology. Vol. 42. pp. 199-202.

Etzel MR. 2004. Manufacture and use of dairy protein fractions. J Nutr. Vol. 134, No. 4, pp. 996S-1002S.

Farnworth, E.R. 2005. Kefir – a complex probiotic. Food Science and Technology Bulletin: Functional Foods. Vol. 2, No. 1, pp. 1-17.

Fink, A.L. 1989. Dans : Enzyme mechanisms. Chapitre 10 : Acyl Group Transfer – The Serine Proteinases. M.I. et Williams, A. Éditeurs. The Royal Society of Chemistry. London. 567 pages.

Foegeding, E.A., Kuhn, P.R. and Hardin, C.C. 1992. Specific divalent cation-induced changes during gelation of β-lactoglobulin. J. Agric. Food Chem. Vol. 40. pp. 2092-2097.

Frengova, G.I., Simova, E.D., Beshkova, D.M., Simov, Z. 2002. Exopolysaccharides produced by lactic acid bacteria of kefir grains. Zeitschrift für Naturforschung. Vol. 57, pp. 805-810.

Fujisawa, T., Adachi, S., Toba, T., Arihara, K., Mitsuoka, T. 1988. Lactobacillus kefiranofaciens sp. nov. Isolated from kefir grains. International Journal of Systematic Bacteriology. Vol. 38, No. 1, pp. 12-14.

Glick, B.R. and Pasternak, J.J. 1998. Molecular Biotechnology; principles and applications of recombinant DNA. Second edition. ASM Press. Washington, 683 pages.

Gonzàlez Siso, M. I. 1996. The biotechnological utilization of chesse whey: a review. Bioresource Technology. Vol. 57. pp 1-11.

Greene, J.D., Klaenhammer, T.R. 1994. Factors involved in adherence of lactobacilli to human Caco-2 cells. Appl. Environ. Microbiol. Vol. 60, No. 12, pp. 4487-4494.

Ha E, Zemel MB. 2003. Functional properties of whey, whey components, and essential amino acids: mechanisms underlying health benefits for active people (review). J Nutr Biochem. Vol. 14, No. 5, pp. 251-8.

Hadziyev, D. 1987. Food chemistry.Chapitre 10, Milk and dairy products. Springer-Verlag. New-York. pp. 377-402.

Han KS, Kim Y, Choi S, Oh S, Park S, Kim SH, Whang KY. 2005. Rapid identification of Lactobacillus acidophilus by restriction analysis of the 16S-23S rRNA intergenic spacer region and flanking 23S rRNA gene. Biotechnol Lett. Vol. 27, No. 16, pp. 1183-8.

Havea P, Carr AJ, Creamer LK. 2004. The roles of disulphide and non-covalent bonding in the functional properties of heat-induced whey protein gels. J Dairy Res. Vol. 71, No 3, pp. 330-9

Havea P, Singh H, Creamer LK. 2002. Heat-induced aggregation of whey proteins: comparison of cheese WPC with acid WPC and relevance of mineral composition. J Agric Food Chem. Vol. 50, No. 16, pp. 4674-81.

Heslot, H. 1996. L'ingénierie des protéines et ses applications. Lavoisier. Paris, 623 pages.

Hines, M.E. and Foegeding, E.A. 1993. Interactions of α-lactalbumin and bovine serum albumin with β-lactoglobulin in thermally induced gelation. J. Agric. Food Chem. Vol. 41. No. 3. pp. 341-346.

Hoffmann, M.A.M., van Mil, P.J.J.M. 1999. Heat-induced aggregation of β-lactoglobulin as function of pH. J. Agric. Food Chem. Vol. 47, pp. 1898-1905.

Holzapfel, W.H., Haberer, P., Geisen, R., Björkroth, J., Schillinger, U. 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. American journal of Clinical Nutrition. Vol. 73. pp. 365-373.

Hongsprabhas, P. and Barbut, S. 1997. Effect of gelation temperature on Ca^{2+}-induced gelation of whey protein isolate. Lebensm.-Wiss. u.-Technol. Vol. 30. pp. 45-49.

Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M. 1985. Genetic manipulation of streptomyces. A laboratory manual. Norwich: The John Innes Foundation.

Huang, X.L., Catignani, G.L., Swaisgood, H.E. 1999. Modification of rheological properties of whey protein isolates by limited proteolysis. Nahrung. Vol. 43, pp. 79-85.

Huffman, L.M., Harper, W.J. 1999. Symposium : marketing dairy value through technology. Maximizing the value of milk through separation tecnologies. J. Dairy. Sci. Vol. 82, pp. 2238-2244.

Huppertz T, Fox PF, Kelly AL. 2004. High pressure-induced denaturation of alpha-lactalbumin and beta-lactoglobulin in bovine milk and whey: a possible mechanism. J Dairy Res. Vol. 71, No. 4, pp. 489-95.

Hurtubise, Y., Shareck, F., Kluepfel, D., Morosoli, R. 1995. A cellulase/xylanase-negative mutant of Streptomyces lividans 1326 defective in cellobiose and xylobiose uptake is mutated in a gene encoding a protein homologous to ATP-binding proteins. Mol. Microbiol. Vol. 17, pp. 367-377.

Ikemura, H., Takagi, H. Inouye, M. 1987. Requirement of Pro-sequence for the production of active subtilisine E in *Escherichia coli.* Journal of Biological Chemistry. Vol. 262., pp. 7859-7864.

Inouye, M.1991. Intramolecular chaperone; the role of the pro-peptide in protein folding. Enzyme. Vol. 45. pp. 314-321.

International Depositary Authority of Canada. National Microbiology Laboratory, Health Canada. 1015 Arlington Street, Winnipeg, Manitoba, Canada. R3E 3R2. Tel: (204) 789-2070. Fax: (204) 789-2097.

Jeyarajah, S. and Allen, J.C. 1994. Calcium binding and salt-induced structural changes of native and preheated β-lactoglobulin. J. Agric. Food Chem. Vol. 42, pp. 80-85.

Ju, Z.Y. and Kilara, A. 1998 (a). Textural properties of cold-set gels induced from heat-denatured whey protein isolates. Journal of Food Science. Vol. 63, No. 2, pp. 288-292.

Ju, Z.Y. and Kilara, A. 1998 (b). Properties of gels induced by heat, protease, calcium salt, and acidulant from calcium ion-aggregated whey protein isolate. J. Dairy Sci. Vol. 81. pp. 1236-1243.

Ju, Z.Y. and Kilara, A. 1998 (c). Effects of preheating on properties of aggregates and of cold-set gels of whey protein isolate. J. Agric. Food Chem. Vol. 46, pp. 3604-3608.

Ju, Z.Y., Otte, J., Madsen, J.S. and Qvist, K.B. 1995. Effects of limited proteolysis on gelation and gel properties of whey protein isolate. Journal of dairy science. Vol. 18, No.10, pp.2119-2128.

Ju, Z.Y., Otte, J., Skriver, A. and Qvist, K.B. 1996. Effects of limited proteolysis on the microstructure of heat-induced whey protein gels at varying ph. Journal of dairy science. Vol. 79, No. 5, pp. 782-790.

Ju, Z.Y., Otte, J., Zakora, M. and Qvist, K.B. 1997. Enzyme-induced gelation of whey proteins: Effects of protein denaturation. Dairy journal. Vol. 7, pp. 71-78.

Keohgh, M. K., Lainé, K. I., O'Connor, J. F. 1995. Rheology of sodium caseinate-carrageenan mixtures. J. Texture Studies. Vol. 26, pp. 635-652.

Kim M, Chun J. 2005. Bacterial community structure in kimchi, a Korean fermented vegetable food, as revealed by 16S rRNA gene analysis. Int J Food Microbiol. Vol. 103, No. 1, pp. 91-6.

Klein, G., Pack, A., Bonaparte, C., Reuter, G. 1998. Taxonomy and physiology of probiotic lactic acid bacteria. International Journal of Food Microbiology. Vol. 41, pp. 103-125.

Kleine, R., Achenbach, S. and Thoss, S. 1995. Whey disposal by deproteinization and fermentation. Acta Biotechnol. Vol. 15m No. 2, pp. 139-148.

Kobayashi, T., Inouye, M. 1992. Functional Analysis of the Intramolecular Chaperone. Mutational Hot Spots in the Subtilisin Pro-peptide and a Second-site Suppressor Mutation within the Subtilisin Molecule. Journal of Molecular Biology. Vol. 226., pp. 931-933.

Kojima, S., Takizawa, S., Tamura, S., Fujinaga, S, Benno, Y., Nakase, T. 1993. An improved medium for the isolation of Lactobacilli from kefir grain. Biosci. Biotech. Biochem., Vol. 57, No. 1, pp. 119-120.

Kontopidis G, Holt C, Sawyer L. 2004. Invited review: beta-lactoglobulin: binding properties, structure, and function. J Dairy Sci. Vol. 87, No. 4, pp. 785-96.

Kulmyrzaev, A., Sivestre, M. P. C., McClements, D. J. 2000. Rheology and stability of whey protein stabilized emulsions with high CaCl2 concentrations. Food Research International. Vol. 33, No. 1, pp. 21-25.

Kwon HS, Yang EH, Yeon SW, Kang BH, Kim TY. 2004. Rapid identification of probiotic Lactobacillus species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. FEMS Microbiol Lett. Vol. 239, No. 2, pp. 267-75.

La Rivière, J.W., Kooiman, P. 1967. Kefiran, a novel polysaccharide produced in the kefir grain by Lactobacillus brevis. Archives of Mikrobiology. Vol. 59, pp. 269-278.

Lefebvre et Masella, R. 1990. Opportunités de valorisation de trois sous-produits de transformation de l'industrie laitière. Centre de recherche STELA, Centre Québécois de Valorisation de la biomasse. Québec, 180 pages.

Lehninger, L.A. 1982. Principles of bichemistry. Worth Publishers inc. New York, 1011 pages.

Lemieux, P, Beaulieu, J. et Simard, E. 2004. Socioeconomic and health benefits of lactoceuticals. Expert Review in Pharmacoeconomics Outcomes Resserch. Vol. 4, No. 2, pp. 199-206.

Lernet, C.G., Kobayashi, T., Inouye, M. 1990. Isolation of Subtilisin Prosequence Mutation that Affect Formation of Active Protease by Localized Random Polymerase Chain Reaction Mutagenesis. Journal of Biological Chemistry. Vol.265., pp. 20085-20086.

Li, Y., Inouye, M. 1996. The Mechanism of Autoprocessing of Propeptide of the Prothiolsubtilisin E: Intramolecular or Intermolecular Event ? Journal of Molecular Biology. Vol. 262., pp. 591-594.

Liuqin, Z., Yongmei, J. 1997. Protein Engineering on subtilisin E. Chinese J. Biotech. Vol. 13, No. 1, pp. 9-15.

Lu, W., Apostol, I., Qasim, M.A., Warne, N., Wynn, R., Zhang, W.L., Anderson, S., Chiang, Y.W., Ogin, E., Rothberg, I., Ryan, K., Laskowski Jr, M. 1997. Binding of amino acid side – chains to S1 cavities of serine proteinases. J. Mol. Biol. Vol. 266, pp. 441-461.

Macrae, R., Robinson, R.K., Sadler, M.J. 1993. Encyclopaedia of food science, food technology and nutrition. Volume seven. Spectroscopy – Z. Academic Press. New York, pp. 4888 - 4892.

Madsen, J.S. and Qvist, K.B. 1997. Hydrolysis of milk protein by a Bacillus licheniformis protease specific for acidic amino acid residues. Journal of Food Scince. Vol. 62, No. 3, pp. 579-582.

Madsen, J.S., Ahmt, T.O., Otte, J., Halkier, T. and Qvist, K.B. 1997. Hydrolysis of β-lactoglobulin by four different proteinases monitored by capillary electrophoresis and high performance liquid chromatography. Int.Dairy Journal. Vol. 7, pp. 399-409.

Maeda, H., Zhu, X., Mitsuoka, T. 2003. New medium for the production of exopolysaccharide (OSKC) by *Lactobacillus kefiranofaciens.* Biosci. Microflora. Vol. 22, pp. 45-50.

Maeda, H., Zhu, X., Omura, K., Suzuki, S., Kitamura, S. 2004A. Effects of an exopolysaccharide (kefiran) on lipids, blood pressure, blood glucose, and constipation. Biofactos. Vol. 22, pp. 197-200.

Maeda, H., Zhu, X., Suzuki, S., Suzuki, S., Kitamura, S. 2004B. Structural characterization and biological activities of an exopolysaccharide kefiran produced by *Lactobacillus kefiranofaciens* WT-2B. Journal of Agricultural and Food Chemistry. Vol. 52, pp. 5533-5538.

Mainville, I., Arcand, Y., Farnworth, E.R. 2005. A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. International Journal of Food Microbiology. Vol. 99, No. 3, pp.287-296.

Mann, E. J. 1986.Whey utilisation, part1. Dairy industries international. London United Trade Press. Vol 51, No.3, pp 11-12.

Marshall K. 2004. Therapeutic applications of whey protein. Altern Med Rev. Vol. 9, No. 2, pp. 136-56.

Matsudomi, N. Rector, D. and Kinsella, J.E. 1991. Gelation of bovine serum albumin and β-lactoglobulin; effects du pH, salts and thiol reagents. Food Chemistry. Vol. 40, pp. 55-69.

McPhalen, C.A., James, M.N.G. 1988. Structural comparaison of two serine proteinase – protein inhibitor complexes : eglin – C – subtilisin Carlsberg and CI – 2 – subtilisin Novo. Biochemistry. Vol. 27, pp. 6582-6598.

Meyrath, J. and Bayer, K. 1979. Microbial Biomass; chapitre 8: Biomass from whey. Academic Press. London, 439 pages.

Micheli, L., Uccelletti, D., Palleschi, C., Crescenzi, V. 1999. Isolation and characterisation of a ropy Lactobacillus strain producing the exopolysaccharide kefiran. Applied Microbiology and Biotechnology Vol. 53, pp. 69-74.

Mitsue, T., Tachibana, K., Hara, T. Fujio, J. 1998 Isolation of kefiran-producing lactic acid bacteria from kefir grain and improvement of kefiran productivity - Note. Seibutsu Kogaku Kaishi. Vol.76, pp. 447-450.

Mitsue, T., Tachibana, K., Hara, T. Fujio, J. 1999 Efficient kefiran production by a mixed culture of lactobacillus kefiranofaciens KF-75 and yeast strains. Seibutsu Kogaku Kaishi. Vol.76, pp. 93-1030.

Monteiro SR, Tavares C, Evtuguin DV, Moreno N, Lopes da Silva JA. 2005. Influence of galactomannans with different molecular weights on the gelation of whey proteins at neutral pH. Biomacromolecules. Vol. 6, No. 6, pp. 3291-9.

Moreira JL, Mota RM, Horta MF, Teixeira SM, Neumann E, Nicoli JR, Nunes AC. 2005. Identification to the species level of Lactobacillus isolated in probiotic prospecting studies of human, animal or food origin by 16S-23S rRNA restriction profiling. BMC Microbiol. Vol. 5, No. 1, pp. 15.

Morr, C.V., Ha, E.Y.W. 1993. Whey protein concentrates and isolates : processing and functional properties. Criticals reviews in food science and nutrition. Vol. 33, No. 6, pp. 431 – 476.

Mukai, T., Toba, T., Itoh, T. Adashi, S. 1990. Structural investigation of the capsular polysaccharide from Lactobacillus kefiranofaciens K1. Carbohydrate Research. Vol. 204, pp. 227-232.

Murofushi, M., Mizuguchi, J., Aibara, K., Matuhasi, T. 1986. Immunopotentiative Effect of Polysaccharide from Kefir Grain, KGF-C, Administred Orrally in Mice. Immunopharmacology. Vol. 12, pp. 29-35.

Murofushi, M., Shiomi, M. Aibara, K. 1983. Effect of orally administred polysaccharide from kefir grain on delayed-type hypersensitivity and tumor growth in mice. Japaneese Journal of Medcine Science and Biology. Vol. 36, pp. 49-55.

Mutilangi, W.A.M., Panyam, D. and Kilara, A. 1995. Hydrolysates from proteolysis of heat-denatured whey proteins. Journal of Food Scince. Vol. 60, No. 5, pp. 1104-1109.

Ness, J.E., Welch, M., Giver, L., Bueno, M., Cherry, J.R., Borchert, T.V., Stemmer, W.P.C., Minshull, J. 1999. DNA shuffling of subgenomic sequences of subtilisin. Nature Biotechnology. Vol. 17. pp. 893-896.

Neysens, P., Messens, W., Gevers, D., Swings, J., De Vuyst, L. 2003. Biphasic kinetics of growth and bacteriocin production with Lactobacillus amylovorus DCE 471 occur under stress conditions. Microbiology. Vol. 49, No. 4, pp. 1073-1082.

Nielsen, W.K., Affertsholt, T. 2003. Global marketing and production of whey products – Focus on high value-added products and derivatives. 3A Bussiness Consulting repport, 190 pages.Nigatu, A. 2000. Evaluation of numerical analyses of RAPD and API 50 CH patterns to differentiate *Lactobacillus platarum, Lact. fermentum, Lact. rhamnosus, Lact. sake, Lact. parabuchneri, Lact. gallinarum, Lact. casei, Weissella minor* and related taxa isolated from *kocho* and *tef.* Journal of Applied Microbiology. Vol. 89, pp. 969-978.

O'Connell, T.P., Day, R.M., Torchilin, E.V., Bachovchin, W.W., Malthouse, J.P.G. 1997. A C-NMR study of role of Asn155 in stabilizing the oxyanion of a subtilisin tetrahedral adduct. Biochem.J. Vol. 326, pp. 861-866.

O'Sullivan, D.B., O'Connell, T.P., Mahon, M.M., Koenig, A., Milne, J.J., Fitzpatrick, T.B., Malthouse, J.P.G. 1999. C-NMR study of how the oxyanion pK_A values of subtilisin and chytrypsin tetrahedral adduct are affected by different amino acid residues binding in enzyme subsites S1-S4. Biochemistry. Vol. 38, pp. 6187-6194.

Oldfield DJ, Singh H, Taylor MW. 2005. Kinetics of heat-induced whey protein denaturation and aggregation in skim milks with adjusted whey protein concentration. J. Dairy Res. Vol. 72, No. 3, pp. 369-78.

Otte, J., Ju, Z.Y. Faergemand, M. and *al.* 1996 (a). Protease-induced aggregation and gelation of whey proteins. Journal of food science. Vol. 61. No. 5, pp. 911-915, 923.

Otte, J., Ju, Z.Y. Skriver, A. and Qvist, K.B. 1996 (b). Effects of limited proteolysis on the microstructure of heat-induced whey protein gels at varying pH. J. Dairy Sci. Vol. 79, pp. 782-790.

Otte, J., Lomholt, B., Ipsen, R., Stapelfeldt, H., Bukrinsky, J.T., and Qvist, K.B. 1997. Aggregate formation during hydrolysis of β-lactoglobulin with Glu and Asp specific protease from *Bacillus licheniformis*. J.Agric.Food Chem. Vol. 45, pp 4889-4896.

Otte, J., Zakora, M. and Qvist, K.B. 1998. Chemical cleavage of bovine β-lactoglobulin B by hydroxylamine. Milchwissenschaft. Vol. 53, No. 11, pp. 613-617.

Pallavicini, C., Pagliarini, E. and Peri, C. 1988. Effect of a proteolytic enzyme preparation (alcalase) on whey proteins. J. Dairy Sci. Vol. 71, pp. 887-892.

Paquin, P. 2004. Étude du potentiel de marché des ingrédients protéiques laitiers au Canada. Université Laval., 84 pages.

Pavlova, S.I., Kilic, A.O., Kilic, S.S., So, J.S., Nader-Macias, M.E., Simoes, J.A., Tao, L. 2002. Genetic diversity of vaginal lactobacilli from women in different countries base don 16S rRNA gene sequences. Journal of Applied Microbiology. Vol. 92, pp. 451-459.

Pearce, R.J. 1992. Dans : Whey and lactose processing. Chapitre 2 : Whey processing. Zadow, J.G. Elsevier applied science. London and New York, pp. 73 – 89.

Pelmont, J. 1995. Enzymes. Catalyseurs du monde vivant. Nouvelle Édition. Presses Universitaires de Grenoble. France, 1040 pages.

Perona, J.J. and Craik, C.S. 1995. Structural basis of substrate specificity in the serine proteases. Protein Science. Vol. 4. pp. 337-360.

Pilote, D. 2002. Optimisation des conditions de fermentation de la souche ES R2C2 pour la valorisation du lactosérum. INRS-Institut Armand-Frappier. Mémoire présenté pour l'obtention du grade de Maître ès sciences (M.Sc.) en microbiologie appliquée. 167 pages.

Prescott, Harley, and Klein. *Microbiologie.* DeBoeck-Wesmael ed, ed. W.C.B. Publishers, Vol. 1. 1995, Bruxelles: DeBoeck-Wesmael. 1014 pages.

Puranik, D.B. and Kanawjia, S.K. 1995. Application of biotechnology in dairy waste treatment. Indian Dairyman. Vol. 49, No. 9, pp. 5-9.

Qin, B.Y., Bewley, M.C., Creamer, L.K., Baker, E.N., Jameson, G.B. 1999. Functional implication of structural differences between variants A and B of bovine β-lactoglobulin. Protein Science. Vol. 8, pp. 75 – 83.

Rawling, N.D., Barrett, A.J. 1994. Methods in enzymology, volume 244. Proteolytic enzymes : serine and cysteine peptidases. Academic Press. Inc. USA.,775 pages.

Regester, G.O., Belford, D.A. 1999. New therapeutics from a dairy byproduct – cheese whey. Drug Development Research. Vol. 46, pp. 286-291.

Rimada PS, Abraham AG. 2001. Polysaccharide production by kefir grains during whey fermentation. J Dairy Res. Vol. 68, No. 4, pp. 653-61.

Rodrigues, K.L., Caputo, L.R., Carvalho, J.C., Evangelista, J., Schneedorf, J.M. 2005A. Antimicrobial and healing activity of kefir and kefiran extract. International Journal of Antimicrobial Agents. Vol. 25, No. 5, pp.404-408.

Rodrigues, K.L., Carvalho, J.C., Schneedorf, J.M. 2005B. Anti-inflammatory properties of kefir and its polysaccharide extract. Inflammopharmacology. Vol. 13, No. 5, pp. 485-492.

Rolland, J. R. 1993. Le lactosérum, ses utilisations. Le producteur de lait québécois. Vol. 13, No. 9, pp. 34-36.

Rossetti L, Giraffa G. 2005. Rapid identification of dairy lactic acid bacteria by M13-generated, RAPD-PCR fingerprint databases. J Microbiol Methods. Vol. 63, No. 2, pp. 135-44.

Santos, A, Mauro, M.S., Sanchez, A., Torres, J.M., Marquina, D. 2003. The antimicobial properties of différent strains of Lactobacillus spp. Isolated from kefir. System. App. Microbiol. Vol. 26, pp. 434-437.

Sarath, G., De La Motte, R. and Wagner, F. 1989. Proteolytic enzymes a pratical approch; chapitre 3 : Protease assay methods. IRL Press at Oxford University Press. Oxford, 255 pages.

Sato, K., Nakamura, M., Nishiya, T., Kawanari, M. and Nakajima, I. 1995. Preparation of gel of partially heat-denatured whey protein by proteolytic digestion. Milchwissenschaft. Vol. 50, No. 7, pp. 389-392.

Satokari RM, Vaughan EE, Smidt H, Saarela M, Matto J, de Vos WM. 2003. Molecular approaches for the detection and identification of bifidobacteria and lactobacilli in the human gastrointestinal tract. Syst Appl Microbiol. Vol. 26, No. 4, pp. 572-84. Review.

Schechter, I., Berger, A. 1967. On the size of the active site in proteases. I. Papain. Biochem. Biophys. Res. Commun. Vol. 27, pp. 157–162.

Schleifer, K.H. 1987. Recent changes in the taxonomy of lactic acid bateria. FEMS Microbiol. Rev. Vol. 46, pp. 201-203.

Schmidt, D.G. and Poll, J.K. 1991. Enzymatic hydrolysis of whey proteins. Hydrolysis of α-lactalbumin and β-lactoglobulin in buffer solutions by proteolytic enzymes. Neth. Milk Dairy J. Vol. 45, pp. 225-240.

Schmitke, J.L., Stern, L.J., Klibanov, A.M. 1998. Comparison of x-ray crystal structures of an acyl-enzyme intermediate of subtilisin Carlsberg formed in anhydrous acetonitrile and in water. Proc. Natl. Acad. Sci. USA. Vol. 95, pp. 12918-12923.

Schmitt, C., Sanchez, C., Desobry-Banon, S., Hardy, J. 1998. Structure and technofunctional properties of protein-polysaccharide complexes : a review. Critical Reviews in Food Science and Nutrition. Vol. 38, No. 8, pp. 689-753.

Shinde, U. Inouye, M. 1993. Intramolecular chaperones and protein folding. Trends in Biochemical Science. Vol. 18., pp. 442-446.

Shinde, U., Inouye, M. 1996. Propeptide-Mediated Folding in Subtilisin : The Intramolecular chaperone concept. Practical Protein Engineering. pp. 147-154.

Shinde, U., Inouye, M. 2000. Intramolecular chaperones : polypeptide extentions that modulate protein folding. Cell & Developmental Biology. Vol. 11. pp. 35-44.

Shiomi, M., Sasaki, K., Murofushi, M., Aibara, K. 1982. Antitumor activity in mice of orally administered polysaccharide from kefir grain. Japaneese Journal of Medcine Science and Biology. Vol. 35, pp. 75-80.

Sienkiewicz, T., Riedel, C.L. 1990. Whey and whey utilization. Verlag Th. Mann. Gelsenkirchen-Buer. Germany, pp.55-66.

Siezen, R.J., Leunissen, J.A.M. 1997. Subtilases : the family of subtilisin-like serine proteases. Protein Science. Vol. 6, pp. 501-523.

Siezen, R.J., M. de Vos, W., Leunissen, J.A.M. and Dijkstra, W. 1991. Homology modelling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteinases. Protein Engineering. Vol.4., No. 7. pp. 719-737.

Silen, J.L., Agard, D.A. 1989. The α-lytic protease pro-region does not require a physical linkage to activate the protease domain in vivo. Nature. Vol. 341., pp. 462-464.

Smoragiewicz, W., Langlet, B. et Boutard, A. 1986. Revue de la valorisation du lactosérum comme moyen efficace de lutte contre la pollution du milieu. Sciences et techniques de l'eau. Université du Québec à Montréal. Vol.19, No. 3, pp. 277-281.

Smyth, M., FitzGerald, R.J. 1999. Relationship between some characteristics of WPC hydrolysates and the enzyme complement in commercially available proteinase preparations. Int. Dairy Journal. Vol. 8, pp. 819-827.

Spellman D, Kenny P, O'Cuinn G, FitzGerald RJ. 2005. Aggregation properties of whey protein hydrolysates generated with Bacillus licheniformis proteinase activities. J Agric Food Chem. Vol. 53, No. 4, pp. 1258-65.

Stackebrandt, E., Goebel, B.M. 1994. Taxonomic note: a place for DNA-DNA reassociation ans 16SrRNA sequence analysis in the present species definition in bacteriology. International Journal of Systematic Bacteriology. Vol. 44, No. 4, pp. 846-849.

Stiles, M.E., Holzapfel, W.H. 1997. Lactic acid bacteria of foods and their current taxonomy. International Journal of Food Microbiology. Vol. 37, pp. 1-29.

Strausberg, S., Alexander, P., Wang, L., Schwarz, F., Bryan, P. 1993. Catalysis of a Protein Folding Reaction : Thermodynamic and Kinetic Analysis of Subtilisin BPN Interactions with Ist Propeptide Fragment. Biochemistry. Vol. 32. pp. 8112-8119.

Svendsen, I., Breddam, K. 1992. Isolation and amino acid sequence of a glutamic acid specific endopeptidase from *Bacillus licheniformis*. Eur. J. Biochem. Vol. 204, pp. 165-171.

Takizawa, S., Kojima, S., Tamura, S., Fujinaga, S., Benno, Y., Nakase, T. 1994. Lactobacillus kefirgranum sp. nov. and Lactobacillus parakefir sp. nov., Two New Species from Kefir Grains. International Journal of Systematic Bacteriology. Vol. 44, pp. 435-439.

Taniguchi, M., Tanaka, T. 2004. Clarification of interactions among microorganisms and development of co-culture system for production of useful substances. Adv Biochem Eng Biotechnol. Vol. 90, pp.35-62.

Theunissen J, Britz TJ, Torriani S, Witthuhn RC. 2005. Identification of probiotic microorganisms in South African products using PCR-based DGGE analysis. Int J Food Microbiol. Vol. 98, No. 1, pp. 11-21.

Toba, T., Abe, S., Arihara, K., Adachi, S. 1986. A medium for the isolation of capsular bacteria from kefir grains. Agriculture Biology and Chemistry. Vol. 50, pp. 2673-2674.

Toba, T., Abe, S., Arihara, K., Adachi, S. 1987. Modification of KPL medium for polysaccharide production by Lactobacillus sp. Isolated from kefir grain. Jpn. J. Zootech. Sci. Vol. 58, No. 11, pp. 987-990.

Toba, T., Uemura, H., Mukai, T., Fujii, T., Itoh, T., Adachi, S. 1991. A new fermented milk using capsular polysaccharide-producing Lactobacillus kefiranofaciens isolated from kefir grains. Journal of Dairy Research. Vol. 58, pp. 497-502.

Tonge, P.J., Carey, P.R. 1990. Length of the acyl carbonyl bond in acyl-serine proteases correlates with reactivity. Bichemistry. Vol, 29, pp.10723-10727.

Turgeon, S. L., Sanchez, C., Gauthier, S. F., Paquin, P. 1996. Stability and rheological properties of salad dressing containing peptidic fractions of whey proteins. International Dairy Journal. Vol. 6, No. 6, pp. 645-658.

Tyagi, R.D., Kluepfel, D., Couillard, D. 1991. Dans : Bioconversion of waste materials to industrial products. Chapitre 10 : Bioconversion of chesse whey to organic acids. Martin, A.M. Elsevier Applied Science. pp. 313 – 333.

Vancanneyt, M., Mengaud, J., Cleenwerck, I., Vanhonacker, K., Hoste, B., Dawyndt, P., Degivry, M.C., Ringuet, D., Janssens, D., Swings, J. 2004. Reclassification of *Lactobacillus kefirgranum* Takizawa et al. 1994 as *Lactobacillus kefiranofaciens subsp. Kefirgranum,* and emended description of *L. kefiranofaciens* Fujisawa et al. 1988.

Vardhanabhuti, B., Foegeding, E.A. 1999. Rheological properties and characterization of polymerized whey protein isolates. J. Agric. Food Chem. Vol. 47, pp. 3649-3655.

Vasala A, Panula J, Neubauer P. 2005. Efficient lactic acid production from high salt containing dairy by-products by Lactobacillus salivarius ssp. salicinius with pre-treatment by proteolytic microorganisms. J Biotechnol. Vol. 117, No. 4, pp. 421-31.

Verheul, M., Roefs, S.P.F.M. 1998. Structure of particulate whey protein gels : effet of NaCl concentration, pH, Heating temperature, and protein composition. J. Agric. Food Chem. Vol. 46, pp. 4909-4916.

Volkov, A., Jordan, F. 1996. Evidence for Intramolecular Processing of Prosubtilisin Sequestered on a Solid Support. Journal of Molecular Biology. Vol. 262. pp., 595-599.

Walkenstrom, P., Nielsen, M., Windthab, E., Hermansson, A.M. 1999. Effects of flow behaviour on the aggregation of whey protein suspensions, pure or mixed with xanthan. Journal of Food Engineering. Vol. 42, pp. 15-26.

Walzem RL, Dillard CJ, German JB. 2002. Whey components: millennia of evolution create functionalities for mammalian nutrition: what we know and what we may be overlooking. Crit Rev Food Sci Nutr. Vol. 42, No. 4, pp. 353-75.

Weinbreck F, de Vries R, Schrooyen P, de Kruif CG. 2003. Complex coacervation of whey proteins and gum arabic. Biomacromolecules. Vol. 4, No. 2, pp. 293-303.

Weinbreck F, Nieuwenhuijse H, Robijn GW, De Kruif CG. 2004a. Complexation of whey proteins with carrageenan. J Agric Food Chem. Vol. 52, No. 11, pp. 3550-5.

Weinbreck F, Tromp RH, de Kruif CG. 2004b. Composition and structure of whey protein/gum arabic coacervates. Biomacromolecules. Vol. 5, No. 4, pp. 1437-45.

Wells JA, Powers DB. 1986. In vivo formation and stability of engineered disulfide bonds in subtilisin. J Biol Chem. Vol. 261, No. 14, pp 6564-70.

Wells, J.A., Estell, D.A. 1988. Subtilisin – an enzyme designed to be engineered. TIBS. Vol. 13, pp. 291 – 297.

Whiting, A.K., Peticolas, W.L. 1994. Details of the acyl-enzyme intermediate and the oxyanion hole in serine protease catalysis. Biochemistry. Vol. 33, pp. 552-561.

Whitley, K., Marshall, V. M. 1999. Heterofermentative metabolism of glucose and ribose and utilisation of citrate by the smooth biotype of Lactobacillus amylovorus NCFB 2745. Antonie van Leeuwenhoek. Vol. 75, pp. 217–223.

Wright, C.T., Kleanhammer, T.R. 1981. Calcium-Induced Alteration of Cellular Morphology Affecting the Resistance of Lactobacillus acidophilus to Freezing. Applied and Environmental Microbiology. Vol. 41, No. 3, pp. 807-815.

Xiong, Y.L., Dawson, K.A. and Wan, L. 1993. Thermal aggregation of β-lactoglobulin: effect of ionic environment, and thiol reagent. J. Dairy Sci. Vol. 76, pp. 70-77.

Yalcin, A.S., 2006. Emerging therapeutic potential of whey proteins and peptides. Curr. Pharm. Des. Vol. 12, No. 13, pp. 1637-43.

Ye A, Hemar Y, Singh H. 2004. Flocculation and coalescence of droplets in oil-in-water emulsions formed with highly hydrolysed whey proteins as influenced by starch. Colloids Surf B Biointerfaces. Vol. 38, No. 1-2, pp. 1-9.

Yeung PS, Kitts CL, Cano R, Tong PS, Sanders ME. 2004. Application of genotypic and phenotypic analyses to commercial probiotic strain identity and relatedness. J Appl Microbiol.; Vol. 97, No. 5, pp.1095-104.

Yeung PS, Sanders ME, Kitts CL, Cano R, Tong PS. 2002. Species-specific identification of commercial probiotic strains. J Dairy Sci.; Vol. 85, No. 5, pp. 1039-51.

Yokoi, H., Fujii, Y., Mukai, T., Toba, T., Adashi, S. 1991. Some taxonomical characteristics of encapsulated Lactobacillus sp. KPB- 167B isolated from kefir grains and characterization of its extracellular polysaccharide. International Journal of Food Microbiology. Vol. 13, pp. 257-264.

Yokoi, H.; Watanabe, T. 1992. Optimum culture conditions for production of kefiran by Lactobacillus sp. KPB-167B isolated from kefir grains. Journal of fermentation and bioengineering. Vol. 74, pp. 327-329.

Zhu, X., Ohta, Y., Jordan, F., Inouye, M. 1989. Pro-sequence of subtilisin can guide the refolding of denatured subtilisin in an intermolecular process. Nature. Vol. 339. pp. 483-484.

Annexe 1 : Autres contributions

Chapitre 7 : Effets anti-inflammatoires de *L.kefiranofaciens*.

Contenu du chapitre : manuscrit déposé en révision.

Manuscrit déposé en révision.

Précourt, L.P., Beaulieu, J., Goyette, P., Simard, E., Dupont, C., Lemieux, P., Shareck, F. 2005.* Protective Effect of Live or Pasteurized *Lactobacillus kefiranofaciens* on Dextran Sulfate Sodium-Induced Intestinal Inflammation. Journal of Dairy Science. Soumission de 28 pages.

Contributions : J'ai effectué l'isolement des souches bactériennes durant ma maîtrise à partir de grains de kéfir. J'ai participé à l'encadrement de Louis-Phillippe Précourt durant sa maîtrise et à la révision du manuscrit.

7.1 Manuscrit déposé en révision

Précourt, L.P., Beaulieu, J., Goyette, P., Simard, E., Dupont, C., Lemieux, P., Shareck, F. 2005.* Protective Effect of Live or Pasteurized *Lactobacillus kefiranofaciens* on Dextran Sulfate Sodium-Induced Intestinal Inflammation. Journal of Dairy Science. Soumission de 28 pages.

Protective Effect of Live or Pasteurized *Lactobacillus kefiranofaciens* on Dextran Sulfate Sodium-Induced Intestinal Inflammation

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ABSTRACT

Probiotic bacteria have been shown to exert several health benefits, and more specifically help in the management of serious illnesses such as inflammatory bowel disease (IBD). The strain *Lactobacillus kefiranofaciens* R2C2 presented herein was isolated from kefir grains. Kefir consumption has traditionally been believed to promote gastrointestinal health, which promted the investigation of the biological contributions of this newly isolated *Lactobacillus* strain. In an animal model of intestinal inflammation, chemically-induced with dextran sulfate sodium (DSS), *L. kefiranofaciens* R2C2 significantly reduced rectal bleedings and diarrhoea by close to 50%. Treatment with *L. kefiranofaciens* R2C2 also significantly reduced weight loss, while facilitating recovery towards normal weight and better colon integrity was observed. Interestingly, bacteria inactivated by pasteurization showed protective effects comparable to those obtained with live bacteria in all observations monitered in the intestinal inflammation model. The overall protective effect of *L. kefiranofaciens* R2C2 was also similar to that of 5-ASA, a widely used drug for the treatment of inflammatory bowel disease. In conclusion, the capacity of *L. kefiranofaciens* R2C2 to improve symptoms of intestinal inflammation makes it a good candidate as a probiotic promoting gastrointestinal health. Moreover, efficacy of pasteurized bacteria suggests new mechanisms of action for probiotics, possibly through recognition of specific components such as CpG motifs in bacterial DNA.

(Key words : *Lactobacillus kefiranofaciens*, probiotic, intestinal inflammation)
Abbreviation key : 5-ASA = 5-amino salicylic acid, **CD** = Crohn's disease, **CMC** = carboxymethyl cellulose, **DSS** = dextran sulfate sodium, **IBD** = inflammatory bowel disease, **PBS** = phosphate buffered saline, **TLR** = Toll-like receptor, **UC** = ulcerative colitis

INTRODUCTION

The pathogenosis of inflammatory bowel disease (**IBD**), including Crohn's disease (**CD**) and ulcerative colitis (**UC**) is still unknown and its etiology is complex and multifactorial. The prevalence of IBD is about 0,2% of the population in industrialized countries (Singh et al., 2001) and yearly, more and more individuals are diagnosed with such health problems. Treatments for IBD are currently mainly targeting symptoms and relapses are occurring in a majority of patients, ultimately leading to surgery in most cases. CD is genetically associated with mutations of the Nod2 gene, which could lead to inflammation by abnormal bacterial sensing (Danese et al., 2004). Toll-like receptors (**TLR**) are also responsible for bacterial sensing, detecting molecular products of microorganisms and initiating inflammatory and immune responses (Rakoff-Nahoum et al., 2004). A disturbed balance of the T helper type 1 vs T helper type 2 immune response, favoring type 1 cells could also lead to IBD (Siegmund et al., 2001).

However, environmental risk factors seem to play a major role in the increased incidence of IBD in occidental population, as described by the "hygiene hypothesis" (Danese, S. et al., 2004). This theory states that the eradication of infectious diseases and better public health measures have led to a reduction of bacterial exposure and consequently to an increased incidence of atopic and autoimmune diseases such as IBD (Shi and Walker, 2004; Strachan, 2000). This theory increases awareness to the importance of host-microbial interactions, particularly in the intestine, in normal human immune system development (Tamboli et al., 2003). Favorable bacterial exposures, obtained with the use of well-selected probiotic bacteria, could protect against IBD by educating the immune sytem to develop tolerance (Shi and Walker, 2004).

Probiotics are generally defined as "nutritional supplements composed of living microorganisms which exert beneficial effects on the host" (Guarner and Shaafsma, 1998), but newer definitions have recently been proposed, such as "microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host" (Vaarala, 2003). The second definition does not specify if the bacteria have to be alive and suggests that only bacterial "components" can exert beneficial effects. Probiotic bacteria have shown various health benefits, particularly for the gastrointestinal tract, and many different strains have been shown to reduce the symptoms of intestinal inflammation in animal models and clinical studies (Sullivan and Nord, 2005).

Knowledge on probiotic bacteria has increased a lot in recent years, but fermented milks such as kefir represent the origins of all the promises of probiotic bacteria. Kefir has been traditionally consumed in Soviet countries and was provided as a clinical treatment for gastrointestinal tract disorders and metabolic diseases (Koroleva, 1988). Kefir grains are composed of many different lactic acid bacteria, acetic acid bacteria, yeasts and other microorganisms (Toba et al., 1990). Kefir is recognized as a product of alternative medecine since most of its health benefits have not been demonstrated in animal or human studies. Kefir has been known and consumed for many years with allegated beneficial effects, but there is still very poor knowledge on the contribution of some of the bacteria it contains. Up to date, only Santos et al. (2003) suggested potential probiotic properties of different *Lactobacillus kefiranofaciens* strains isolated from kefir grains. Recently, a new strain was isolated from kefir grains, called R2C2, and identified as *Lactobacillus kefiranofaciens* based on the 16S DNA sequence. Traditional knowledge of kefir benefits on gastrointestinal health has led to a further investigation of the possible

protective effect of the bacteria *Lactobacillus kefiranofaciens* R2C2 in an animal model of chemically-induced intestinal inflammation.

The present study was performed to investigate the potential protective effect of this newly isolated *L. kefiranofaciens* strain in the dextran sulfate sodium (**DSS**)-induced intestinal inflammation animal model. *Lactobacillus kefiranofaciens* R2C2 was fed as live and as pasteurized bacteria to laboratory animals to investigate the effect of both live and dead bacteria in that model. Furthermore, protective effect of the strain against intestinal inflammation was compared to the effect obtained with a commonly used drug in patients suffering from IBD, 5-amino salicylic acid (**5-ASA**).

MATERIALS AND METHODS

Animals and Treatments

For the experiment, female C57BL/6 mice, 6 to 8 weeks old were purchased from Charles River Canada (Saubermann et al. 2002). Mice were randomized according to body weight, in 5 different groups. All animals consumed standard diet and received water *ad libitum*. They were housed under specific pathogen-free conditions and maintained in a 12 hours light/dark cycle. Experiments were repeated at least twice, with 5 to 10 mice per group. The data displayed provides a summary of the results.

The bacterial strain investigated in this study for its potential anti-inflammatory properties was isolated from kefir grains. The strain R2C2 was identified as a *Lactobacillus* of the *kefiranofaciens* specie using the ribosomal 16S DNA sequence. The strain was adapted to grow in whey in order to produce a fermented product.

Before induction of intestinal inflammation, animals were pre-treated *per os* once daily for 7 days with various products : 100 uL of phosphate buffered saline (**PBS**) (Invitrogen) (for the 2 control groups, with or without DSS in drinking water), 100 uL of

a bacterial suspension of live *Lactobacillus kefiranofaciens* R2C2 in PBS (containing 10^9 bacteria/mL), 100 uL of a bacterial suspension of pasteurized *Lactobacillus kefiranofaciens* R2C2 in PBS (containing 10^9 bacteria/mL) or the drug 5-ASA (at a dose of 150 mg/kg) (MP Biomedicals) prepared in 0,5% of carboxymethyl cellulose (**CMC**) (Sigma). (Rumi et al., 2004) Feedings with the different products continued during 7 days of exposure to DSS and were then stopped for an 8-day post-inflammatory recovery period. At the end of the experiment, animals were killed by cervical dislocation under anesthesia.

The bacterial strain *Lactobacillus kefiranofaciens* R2C2 was routinely cultured in Man Rogosa Sharpe broth (BD Biosciences) at 37°C for a period of 24h. Bacteria were then pelleted by centrifugation at 4000×g for 8 minutes and re-suspended at a concentration of 10^9 cells/mL in sterile PBS (Invitrogen), using a standard curve of optical density at 640 nm. For the pasteurization process, bacteria were heated at 75°C for 30 minutes and immediately placed at 4°C. All procedures for the feedings and for the induction of intestinal inflammation with DSS were in accordance with the institution's guide for the care and use of laboratory animals and accepted by the ethic committee.

Induction of Intestinal Inflammation

Intestinal inflammation was chemically-induced using DSS (molecular weight=36000-50000) (MP biomedicals). Following 7 days of pre-treatment with the different products, DSS was dissolved in drinking water at a concentration of 2,5% (wt/vol) and animals had free access to it for 7 days (Hibi et al., 2002; Pizzaro et al., 2003). Water supplemented with DSS was then replaced by sterile water and treatments were stopped for a recovery period of 8 days.

Observation of Clinical Signs

The severity of intestinal inflammation was assessed daily by following multiple observations. The clinical signs followed are comprehensive functional measures that are analogous to clinical symptoms observed in human CD and UC (Osman et al., 2004). DSS-induced intestinal inflammation is associated with a rapid weight loss and bloody diarrhoeas (Pizzaro et al., 2003). Consequently, weight variation, rectal bleedings and feces consistency were monitered daily. Hemoccult II (Beckman Coulter) serial test slides of routine screening for fecal occult blood were used to evaluate rectal bleedings on a scale of 0-4, defined as follows : **0**- No blood, **4**- Feces like blood. Feces consistency was also evaluated on a scale of 0-4, defined as follows : **0**- Normal consistency, **4**- Liquid feces (Osman et al., 2004). Feces consistency and rectal bleedings values were combined and defined as the "clinical score". Scores were given by a blinded evaluator. At the end of the recovery process, other measurements were taken to assess the severity of the disease. Postmortem, the entire colon was removed, from the caecum to the anus, and it was measured as a marker of the deterioration caused by inflammation (Siegmund et al., 2001) and myeloperoxidase activity was determined.

Determination of Myeloperoxidase Activity

Colons were snap-frozen and conserved at -80°C before analysis of myeloperoxidase (MPO) activity. Tissues were weighed while still frozen, cut into small pieces and placed in 1 mL of 50 mM potassium-phosphate buffer (pH 6.0) containing 0,5% of hexadecyltrimethylammonium bromide. Tissues were then sonicated on ice 3 times for 10 seconds, with a 30 seconds pause between each sonication. Tissues were

freeze-thawed 3 times before 2 other sonication cycles. A centrifugation of 15 minutes at 10 000×g was performed and an aliquot of the supernatant was taken for determination of the MPO activity according to the method described by Bradley et al. (1982).

Statistical Analysis

Statistical analysis was performed using the Student's t test. P values < 0.05 were considered significant. Error bars represent ± standard error means.

RESULTS

Weight Loss and Recovery

All DSS-treated animals started consistently losing weight after 4 days of exposure. Weight variation was comparable for all groups until day 7, the last day of exposure to DSS, where treatments with live R2C2 ($P < 0.01$, -7.19 ± 0.88%), pasteurized R2C2 ($P < 0.01$, -7.64 ± 0.81%) or 5-ASA/CMC ($P < 0.01$, -8.06 ± 0.50%) significantly prevented weight loss, compared to PBS-DSS controls (-12.60 ± 1.03%). Weight loss stopped on day 10 for groups treated with live R2C2, pasteurized R2C2 or 5-ASA/CMC, 3 days earlier than for the PBS-DSS control group. Recovery was comparable for groups treated with live R2C2, pasteurized R2C2 or 5-ASA/CMC, no significant difference was found. However, animals in these groups recovered more rapidly compared to the PBS-DSS control group, each showing statistically significant ($P < 0.01$) weight variation through days 8 to 15.

Clinical Score

The clinical score represents combined results of rectal bleedings and diarrhoea. Detectable occult blood appears after 4 days of exposure to DSS, when feces also start to be watery. Protection was observed as soon as day 4 of exposure to DSS for groups treated with live R2C2 ($P < 0.05$, 1.71 ± 0.16), pasteurized R2C2 ($P < 0.05$, 1.69 ± 0.21) or 5-ASA/CMC ($P < 0.01$, 1.11 ± 0.31), compared to the PBS-DSS control group (2.29 ± 0.22). Clinical score was significantly lower on day 5 for the live R2C2 group ($P < 0.01$, 2.14 ± 0.24), pasteurized R2C2 group ($P < 0.01$, 2.69 ± 0.24) and 5-ASA/CMC group ($P < 0.05$, 2.63 ± 0.50), compared to PBS-DSS controls (3.92 ± 0.29). On day 6, scores were still lower for groups treated with live R2C2 ($P < 0.01$, 3.92 ± 0.32), pasteurized R2C2 (*P*

< 0.01, 3.00 ± 0.37) or 5-ASA/CMC (P < 0.01, 4.13 ± 0.23), compared to the PBS-DSS control group (5.69 ± 0.26). On day 7, scores were significantly lower for groups treated with live (P < 0.01, 5.25 ± 0.22) or pasteurized R2C2 (P < 0.01, 4.83 ± 0.30), but not for the 5-ASA/CMC group (5.88 ± 0.35), compared to PBS-DSS controls (6.58 ± 0.25). Statistical differences were observed between the 5-ASA/CMC groups and both R2C2 groups on day 4, R2C2 groups being slightly higher, and on day 7, R2C2 groups being slightly lower. On day 6, the pasteurized R2C2 groups was significantly lower than groups treated with live R2C2 or 5-ASA/CMC. However, the general tendency showed a significant and similar protection confered by treatments with live R2C2, pasteurized R2C2 or 5-ASA/CMC, compared to PBS-DSS controls.

Colon Length

Shortening of the intestine is a common feature of the DSS-induced intestinal inflammation animal model and correlates with the intensity of the disease (Siegmund et al., 2001). Colon length was thus measured at the end of the recovery period as a marker of inflammation. Normal colon length of 6-8 weeks old C57BL/6 mice is represented by the PBS control group (7.17 ± 0.14 cm). Colon length was dramatically reduced after 7 days of exposure to DSS and 8 days of recovery, particularly for the PBS-DSS control group (4.79 ± 0.22 cm). Colon length reduction seemed partially prevented in groups treated with live R2C2 (P < 0.01, 5.86 ± 0.13 cm), pasteurized R2C2 (P < 0.01, 6.06 ± 0.16 cm) or 5-ASA/CMC (P < 0.01, 5.81 ± 0.13 cm) compared to PBS-DSS controls. No statistical difference was observed between groups treated with live R2C2, pasteurized R2C2 or 5-ASA/CMC.

MPO Activity

MPO is a naturally occurring constituent of neutrophils and its quantification is representative of the intensity of the disease process (Bradley et al., 1928). MPO activity was measured at the end of the experiment, after an 8-day recovery period, so values were expected to be low or equal to the healthy mice group. But even after 8 days of recovery, differences were observed between groups. Mice that received live R2C2 ($P < 0.05$, 0.082 ± 0.025 units/g of tissue), pasteurized R2C2 ($P < 0.05$, 0.066 ± 0.024 units/g of tissue) or 5-ASA/CMC ($P < 0.05$, 0.043 ± 0.004 units/g of tissue) had levels significantly lower compared to that of the PBS-DSS group (0.226 ± 0.088 units/g of tissue), but similar to that of the PBS control group (0.056 ± 0.007 units/g of tissue).

DISCUSSION

There are many reports indicating the efficacy of several probiotic bacteria to protect or reduce symptoms in animal models of experimental colitis (Osman et al., 2004; Kamada et al., 2005). However, there are no report on the anti-inflammatory potential of bacterial strains of *L. kefiranofaciens*. Only one publication is showing probiotic characteristics of a *kefiranofaciens* strain, *L. kefiranofaciens* CYC 10058, which showed good adhesion, resistance to acidic pH values and bile salts and inhibition of some pathogenic bacteria (Santos et al., 2003). The strain *Lactobacillus kefiranofaciens* R2C2 was isolated from kefir, which has been traditionally provided for clinical treatment of gastrointestinal tract in eastern Europe (Koroleva, 1988). The effects of R2C2 in intestinal inflammation were evaluated and dead cells were also tested in order to clarify mechanisms of action.

The DSS-induced intestinal inflammation seems to be a fast and reliable animal model that recapitulates events that lead to acute mucosal injury (Pizzaro et al., 2003). The model is characterized by epithelial disruption resulting in luminal bacterial translocation, mucosal ulcers and infiltration of neutrophils and other acute immune cells in the intestinal wall. DSS-induced intestinal inflammation is also associated with rapid body weight loss, bloody diarrhoea and shortening of the intestine (Hibi et al., 2002). It allows a close survey of many events occurring in human IBD (Pizzaro et al., 2003). This animal model has shown useful in providing proof of concept for therapeutic interventions in a simple and relatively inexpensive setting (Pizzaro et al., 2003), particularly with probiotic bacteria (Shibolet et al., 2002; Osman et al., 2004).

Reduction of Symptoms by Live or Pasteurized R2C2

Live *L. kefiranofaciens* R2C2 fed as treatment against chemically-induced intestinal inflammation significantly reduced weight loss and facilitated post-inflammatory recovery. After 7 days of exposure to DSS, weight loss was less pronounced in the group treated with live R2C2, demonstrating a protective effect during the acute phase of inflammation. Clinical scores of rectal bleedings and diarrhoea throughout exposure to DSS were also significantly lower than in the placebo group, again suggesting a reduction of DSS-induced injury to the intestinal wall. Human acute colitis or relapses of CD and UC are characterized by weight loss and bloody diarrhoea. In this animal experiment of intestinal inflammation, live R2C2 reduced both these symptoms significantly. Results of the clinical score during the recovery period were not presented because they diminished rapidly for all groups (data not shown). During the post-inflammatory recovery period, weight gain started 3 days earlier in the live R2C2 group and continued significantly more rapidly than for the PBS-DSS control group. DSS exposure thus could probably not create an intestinal injury as pronounced when animals were treated with live R2C2. Since no more probiotic feedings were given during the post-inflammatory recovery period, the protection conferred by R2C2 during the acute phase of intestinal inflammation is confirmed. A better recovery process is also demonstrated by post-mortem colon length. Thickening and shortening of the colon is a common feature of intestinal inflammation (Siegmund et al., 2001). Colons of the live R2C2-treated group were shorter than those of healthy animals but were significantly longer than those of the PBS-DSS control group, suggesting a less intense inflammation. MPO activity, representative of the infiltration of inflammatory cells in the colonic wall, was slightly higher in the live R2C2-treated group

than in the healthy mice group, but no statistical difference was observed, suggesting a near finished inflammatory process. Contrastingly, MPO levels were significantly higher in the PBS-DSS control group, indicating that the inflammatory process was still active after 8 days of recovery. Taken together, these results suggest that treatment with live R2C2 partially protected from DSS-induced intestinal inflammation, significantly attenuating many important symptoms. The recovery process was possibly facilitated because of the reduced intestinal injury.

Interestingly, pasteurized *L. kefiranofaciens* R2C2 fed as treatment against chemically-induced intestinal inflammation significantly reduced weight loss and facilitated post-inflammatory recovery, with no statistical difference with live R2C2. Similarly to the live R2C2-treated group, mice treated with pasteurized R2C2 showed a significant reduction in clinical scores and better post-mortem colon integrity demonstrated by its length and reduced MPO activity.

Few studies have demonstrated the efficacy of dead bacteria to protect against intestinal inflammation. Rachmilewitz et al. (2004) showed that live or irradiated VSL#3 reduced symptoms of DSS-induced intestinal inflammation, but the heat-killed (30 minutes, 100°C) probiotic mixture had no effect. They determined that purified bacterial DNA from the VSL#3 mixture had a comparable efficacy than that obtained with live bacteria (Rachmilewitz et al., 2004). Kamada et al. (2005) demonstrated that the protective effect of *Escherichia coli* Nissle1917 in DSS-induced intestinal inflammation could be obtained by using heat-killed (30 minutes, 60°C) bacteria. In that study, purified bacterial DNA also attenuated colitis. However, live *Lactobacillus crispatus* M247 reduced the severity of DSS colitis, but heat-killed (time and temperature not precised) bacteria had no effect (Castagliuolo et al., 2005). Arguably, treatment at 100°C might

expose bacterial DNA to a too intense heat, which might explain the loss of the protective effect, in comparison to the study of Kamada et al. (2005) and the experiment presented herein, where bacteria was heated at 75°C for 30 minutes.

Results of these studies and the efficacy of live as well as pasteurized R2C2 to protect against chemically-induced intestinal inflammation suggest that beneficial effects produced by probiotic consumption can be mediated by specific components, and thus dead bacteria can be efficient, while in other cases, viability seems to be a major factor (Mottet and Michetti, 2005). Consequently, for specific uses, probiotic strains do not necessarily have to survive stomach acidity, digestive and pancreatic enzymes and bile salts. Moreover, probiotic bacteria might not have to adhere to intestinal epithelial cells and colonize the gastrointestinal tract, because recognition of cell components could trigger immunological processes possibly responsible for anti-inflammatory effects.

Precise mechanisms of action of probiotics remain unclear and the efficacy of dead bacteria is still controversial. Conventional mechanisms of action of probiotic bacteria to protect against intestinal inflammation include exclusion of pathogenic species by competition for attachment sites and nutrients or production of antibacterial factors (O'Mahony et al., 2001). A reduction in fecal coliforms and enterococci levels in a probiotic-fed group was found to reduce intestinal inflammatory activity, showing that the modulation of the microflora might help reduce symptoms of colitis (O'Mahony et al., 2001). Viability is logically a major factor for such effects to take place. In the present experiment, live R2C2 demonstrated the capacity to reduce fecal coliforms, while pasteurized R2C2 did not but still reduced symptoms of intestinal inflammation (data not shown). Modulation of the microflora is certainly a desirable feature of R2C2, but does not seem to be necessary for its beneficial impact on DSS-induced colitis.

The efficacy of pasteurized R2C2 to reduce symptoms of intestinal inflammation suggests that specific components of the bacteria interact with immune or epithelial cells along the gastrointestinal tract, possibly regulating the expression of inflammatory cytokines or restoring the Th1/Th2 balance (Siegmund et al., 2001; O'Mahony et al., 2001). Moreover, new mechanisms of action were recently identified, suggesting beneficial impact of dead probiotic bacteria in intestinal inflammation through immunomodulation mediated by its own DNA (Rachmilewitz et al., 2004; Kamada et al., 2005). Unmethylated CpG dinucleotides within consensus sequences, called CpG motifs, are present in high number on bacterial DNA, but are underrepresented in mammalian DNA (Rachmilewitz et al., 2004). Recognition of bacterial components, such as CpG motifs, by Toll-like receptors (TLR) is partially responsible for maintenance of intestinal homeostasis and necessary for the healing process of injured epithelium (Abreu et al., 2005; Rakoff-Nahoum et al., 2004). Recognition of bacterial CpG motifs by TLR9 could potentially lead to an increased activity of regulatory T cells, that can control inflammation by expression of anti-inflammatory cytokines such as IL-10 and TGFβ (Singh et al., 2001), through production of IFNα by dendritic cells (Waston and McKay, in press).

Comparison of the Anti-Inflammatory Effect of *L. kefiranofaciens* R2C2 to that of 5-ASA

5-ASA is a standard anti-inflammatory drug for human IBD and its beneficial effects were demonstrated in DSS-induced colitis in animals (Rumi et al., 2004). 5-ASA has shown the capacity to prevent body weight loss, colon shortening, diarrhoea and

rectal bleedings (Rumi et al., 2004). In the present experiment, 5-ASA significantly prevented weight loss and facilitated post-inflammatory recovery, reduced clinical scores based on rectal bleedings and diarrhoea and better colon integrity was observed, based on length and MPO activity. The protective effects observed with live or pasteurized *L. kefiranofaciens* R2C2 were comparable to those of 5-ASA, for all clinical signs monitored. These results demonstrated that probiotic supplementation, in laboratory animals, could have a similar impact to a commonly used drug in human.

In conclusion, supplementation with live or pasteurized *Lactobacillus kefiranofaciens* R2C2 demonstrated a protective effect on DSS-induced intestinal inflammation. Live or pasteurized R2C2 reduced weight loss, facilitated post-inflammatory recovery, reduced rectal bleedings and diarrhoea and partially preserved colon integrity. Moreover, the protective effect obtained with R2C2 was comparable to that of 5-ASA. Efficacy of pasteurized bacteria against IBD suggests new mechanisms of action for probiotics, including immunomodulation by specific components such as CpG motifs in bacterial DNA.

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REFERENCES

Abreu, M.T., M. Fukata and M. Arditi. 2005. TLR signaling in the gut in health and disease. J. Immunol. 174(8):4453-4460.

Bradley, P.P., D.A. Priebat, R.D. Christensen and G. Rothstein. 1982. Measurement of cutaneus inflammation : estimation of neutrophil content with an enzyme marker. J. Invest. Dermatol. 78(3):206-209.

Castagliuolo, I., F. Galeazzi, S. Ferrari, M. Elli, P. Brun, A. Cavaggioni, D. Tormen, G.C. Sturniolo, L. Morelli and G. Palu. 2005. Beneficial effect of auto-aggregating *Lactobacillus crispatus* on experimentally induced colitis in mice. FEMS Immunol. Med. Microbiol. 43(2):197-204.

Danese, S., M. Sans and C. Fiocchi. 2004. Inflammatory bowel disease : the role of environmental factors. Autoimmun. Rev. 3(5):394-400.

Guarner, F. and G.J. Shaafsma. 1998. Probiotics. Int. J. Food Microbiol. 39(3):238-239.

Hibi, T., H. Ogata and A. Sakuraba. 2002. Animal models of inflammatory bowel disease. J. Gastroenterol. 37(6): 409-417.

Kamada, N., N. Inoue, T. Hisamatsu, S. Okamoto, K. Matsuoka, T. Sato, H. Chinen, K.S. Hong, T. Yamada, Y. Suzuki, N. Watanabe, K. Tsuchimoto and T. Hibi. 2005. Nonpathogenic *Escherichia coli* strain Nissle1917 prevents murine acute and chronic colitis. Inflamm. Bowel Dis. 11(5):455-463.

Koroleva, N.S. 1988. Technology of kefir and kumys. Fed. Int. Laiterie Int. Dairy Fed. Bull. 227:96-100.

Marteau, P. and F. Shanahan. 2003. Basic aspects and pharmacology of probiotics : an overview of pharmacokinetics, mechanisms of action and side-effects. Best Pract. Res. Clin. Gastroenterol. 17(5):725-740.

Mottet, C. and P. Michetti. 2005. Probiotics: wanted dead or alive. Dig. Liv. Dis. 37(1):3-6.

O'Mahony, L., M. Feeney, S. O'Halloran, L. Murphy, B. Kiely, J. Fitzgibbon, G. Lee, G. O'Sullivan, F. Shanahan and J.K. Collins. 2001. Probiotic impact on microbial flora, inflammation and tumor development in IL-10 knockout mice. Aliment. Pharmacol. Ther. 15(8):1219-1225.

Osman, N., D. Adawi, S. Ahrne, B. Jeppsson and G. Molin. 2004. Modulation of the effect of dextran sulfate sodium-induced acute colitis by the administration of different probiotic strains of *Lactobacillus* and *Bifidobacterium*. Dig. Dis. Sci. 49(2):320-327.

Pizzaro, T.T., K.O. Arseneau, G. Bamias and F. Cominelli. 2003. Mouse models for the study of Crohn's disease. Trends Mol. Med. 9(5):218-222.

Rachmilewitz, D., K. Katakura, F. Karmeli, T. Hayashi, C. Reinus, B. Rudensky, S. Akira, K. Takeda, J. Lee, K. Takabayashi and E. Raz. 2004. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. Gastroenterology. 126(2):520-528.

Rakoff-Nahoum, S., J. Paglino, F. Eslami-Varzaneh, S. Edberg and R. Medzhitov. 2004. Recognition of commensal microflora by Toll-like receptors is required for intestinal homeostasis. Cell. 118(2):229-241.

Rumi, G., R. Tsubouchi, M. Okayama, S. Kato, G. Mozsik and K. Takeuchi. 2004. Protective effect of lactulose on dextran sulfate sodium induced colonic inflammation in rats. Dig. Dis. Sci. 49(9):1466-1472.

Santos, A., M. San Mauro, A. Sanchez, J.M. Torres and D. Marquina. 2003. The antimicrobial properties of different strains of *Lactobacillus* spp. isolated from kefir. System. Appl. Microbiol. 26(3):434-437.

Saubermann, L.J., A. Nakajima, K. Wada, S. Zhao, Y. Terauchi, T. Kadowaki, H. Aburatani, N. Matsuhashi, R. Nagai and R.S. Blumberg. 2002. Peroxisome proliferator-activated receptor gamma agonist ligands stimulate a Th2 cytokine response and prevent acute colitis. Inflamm. Bowel Dis. 8(5):330-339.

Shi, H.N. and A. Walker. 2004. Bacterial colonization and the development of intestinal defences. Can. J. Gastroenterol. 18(8):493-500.

Shibolet, O., F. Karmeli, R. Eliakim, E. Swennen, P. Brigidi, P. Gionchetti, M. Campieri, S. Morgenstern and D. Rachmilewitz. 2002. Variable response to probiotics in two models of experimental colitis in rats, Inflamm. Bowel Dis., 8(6):399-406.

Siegmund, B., G. Fantuzzi, F. Rieder, F. Gamboni-Robertson, H.A. Lehr, G. Hartmann, C.A. Dinarello, S. Endres and A. Eigler. 2001. Neutralization of interleukin-18 reduces severity in murine colitis and intestinal IFN-γ and TNF-α production. Am. J. Physiol. Regul. Integr. Comp. Physiol. 281(4):R1264-R1273.

Singh, B. S. Read, C. Asseman, V. Malmstrom, C. Mottet, L.A. Stephens, R. Stepankova, H. Tlaskalova and F. Powrie. 2001. Control of intestinal inflammation by regulatory T cells. Immunol. Rev. 182:190-200.

Strachan, D.P. (2000) Family size, infection and atopy : the first decade of the "hygiene hypothesis". Thorax. 55(Suppl. 1):S2-S10.

Sullivan, A. and C.E. Nord. 2005. Probiotics and gastrointestinal diseases. J. Intern. Med. 257(1):78-92.

Tamboli, C.P., C. Caucheteux, A. Cortot, J.F. Colombel and P. Desreumaux. 2003. Probiotics in inflammatory bowel disease : a critical review. Best Pract. Res. Clin. Gastroenterol. 17(5):805-820.

Toba, T., K. Arihara, and S. Adachi. 1990. Distribution of microorganisms with particular reference to encapsulated bacteria in kefir grains. Int. J. Food Microbiol. 10(3-4):219-224.

Vaarala, O. 2003. Immunological effects of probiotics with special reference to lactobacilli. Clin. Exp. Allergy. 33(12):1634-1640.

Watson, J.L. and D.M. McKay. Article in press. The immunophysiological impact of bacterial CpG DNA on the gut. Clin. Chim. Acta.

Figure 1. Body weight variation for groups treated with live or pasteurized *Lactobacillus kefiranofaciens* R2C2 compared to the PBS-DSS control group and the 5-ASA/CMC group, during 7 days of exposure to 2,5% of DSS in drinking water and 8 days of post-inflammatory recovery. Animals were pre-treated for 7 days and feedings continued during exposure to DSS, but stopped at the beginning of the recovery period. Body weight is shown as percentage of the body weight just before DSS administration. **, Statistically significant, $P < 0.01$, for both R2C2-treated groups and the 5-ASA-treated group, compared to the PBS-DSS control group (using the Student's t test). No statistical difference was observed between the groups treated with live R2C2, pasteurized R2C2 or 5-ASA/CMC.

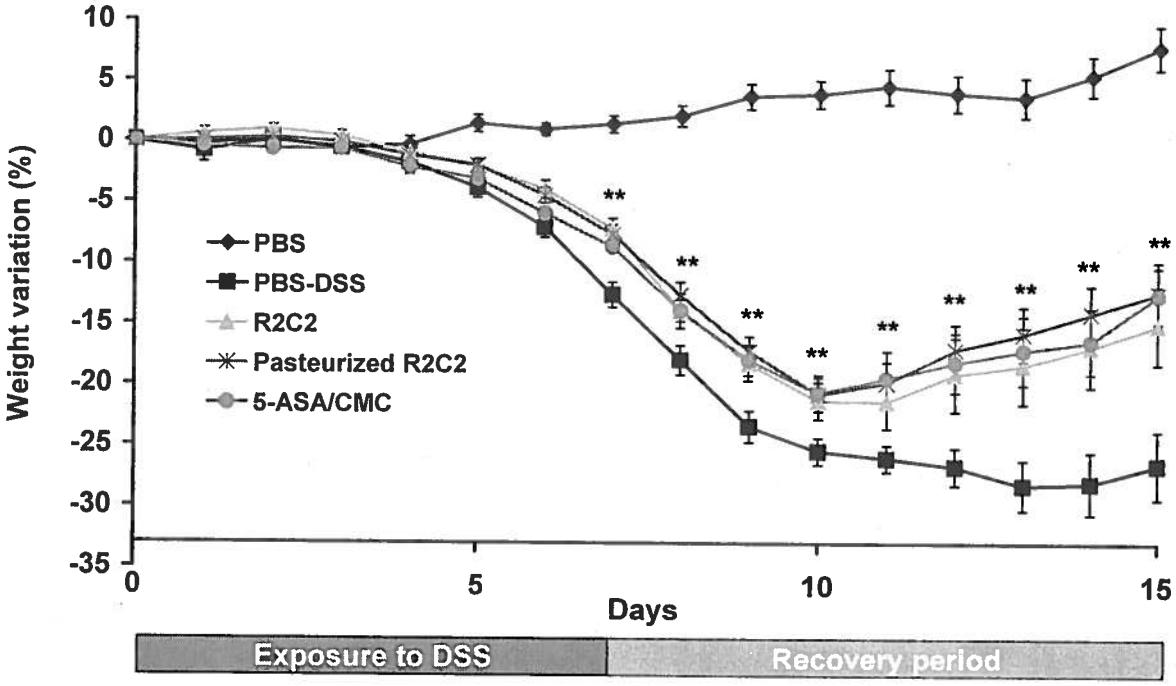

10
5
0
-5
-10
-15
-20
-25
-30
-35
Weight variation (%)
PBS
PBS-DSS
R2C2
Pasteurized R2C2
5-ASA/CMC
**
0
5
10
15
Days
Exposure to DSS
Recovery period

Figure 2. Clinical score (rectal bleedings and feces consistency) for animals treated with live *Lactobacillus kefiranofaciens* R2C2, pasteurized R2C2 or 5-ASA/CMC and compared to the PBS-DSS control group (days 4, 5, 6 and 7 of exposure to DSS). *, $P < 0.05$, **, $P < 0.01$ Statistically significant compared to the PBS-DSS control group (using the Student's t test).

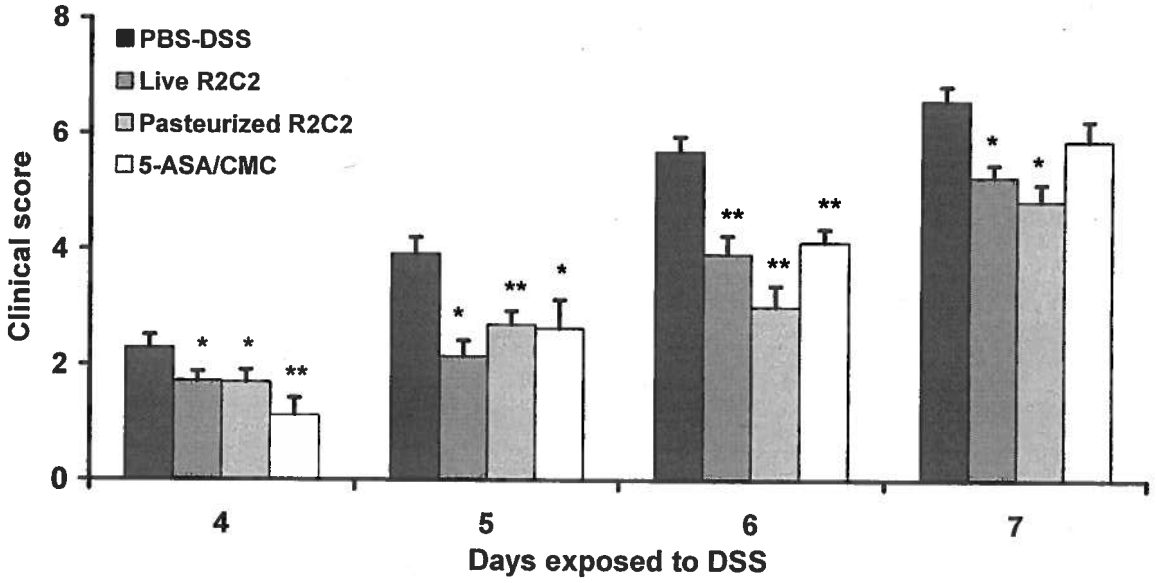

PBS-DSS
Live R2C2
Pasteurized R2C2
5-ASA/CMC
Clinical score
0
2
4
6
8
4
5
6
7
Days exposed to DSS
*
**

Figure 3. Colon length for groups treated with live or pasteurized *Lactobacillus kefiranofaciens* R2C2 and compared to the PBS-DSS control group. Colon length is measured at the end of the experiment, after the recovery period. The length of a healthy colon from a C57BL/6 mice, from the caecum to the anus, is represented by the PBS control group. No statistical difference was observed between R2C2-treated groups and the 5-ASA/CMC group. **, $P < 0.01$ Statistically significant compared to the PBS-DSS control group (using the Student's t test).

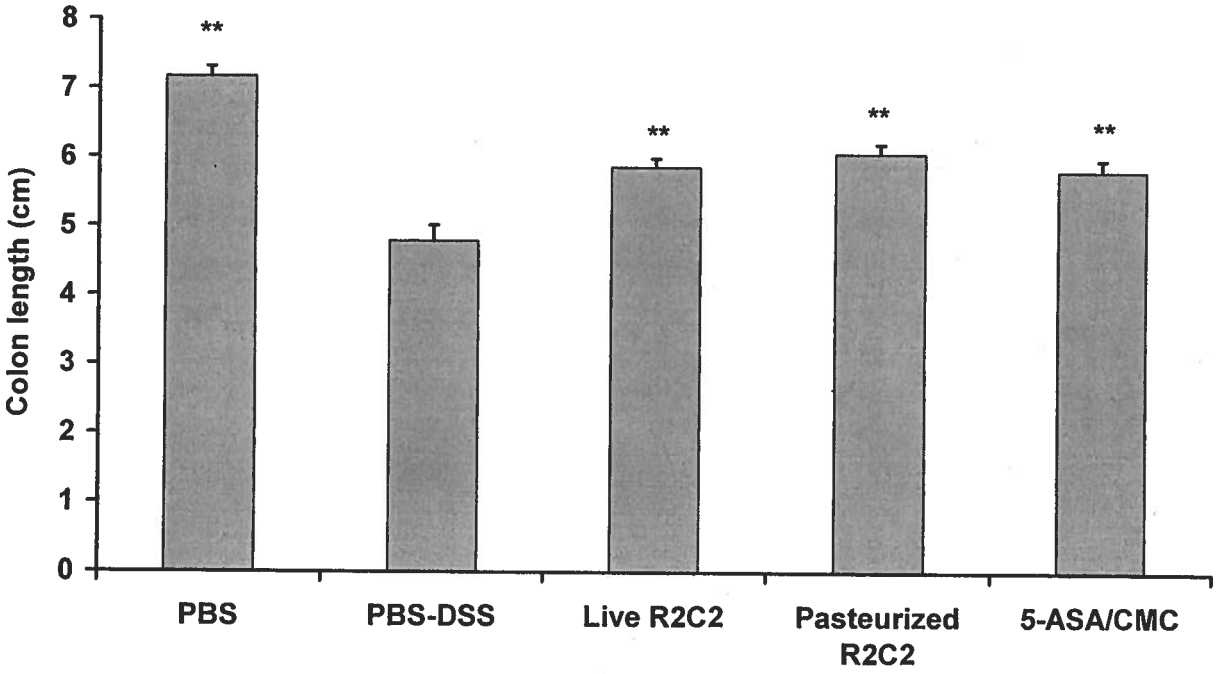
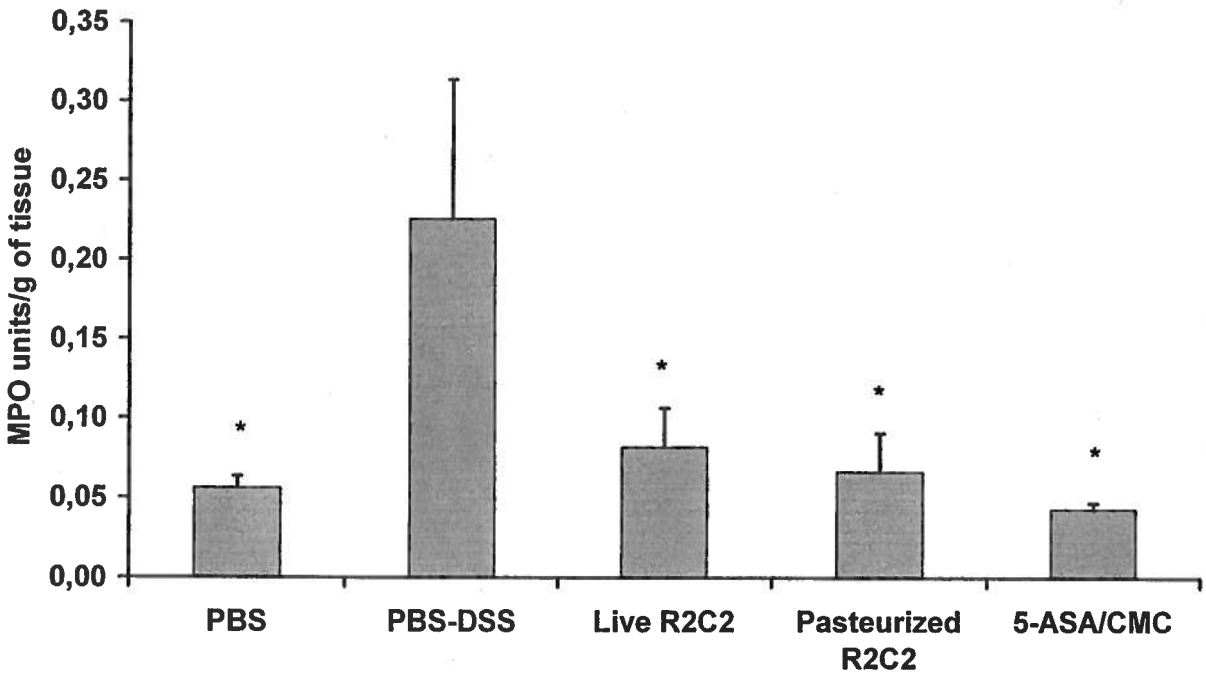

Colon length (cm)
8
7
6
5
4
3
2
1
0
**
**
**
**
PBS
PBS-DSS
Live R2C2
Pasteurized R2C2
5-ASA/CMC

Figure 4. Myeloperoxidase (MPO) activity in the colon at the end of the experiment, after 7 days of exposure to DSS and 8 days of post-inflammatory recovery. The MPO activity represents the extent of inflammatory cells infiltration in the colonic wall. No statistical difference was observed between *Lactobacillus kefiranofaciens* R2C2-treated groups, the 5-ASA/CMC group and the PBS control group, but MPO activity was significantly higher in the PBS-DSS group. *, $P < 0.05$, Statistically significant compared to the PBS-DSS control group (using the Student's t test).


MPO units/g of tissue
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
*
*
*
*
PBS
PBS-DSS
Live R2C2
Pasteurized R2C2
5-ASA/CMC

Chapitre 8 : Intérêts commerciaux et sociaux-économiques des propriétés technologiques et biologiques identifiées.

Contenue du cahpitre : article publié.

Lemieux, P. Beaulieu, J. et Simard, E. 2004. Socioeconomic and health benefits of lactoceuticals. Expert review in pharmacoeconomics outcomes research. 4 (2). 199-206.

Contributions : J'ai défini initialement la structure de l'article et j'ai eu une contribution majeure à la rédaction tout en effectuant une partie significative de la revue de littérature. Pierre Lemieux a coordonné la rédaction, j'ai aidé à la rédaction et à la revue de littérature et Josée Beaulieu a participé à la revue de littérature par la recherche des produits et de certains effets santé.

8.1 Article publié.

Lemieux, P. Beaulieu, J. et Simard, E. 2004. Socioeconomic and health benefits of lactoceuticals. Expert review in pharmacoeconomics outcomes research. 4 (2). 199-206.

Expert Review in Pharmacoeconomics and Outcomes Research

Title: Socioeconomic and health benefits of lactoceuticals

Principal author : Pierre Lemieux*, Ph.D.

with the help of the following co-authors : Josée Beaulieu, M.Sc. and Eric Simard

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Table of contents

1. Abstract

Skyrocketing increase of health care and medication costs is a growing problem for industrialised countries. Several factors contribute to that problem and solutions are therefore imperatives to help this situation. We introduce in this manuscript the concept of lactoceuticals and explain why that class of product could have an impact in reducing health care and medication costs. The term lactoceuticals will be defined and we will present the key players already involved in this field. This manuscript will also summarize and list lactoceuticals that have reached a certain market maturity as well as innovative lactoceuticals that will soon appear on the market following proper clinical investigation and regulatory homologations.

2. Introduction to the health care and medication costs problematic.

The phenomenon of consistently increasing health care and medication costs is now spreading all over the world especially to occidental countries. In the United States and Canada, the spending reached 17 % between january 2001 to 2002. This increase is worrying not only to governmental health care systems but also for private health insurers. Companies offering collective insurance benefits to their employees see their premium go up by 15 to 20% each year. This increase in costs is mainly due to the following reasons: 1) appearance of new medication on the market taping unmet needs, 2) aging of the population, 3) overpresciption of drugs used prophylactically and 4) poor diet adversely affecting the health of

the general public. This last point but not the least now seems to be the easiest to tackle and seems to be a logical starting point to help with the reductions of health care and medication costs. A healthy diet and frequent consumption of fruit and vegetables along with reducing tobacco consumption and early detection through screening have been known to be among identified ways to help reduce cost of health care. The Canadian government is unanimous to say that effort should be done to encourage the optimal use of medication not only by the pharmaceutical companies and physicians but also by the consumers. A better food consomption and healthy lifestyle is therefore desirable. The latter option is rather easy when we think that a healthy diet is the key to long lives. Development of food that would not only feed but maintain and/or correct health by having features that are desirable is then imperative and gaining in popularity.

3. Diseases driving health care and medications costs and new trends.

Among health problems that are often heard of and that we are the most concerned about are cancer, obesity and diabetes leading to cardiovascular conditions. Cancer rates could increase by 50 per cent by 2020, according to new figures released by the World Health Organisation. The 'World Cancer Report' also suggests that healthy lifestyles and government initiatives could stem this trend, and prevent as many as one third of cancers worldwide. Furthermore, as each day passes it would seem that new initiatives are taking place in the western world to tackle the increasing problem of obesity and

diabetes. This year, George W. Bush, US president, announced that the fiscal year 2004 budget plan will include an increase of $25m (€23m) - to $125 million - for a new project to prevent diabetes, obesity and asthma. According to the US government, healthier lifestyles for the Americans are to be encouraged through community initiatives. This kind of intervention will truly stem the epidemic of preventable diseases that threaten too many Americans. Not only the USA but many industrialzed countries need to move from a health care system that treats disease to one that avoids disease through wiser personal choices. This new initiative will support community programmes aimed at getting results and helping those at risk to avoid these diseases through proven prevention methods.

The incidence of diabetes and obesity among Americans has increased sharply in the past decade, putting millions more Americans at higher risk for heart disease, stroke and other related medical conditions. Diabetes alone costs the US nearly $100 billion each year in direct medical costs as well as indirect economic costs, including disability, missed work and premature death. Not only do these preventable diseases take a terrible toll on the lives of individual Americans, but they also contribute to skyrocketing health care costs. Hence, new trends will have to do more to encourage individual Americans to take personal responsibility for their health choices and to create a sense of social responsibility to ensure that policymakers support the kinds of programmes that foster healthy activity and prevention. A part of the 'Steps to a Healthier US' initiative, the

government is aiming to prevent diabetes for at least 75,000 Americans and obesity for at least 100,000 individuals.

In terms of new trend, we see large companies announcing that they will try to help in that sense. Kraft, Nestlé and McDonald's are saying that they will offer better food and to cut on the content of some ingredients having bad press like like fat, salt and sugar contained in their prepared food. Recently, the International Obesity Task Force (IOTF) as produced a report highliting the need for the food industry to be part of the solution rather than part of the problem when in comes to growing levels of obesity in the US and other countries. The organization is asking for a clear and more restrictive labeling of food products.

The US fast food group McDonald's have been associated with the negative image that fast food is inextricably linked to obesity – a serious health risk. McDonald's which recently reported its first ever quarterly losses, is the latest multinational to jump onto the organic bandwagon in the UK. The international fast food chain announced earlier this month that it is to start selling organic milk in its British restaurants starting in 2003. McDonald's is aiming to buck this trend by using also healthier ingredients. It has introduced lower-fat sauces, diet drinks, sweeteners and promotional salads. Its switch to organic milk is part of a strategy to give the fast food chain a healthier and more environmentally-friendly image. Semi-skimmed organic milk will be available in 250ml bottles and certification will be provided by the UK organic certification body, the Soil

Association (Food and Drink Europe.com). Polls carried out with consumers report that 60 per cent of respondents are eating less fast food than they were a year ago, and that a third of those who participated feel that chains like McDonald's and Burger King will be forced to downsize due to the declining appeal of their trademark products. These companies must make enormous and important changes in their approach to food, to consumers, and to the health-related issues. They need to be proactive about questions regarding food safety and food security, and will have to deal with the issue of childhood obesity in a way that is both responsible and responsive.

With US obesity levels higher than ever – and with the UK catching up fast – the fast food chains are increasingly being seen as the culprits in markets where they have traditionally been popular. This wave of anti-fast food sentiment threatens to engulf the industry unless it is prepared to act quickly and decisively.

It is hard to imagine a world without fast food – modern lifestyles demand that food becomes even more convenient, in fact – but ten years from now we may see many more salad bars and heath food outlets alongside the burger, pizza and taco outlets, and for many people that will be a major improvement.

Thus, it is the beginning of an era for innovative products that will feed masses but also have health benefits that will impact on the outcome of health status of the general public. We can ask ourselves what would be the product that would have the greatest potential and the most impact to help maintain or correct health of the general public? It has to be a product that the consumer is used to see in the everyday life and that would be convenient for consomption. It also has to be associated with products that are already known to have health benefits but that would be innovative and that will exploit a wide range of health benefits. Milk-based products anwser to that demand and has all the prerequesite to become the basis for multiple products having a variety of health benefits. In this context, it is noteworthy to say that milk products have the following 3 important advantages: 1) have exceptional nutritional value; milk is the only food product aimed at feeding from a biological point of view, 2) are well-positioned to allow the development of novel functional products already being extremely well implanted in our dietery habits and 3) are supported by a strong and well-established industry on a global scale. It is not a coincidence that MacDonald's has chosen to offer organic milk to attract attention on their intention to better feed people. Milk has a history of being noble and has been linked to multiple health benefits since our young age. The litterature is rich of clinical and laboratory data supporting the health benefits of milk and related products. We thus decided to write a review to summarize the work that has been done with milk to develop novel and innovative products having measurable

in vivo biological and physiological effects that could help the health status of the general public and therefore help reduce the burden of health care and medication costs on our modern societies. We thus have decided to called this new class of products, lactoceuticals.

4. Definition and categories of lactoceuticals and their components.

In order to find and to be able to construct a list of the most interesting lactoceuticals, various internet searches were performed through engines such as Yahoo and on websites of companies known to develop milk protein-based ingredients while the search for health benefits of lactoceuticals was performed using sites like Medline, Pubmed, and the NCBI library. To help stimulate the interest of the reader, colostrum (although difficult to find commercially) and milk fortified with vitamin D can be considered as the first lactoceuticals. Milk in this case was used as a vehicle for vitamin D to synergize and increase calcium absorption in order to prevent rickets, a bone disease seen in children. Prior to the fortification of milk products in the 1930s, rickets was a major public health problem in the United States. Milk in the United States is now fortified with 10 micrograms (400 IU) of vitamin D per quart, and rickets is now uncommon in the US (1,2). One cup of vitamin D fortified milk supplies about one-fourth of the estimated daily need for this vitamin for adults. Although milk is fortified with

vitamin D, dairy products made from milk such as cheese, yogurt, and ice cream are generally not fortified with vitamin D.

Before reviewing and speculating of the multiple and potential health benefits of lactoceuticals from an economic and health perspectives, we should properly define the term itself. To our knowledge lactoceuticals is a novel term which can be considered as a subgroup of the large and growing family of products called nutraceuticals. In this manuscript, we refer to lactoceuticals to: *a product comprising any milk proteins from various sources that are either intact or modified using soft treatments that have superior nutritional value than current milk-based products and exhibiting exceptional and scientifically proven health benefits.* The treatments described above that can be applied to milk proteins are usually fermentations, purification systems of different sort (eg. ultrafiltration, HPLC), and enzymatic hydrolysis. The treatments can be performed separately or applied in combination. Lactoceuticals can be a mixture of milk proteins and do not necessarily need to be highly purified or manufactured according to pharmaceutical standards. The active principles found in lactoceuticals can comprise: milk proteins like caseins, whey proteins, bioactives peptides originating from the fermentation process, bacteria that are used in the fermentation process, and the various nutraceutical components secreted during the fermentation process (eg. Exopolysaccharide, antioxidant, vitamin, bacteriocin, antibiotics, etc...). It is important to note here that bacteria used in

the fermentation process to alter or modify milk proteins can have a probiotic status or not. In addition, all extra components that can be exogenously added to the lactoceuticals, like calcium and vitamin D, are also considered lactoceuticals if associated with a health benefit (eg. vitamin D-rickets, calcium-osteoporosis).

The manuscript is intended to list only lactoceuticals already being sold on the market or under development that are the most promising and likely to go towards future clinical trial settings while reviewing solely recent clinical/*in vivo* findings reported in the last 5 years supporting measurable health enhancing properties of lactoceuticals. Common nutritional qualities of milk proteins-based products like yogourts found on the market will not be considered in this review unless exceptional. Although the concept of probiotics, and prebiotics is undenieably an important contributing factor to the health benefits of lactoceuticals, this manuscript is not intended to neither cover the aspects and the variety of health benefits of probiotics not prepared in milk nor to cover the biological activities of isolated milk proteins unless developed as a stand-alone with scientifically proven facts. The manuscript is rather intented to cover the health benefits coming from the intuitive synergy (Synbiotism) of several components of milk transformed by means of various processes and treatments. Excellent reviews have been written on both components (probiotics and

prebiotics) and the following reviews can be consulted (3, 4, 5). Finally, the reader can find in Table I, a list of lactoceuticals that we classified according to the following 5 categories; 1) fermented milks, 2) fermented whey, 3) milk and whey proteins, 4) bioactive peptides from milk or whey, and 5) milk or whey as vehicle or protective matrix for active ingredients.

5. Therapeutic targets of lactoceuticals and targeted populations.

Lactoceuticals have the potential to target various systems such as, the nervous system, the cardiovascular system, the urogenital sytem, the immune system and most importantly the digestive system which is probably the first biological sytem in contact with lactoceuticals. Having multiple targets, lactoceuticals lead to the following health benefits and improve several conditions such as; heart diseases and all corresponding cardiovascular conditions such as, hypertension, atherosclerosis caused by abnormal circulating lipid levels; stimulation of the immune system, downregulation of inflammation, thrombotic activity, opioid-like effect, and reducton of allergies, cancer, obesity and osteoporosis.

The first system to be in contact with ingested lactoceuticals is the digestive system. Growing attention is focusing on the latter and with reasons. We tend to forget that the digestive system is complex and probably the main entry of a variety of environmental and food material. This entry is approximately 8-9

metres long. It begins at the mouth and ends at the anus. Food takes between 1 and 3 days to travel its entire length. The digestive system is lined with bacteria and other microorganisms known as the intestinal flora. Intestinal flora refers to all the microorganisms that live in the human digestive system. Without these our digestive system would not function efficiently. The intestine is a warm home to our largest collection of microbes. Although the true extent of biodiversity is not known, it appears that the gut contains at least 1,000 different species of bacteria, and that their collective genomes ('the microbiome') contains 100-fold more genes than the human genome. These bacteria provide certain metabolic capabilities that humans lack, including the ability to process nutrients that human genes cannot break down. The digestive system has four main functions: 1) *Transportation:* movement of food through the digestive system, 2) *Secretion:* release of fluids such as enzymes and acids into the digestive system to assist digestion, 3) *Digestion:* process of breaking down food to allow release of nutrients (carbohydrate, protein, fat, vitamines, minerals), in the form of their smallest components, for absorption into the body, 4) *Absorption*: process of nutrients being transported through the digestive system wall to the bloodstream. In the average human lifetime, the digestive system handles approximately 65 tons of food and drink. This is equal to the weight of 12 elephants! Probiotic products that contain live microbial cultures, prebiotic products that selectively stimulate the growth of probiotics, and synbiotic

products that combine probiotics and prebiotics all exert a positive effect on digestive health and overall wellbeing (6).

It is important to note that the role of the digestive tract goes beyond the four functions described above. For instance, there is a clear connection with the immune function and the intestinal microflora which attracts research and consumer interest, and significant advance is being made currently (7). However, most mass market consumers appear to lack a clear understanding of the specific nature of gastrointestinal functioning and the concept of probiotic action. This dearth of knowledge tends to be even more evident in the case of prebiotic and synbiotic concepts. This situation poses the same critical challenge for the lactoceutical industry, there are encouraging signs that growing consumer awareness about the health benefits offered by lactoceuticals will boost consumption levels and their impact on health care and medication costs. Across the world, there has been a general upswing in consumer interest regarding functional foods, bolstered by positive media coverage of probiotic health benefits. It is expected that the increasing number of lactoceuticals available and advertised in the market will improve consumer understanding of the concepts and the functionality involved. Lactoceuticals will enjoy better acceptance in the future through this improved understanding. Demand for lactoceuticals will also be driven by a rapidly ageing population. Lactoceuticals will become more important since they offer the prospect of averting or, at the very least, delaying

age-associated degenerative diseases and poor dieting-self-inflicted conditions. Lactoceuticals are emerging at the forefront in the search for food-based drug substitutes to combat lifestyle-related diseases. Their role in controlling high blood pressure and obesity, two key risk factors for cardiovascular disease, is well documented (11, 21). They are also relevant in the battle against colon cancer and type 2 diabetes. And they are thought to boost the immune system, thereby reducing the effects of stress on health.

The interest in natural alternatives to replace conventional medicine will also affect the rise in the consumption of lactoceuticals. This is expected to parallel the shift by health-conscious consumers from a negative approach, i.e. removing ingredients through reduced salt, fat, and sugar, to a more positive approach, i.e. fortifying food through vitamines, supplements, minerals, and probiotic and prebiotic ingredients. The widespread and growing popularity of yoghurt, driven partly by convenience and health considerations, will also be applicable and to be key to market growth of lactoceuticals. Not surprisingly, then, probiotic yoghurt constitutes the largest segment in the probiotic dairy foods market, accounting for 82 per cent of the total probiotic dairy product volumes. This dominance is forecast to continue over the medium term.

6. Players in the field of lactoceuticals and their innovative products.

This section will highlight the principal players in this field and will summarize the various interesting lactoceuticals that can be found on the market or being developed by the industry and/or research laboratory (Table I). But before it is important to note that we are seeing a trend with regards to the formation of groups and ventures uniting forces for the development and the creation of innovative lactoceuticals. In order to develop innovative products, research and development activities will have to go through state-of–the-art techniques and methods developed and applied by the pharmaceutical industry. For instance, techniques of purification like High Pressure Liquide Chromatography and ultrafiltration, recombinant DNA technology (not used to produce genetically-modified lactoceuticals) and tools that can help with the rapid screening of wide array of novel lactoceuticals like those used in the genomic era are now being applied to nutrition (nutrigenomics) thus helping with the developement of lactoceuticals. This approach will dissect the effect of multiple components of lactoceuticals like peptides and various fermentation products. For instance, we witnessed the creation of a venture called LactoPharma to achieve that goal. This venture is getting funding for research on milk health benefits. This is joint venture between New Zealand dairy company Fonterra and the University of Auckland, that will focus on the use of milk to enhance bone growth and in the treatment of cancer and inflammatory diseases. New Zealand's Foundation for

Research Science and Technology will match funding from Fonterra over the next seven years, to reach a total of around €14.2 million.

In Spain, there is Puleva Biotech researching milk proteins for functional foods. Puleva Biotech, the biotechnology unit of Spanish food company Ebro Puleva said that it has started researching the production of proteins with health benefits. As part of the project, the company said it plans to obtain products of nutritional or pharmacological value, which could then be incorporated as "functional ingredients" in the food products of Ebro Puleva or other companies. Dairy product sales at Spain's Ebro Puleva group reached €522.2 million in 2002, helped by the successful launch of a range of functional dairy products and dairy drinks. Ebro also claimed that its Puleva brand of milk was the only one in the Spanish market to show any growth in 2002, with its functional Puleva Calcio and Puleva Omega 3 functional brands showing the most growth. Puleva Biotech, said that it has lodged two international patent applications for the treatment of Alzheimer's disease and cardiovascular illness. Puleva Biotech said it has discovered a natural product that inhibits the "cellular death in the central nervous system." The product acts directly on neurons and protects them against different kinds of cellular damage.

In Japan, Dr. Shirota wanted to make the beneficial bacteria easily available to everyone. He developed a fermented milk drink as a vehicle for the bacteria. To

make it accessible to everyone in the world, he called it Yakult, inspired by the Esperanto word for yoghurt. Yakult is a fermented milk drink that contains a very high concentration of a unique, beneficial bacterium called *Lactobacillus casei* Shirota strain. This bacteria was named after Dr Shirota and is exclusive to Yakult. Each one of us has around 100 trillion bacteria in our intestines. Some of these are beneficial and some are potentially harmful to our health. To work efficiently our digestive system needs a healthy balance of beneficial and potentially harmful bacteria. The unique beneficial bacteria in Yakult are strong enough to survive the journey through the stomach's gastric juices to reach the small intestine alive, where they help maintain an ideal balance of beneficial bacteria. Each 65ml bottle contains hundreds of millions of these highly acid resistant bacteria, which are exclusive to Yakult. Yakult even began creating and sell pharmaceuticals in 1962. Most of which contained live friendly bacteria. Ten years later, Yakult started creating and sell cosmetics using their knowledge of bacteria to make unique make-up and skin care products. Now, Yakult Honsha, producer of the Yakult probiotic drink, has said that an increase in royalty payments from strong worldwide sales of the drink boosted its results for 2001, reports Dairy Industries International. For the 12 months, profit was up 5.5 per cent on 2000 to ¥22.77 billion (€196.6m). The company said that income included €37.5 million in royalty revenue and €40.8 million profit from investment in its foreign affiliates. Yakult Honsha was formed in 1955 and its drink is now sold in 23 countries.

In Canada, we find Technologie Biolactis Inc., a biotechnology company originating from the need to find a solution to the heavy production of whey by local and mide-size cheese makers. A local cheese manufacturer (Fromagerie Boivin) has funded R&D activities to produce a new generation of lactoceuticals based on fermented whey proteins. Several strains of bacteria have been adapted to grow in whey to generate a line of innovative lactoceuticals having a cream-like appearance with no fat and low in calories. The product can be described as a malleable protein matrix that can be used as a vehicle to formulate and synergize with bioactive ingredients but also as novel bioactive ingredients leading to the formulation of functionnal foods. The indredients exhibit a wide array of biological activities such as the stimulation of the immune system (Immunolactis), protection against intestinal inflammation (Intestilactis), control of body weight (Controlactis) and the blood lipids (Lipidolactis), increase bioavailability of calcium (Osteolactis), and the control of hypertension (Cardiolactis) The first product to be developed is Nutrilactis which is used to reduce fat in butter and whipped cream but also to fortify those products with extra proteins.

US supplier of applied protein sciences Proliant and Trega Foods, latterly known as Weyauwega Milk Products, a manufacturer of cheese and dairy-based ingredients, announced a joint venture for the production, sales and marketing of whey protein isolates. According to the two companies the joint venture unites

Proliant's worldwide sales, marketing, research & development and application research with Trega Foods' manufacturing capabilities. In 1991, Proliant extended its line of protein products to include dairy proteins thanks to a joint venture with the Hilmar Cheese company. The joint venture with Trega Foods is a way for Proliant's Dairy Ingredients Division to extend and build upon the success of their existing dairy protein business with Hilmar Cheese company, offering additional dairy ingredients and exciting, new, proprietary manufacturing capabilities. Proliant's Dairy Ingredients Division provides speciality dairy proteins to the food industry.

Davisco Foods International is a privately held, family-owned business with an aggressive, entrepreneurial vision. Three generations of confident, risk-taking expansion have made of Davisco a leader in the dairy industry. Meticulous about quality control and excellent customer service, Davisco has made it a mission to lead the industry in food technology by producing innovative proteins for health and nutrition. A pioneer in whey protein isolate research, Davisco produces 10 million lbs. of whey protein isolates annually. Davisco is the industry leader in technology and production, accounting for 65% of whey protein isolates sold worldwide. Whey protein isolates are found in 50% of grocery products today, including sports drinks, reduced fat candies, low fat salad dressings, infant formula, yogurts, dips, shelf-stable baking mixes, and low fat cheese sauces. Davisco produces a variety of customized whey protein products and a full line of

whey protein products, including products falling under our definition of lactoceuticals: BiPRO®, BioZate®, BioPURE-GMP®, BioPURE-Alphalactalbumin™ (See table I).

Finally, French homeopathy specialist Dolisos is launching a milk protein-based supplement, designed to treat stress, into the UK market this month. Seriane contains a new molecule produced from casein decapeptide derived from hydrolysed milk proteins. It is the first Dolisos product available in the UK. The company did not reveal plans to roll out the product in other markets. Studies indicate that the peptide may help those who suffer from occasional stress, according to the Toulouse-based company. It adds that the product has a soothing effect and does not lead to addiction or other side effects such as weight gain. Priced at £12.95 (€18.80) per pack of 20 capsules, Seriane is currently only available via mail order. It is the first 'stress' supplement in the Dolisos range.

Table I: Categories of lactoceuticals

Category	Name of product or protein/peptides	Action	Company or developpers	Species tested	Refs
Fermented milks	Yakult	Triglyceride-lowering	Yakult Honsa	hamsters	8
	L. delbrueckii subsp. bulgaricus OLL1073R-1	Reduction of arthritis	Meiji Dairy Co.	rats	9
	Kefram-Kefir	Insulin-Responsiveness	Nihon Kefir Co., Fujisawa	mice	10
	Calpis (L. Helviticus)	Anti-hypertensive	Calpis Food Industry Co, Ltd	human	11
	Evolus (L. Helviticus K16H)	Anti-hypertensive	Valio, Ltd	human	12
	Actimel	Control acute diarrhea	Danone	human	13
	Biola (L. GG)	Stomach disorders	Tine	human	14
	Gaio	Cholesterol control	Gaio	human	15
	L. Helveticus R389	Immunestimulation	Institut Rosell	mice	16
	Lactobacillus casei TMC0409 and Streptococcus thermophilus TMC 1543	Control of Serum lipids and hypertension	Takanashi Milk Products Co., Ltd	human	17
Fermented whey	NutractisTM	Multifunctional	Technologie Biolactis Inc.	human	18
Milk or whey proteins	Lactoferrin	Immunostimulator, Multifunctional	DMV International, Glanbia	human	19
	Lactoperoxidase	Anti-infective	DMV International, Glanbia	human	20
	HMS90 (Immunocal)	Glutathion modulation	Immunotec Research Ltd	human	21
	Prodiet 352 EFA(alphalactalbumin)	Anti-bacterial, Fixes minerals, can boost immune system	Ingredia	human	22
	Volactive	Anti-oxidant and anti-bacterial activity and immunomodulatory, regulation of mucle protein	Volac International	human	23
	Imuplus	Immune Stimulator	Biogene	human	24
Bioactive peptides from milk or whey proteins	Prodiet F200	Stress-releving	Ingredia Advitech	Rat, human	25
	BioPURE-GMP®	Satiety, protection against toxins, bacteria, and viruses	Davisco, Land O'Lakes	human	26

	BioZate®	and modulation of the immune system Anti-hypertensive	Davisco	human	27
Milk or whey proteins as vehicle or protective matrix for active ingredients	Lipidolactis (synergy with B vitamins)	Triglyceride-lowering	Technologie Biolactis Inc.	Rats	28
	Lactoval (Calcium, Phosphate, Magnesium)	Bone health, Osteoporosis	DMV International	human	29
	TruCal (Calcium)		Glanbia		
	Omega-3 (CV-17)	Prevents heart disease (cholesterol-lowering)	Puleva Biotech	human	30
	Vitamin D	Bone diseases	Milk companies	humans	1,2

7. Perspectives and challenges for the developpment of lactoceuticals.

The market size for the lactoceuticals listed above is huge since they cover most of the disease that most people are either concerned about today or afflicted somehow. Despite their great potential, it is difficult to evaluate the market of lactoceuticals but alone the market of functionnal food is estimated at 50 billions USD in 2002 to reach 75 billions USD in 2007. The potential economical benefits for the society at large and for developpers is fairly significant since the raw material is relatively cheap and that the costs to support clinical trials to prove health benefits in nutrition are fairly reasonable compared to the developoment of pharmaceutical products. Lactoceuticals as defined above can be a mixture of milk proteins (a complex biological mixture) and can therefore be offered as a functional food and not necessarily as a nutraceutical (tablet or capsule) thus simplifying the manufacturing and lowering the production costs. This will an impact on reducing the cost of the product and to make it more available to the

consumers Even though health claims legislation and regulatory affairs with regards with lactoceuticals may appear easy, the regulatory status of lactoceuticals is complex and depends on the extent of the claims (31). Each country has its own rules or laws that are being written. This issue is discussed in each country as well at Codex Alimentarius Commission making it an international effort. Finally, most of the costs to see lactoceuticals appear on the market will come from the marketing of such products. To bring to the market innovative lactoceuticals or any novel product for that matter takes time, money and patience unless there is a clear commitment from governments to ease the time to market by helping with the promotion of such product. According to the American Council on Science and Health, lactoceuticals are for most of them linked to probiotics and the health benefits support an effect on gastrointestinal health and immunity boost. Furthermore, the council jugdes that the evidence to support health benefits of lactoceuticals is weak and too often limited to in vitro and in vivo data (animal data) rather than clinical data in constrast to other functional food product like soy-based, plant sterol-based, psyllium-based and whole oat-based products (32).

The cost to market and the marketing strategy of lactoceuticals will probably be similar to what was witnessed with prebiotics, probiotics and yoghurts. At present, several yoghurt manufacturers use probiotics in their products. However, very few are actually marketing their products to consumers from the

probiotic angle. Among the yoghurt brands highlighting the probiotic component, Jalna (Australia) has the largest market share followed by Paul's Vaalia. Within the fermented milk drink category, Yakult is the acknowledged leader. Chr. Hansen is the leading probiotic culture supplier to participants in various dairy foods market, with other prominent suppliers including DSM, Rhodia and Danisco. On the other hand, prebiotic ingredients are being used in dairy products principally as a fat replacement solution and as fibres, rather than for their prebiotic functionality. And while synbiotic products are available, they are not being positioned as such due to low levels of consumer awareness and understanding of synbiotics. A key to future success of lactoceuticals will therefore lie in communicating more effectively with mainstream consumers while ensuring the same safety and innocuity that we associate to food products.

8. Expert opinion and Five-year view.

In order to help the development of innovative lactoceuticals, there will be a need to bridge the gap between the food industry and the pharmaceutical/cosmetics industry. The food industry has been relying on the nutritive aspects of their products and are now at the crossroads to exploit the active principle of their products that have been acting in their products for years. In order to do so, the food industry will have to learn the following: 1) work together with research and development providers 2) turn to research

activities of functional nutrition 3) inspire themselves with the experimental approach applied in the pharmaceutical/cosmetics industry in order to dissect and identify active molecules and ingredients. We are already seeing some moves in that sense to achieve that goal. Nestlé and L'Oreal have announced the creation of Laboratoires Innéov, a new joint venture, to launch a first cosmetic food containing a lactoceutical to help restore firmness of the skin. This approach will be applicable to the area of lactoceuticals from both pharmaceuticals companies and cosmetics groups - well-established industries with huge research and development budgets. Food industry participants could do well to consider joint ventures with companies from these other sectors, thus avoiding some of the potential competition for the development of lactoceuticals.

In the same line of thoughts, in the next 5 years, lactoceuticals having functional properties will witness the strongest growth among the large family of nutraceuticals at large and will appear gradually on the market.

From the consumer perspective, we will see major changes. Consumers are more and more informed on their needs for a better nutrition which is dependent on age and their personal medicals needs. In addition, physicians recommend to their patients a change in their lifestyles habits, but also in their dietary habits. With this new trend, consumers will have the opportunity to choose functional

foods including lactoceuticals adapted to their age, lifestyles, and health status. Lactoceuticals will definitely play a role in the establishment of those news trends. Having on one hand, consumers better informed and also willing to improve their health and on the other, products like lactoceuticcals answering to various health problems encountered will potentially contribute to the prevention thus leading to the reduction of health care and medications costs.

9. Key issues

- Since health care and medications costs give vertigo, there is a need to reduce costs.
- Preventative and soft medicine such as functional food and nutraceuticals have the potential to reduce costs.
- Lactoceuticals are a subclass of nutraceuticals and have multiple advantages that can benefit a situation of increasing health care and medications costs.
- The term lactoceutical was in this manuscript for the first time proposed and defined.
- *Lactoceutical: a product comprising any milk proteins from various sources that are either intact or modified using soft treatments that have superior nutritional value than current milk-based products and exhibiting exceptional and scientifically proven health benefits.*

10. References

*** of interest**

**** of considerable interest**

1. Institute of Medicine, Food and Nutrition Board. Dietary Reference Intakes: Calcium, Phosphorus, Magnesium, Vitamin D and Fluoride. National Academy Press, Washington, DC, 1999.

2. Holick MF. Vitamin D. In: Shils M, Olson J, Shike M, Ross AC, ed. Modern Nutrition in Health and Disease, 9th ed. Baltimore: Williams and Wilkins, 1999.

3. Probiotics: an overview of beneficial effects.
Arthur C. Ouwehand, Seppo Salminen and Erika Isolauri. (2002) *Antonie Van Leuwenhoek*. Vol. 82. p.279-289.
** this paper described well all the potential of probiotics and is a nice introduction to this field.

4. Probiotics: potential pharmaceutical applications.
Indu Pal Kaur, Kanwaljit Chopra and Amarpreet Saini. (2002) *European journal of pharmaceutical Sciences*. Vol. 15. p.1-9.

5. Ewan Ha and Michael B. Zernel. Functional properties of whey, whey components, and essential amino acid:mechanisms underlying health benefits for active people (review) (2003) *Journal of nutritional biochemistry*. Vol. 14 p. 251-258.

6. Schneeman BO. Gastrointestinal physiology and functions. Br J Nutr. 2002 Nov;88 Suppl 2:S159-63. Review.

7. Bourlioux P, Koletzko B, Guarner F, Braesco V. The intestine and its microflora are partners for the protection of the host: report on the Danone Symposium "The Intelligent Intestine," held in Paris, June 14, 2002. Am J Clin Nutr. 2003 Oct;78(4):675-83.

8. Matsuzaki T, Chin J., Modulating immune responses with probiotic bacteria. Immunol Cell Biol. 2000 Feb;78(1):67-73.

9. Hiroshi Kano, Orie Mogami and Masayuki Uchida. Oral administration of milk fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1037R-1 to DBA/1

mice inhibits secretion of proinflammatory cytokines. Cytotechnology. 2002.Vol. 40 p.67-73.

10. Kiichiro Teruya, Maiko Yamashita, Rumi Tominaga, Tsutomu Nagira, Sun-Yup Shim, Yoshinori Katakura, Sennosuke Tokumaru, Koichiro Tokumaru, David Barnes and Sanetaka. Fermented milk, Kefiran-kefir enhances glucose uptake into insulin-responsive muscle cells. Cytotechnology. 2002. Vol. 40 p.107-116.

11. Takano T. Anti-hypertensive activity of fermented dairy products containing biogenic peptides. Antonie Van Leeuwenhoek. 2002 Aug;82(1-4):333-40.

12. Seppo L, Jauhiainen T, Poussa T, Korpela R. A fermented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects. Am J Clin Nutr. 2003 Feb;77(2):326-30.
** This is a very good example of the potential of a fermented milk. A simple product that can benefit many people!

13. Agarwal KN, Bhasin SK. Feasibility studies to control acute diarrhoea in children by feeding fermented milk preparations Actimel and Indian Dahi. Eur J Clin Nutr. 2002 Dec;56 Suppl 4:S56-9.

14. Gorbach SL. Probiotics and gastrointestinal health. Am J Gastroenterol. 2000 Jan;95(1 Suppl):S2-4.
A paper with an excellent overview of the relatioship between the immune system, probiotics and gut health.

15. Agerholm-Larsen L, Bell ML, Grunwald GK, Astrup A. The effect of a probiotic milk product on plasma cholesterol: a meta-analysis of short-term intervention studies. Eur J Clin Nutr. 2000 Nov;54(11):856-60.

16. Matar C, Valdez JC, Medina M, Rachid M, Perdigon G. Immunomodulating effects of milks fermented by Lactobacillus helveticus and its non-proteolytic variant. J Dairy Res. 2001 Nov;68(4):601-9.

17. Kawase M, Hashimoto H, Hosoda M, Morita H, Hosono A. Effect of administration of fermented milk containing whey protein concentrate to rats and healthy men on serum lipids and blood pressure. J Dairy Sci. 2000 Feb;83(2):255-63.

18. Simard et al. Nutrilactis as a novel milk ingredient. Submitted to Br J Nutrition. Europeen and US Patents pending.

19. Steijns JM, van Hooijdonk AC. Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. Br J Nutr. 2000 Nov;84 Suppl 1:S11-7.

Lactoferrin is a potent and multifunctional protein that has a bright commercial future. This paper describes well its various facettes.

20. Kussendrager KD, van Hooijdonk AC. Lactoperoxidase: physico-chemical properties, occurrence, mechanism of action and applications. Br J Nutr. 2000 Nov;84 Suppl 1:S19-25.

21. Bounous G, Molson JH. The antioxidant system. Anticancer Res. 2003 Mar-Apr;23(2B):1411-5.

22. Lonnerdal B, Lien EL. Nutritional and physiologic significance of alpha-lactalbumin in infants. Nutr Rev. 2003 Sep;61(9):295-305.

23. Robert R. Wolf. Regulation of muscle protein by amino acids. J Nutr 132:3219S-3224S, 2002.

24. See D, Mason S, Roshan R. Increased tumor necrosis factor alpha (TNF-alpha) and natural killer cell (NK) function using an integrative approach in late stage cancers. Immunol Invest. 2002 May;31(2):137-53.

25. Lefranc C. ProdietTM F-200: An innovative stress-relieving ingredient. Agro-Food Industry Hi-Tech. 13, 4, p. 9-11, 2002.
F200 is probably the most advanced active milk ingredient and so this paper introduces its story and all the potential application of the product.

26. Brody EP. Biological activities of bovine glycomacropeptide. Br J Nutr. 2000 Nov;84 Suppl 1:S39-46.

27. Pins, J.J. and Keenan J.M. Hydrolyzed whey protein isolate BioZate1. Cardiovascular Drugs and Therapy 2002. Volume 16 S.1:68.

28. Lemieux et al. Malleable protein matrices as a vehicle of active ingrdients. Submitted to J. Nutr. And patent pending PCT/CA 02/01988 WO 03/053158 A2.

29. Tucker KL. Dietary intake and bone status with aging. Curr Pharm Des. 2003;9(32):2687-704.

30. Baro L, Fonolla J, Pena JL, Martinez-Ferez A, Lucena A, Jimenez J, Boza JJ, Lopez-Huertas E. n-3 Fatty acids plus oleic acid and vitamin supplemented milk consumption reduces total and LDL cholesterol, homocysteine and levels of endothelial adhesion molecules in healthy humans. Clin Nutr. 2003 Apr;22(2):175-82.

31. Berner, L.A., O'Donnell, J.A. Functional foods and health Claims legislation: Applications to Dairy Products. Int. Dairy Journal. 1998. 355-362.

32. Hasler CM. Functional foods: benefits, concerns and challenges-a position paper from the american council on science and health. J Nutr. 2002 Dec;132(12):3772-81.
** A good introduction of what is going on in terms of legislation on health claims. The reader will discover that the analysis of health claims is not an easy task.

Chapitre 9 : Applications particulières des exopolysaccharides.

Contenu du chapitre : brevet en phase internationale sur l'utilisation du kefiran en formulaion de médicament et brevet en phase internationale sur une nouvelles approche de traitment du cancer.

Beaudet, N., Dupont, C., Goyette, P., Lemieux, P., Simard, E. 2002. International 2003. An exopolysaccharide delivery system for active molecules. PCT/CA2003/001899. 31 pages.

et

Simard, E. Goyette, P. Lemieux, P. 2002. International 2003. Chemotherapeutic agents as anti-cancer vaccine adjuvants and therapeutic methods thereof. PCT/CA2003/000434. 29 pages.

Contributions : J'ai élaboré l'idée originale de l'utilisation des EPS comme véhicule de formulation de médicament. La méthode de production du kéfiran élaborée à la maîtrise est demeurée un secret industriel. La méthode de purification développée par Nicolas Beaudet est aussi mon idée et les grains de kéfir utilisés pour ces travaux sont ceux que j'ai produits.

9.1 Brevet en phase internationale

Beaudet, N., Dupont, C., Goyette, P., Lemieux, P., Simard, E. 2002. International 2003. An exopolysaccharide delivery system for active molecules. PCT/CA2003/001899. 31 pages.

Ce brevet fut déposé le 4 décembre 2002 en dépôt provisoire au État-Unis. Le dépôt international fut effectué le 4 décembre 2003 pour les Etats-Unis, l'Europe, le Canada.

J'ai eu une contribution majeure à la revue des brevets existants, à la revue de littérature et à la rédaction. J'ai eu une contribution mineure à la collecte des données.

AN EXOPOLYSACCHARIDES DELIVERY SYSTEM FOR ACTIVE MOLECULES

ABSTRACT OF THE INVENTION

The present invention relates to a delivery system for delivery of an active molecule to a patient, the delivery system comprising a population of exopolysaccharide micelles, each micelle defining a core for containing the active molecule.

AN EXOPOLYSACCHARIDES DELIVERY SYSTEM FOR ACTIVE MOLECULES

BACKGROUND OF THE INVENTION

(a) Field of the Invention

This invention relates to exopolysaccharide delivery system of active molecules into a patient and/or increase the activity of the active molecules.

(b) Description of Prior Art

Encapsulation of bioactive compounds in natural or synthetic matrices has been extensively studied over the past decades. Advantages of encapsulation are numerous. Firstly, it provides protection from the inactivation or degradation of the bioactive compound. Secondly, it controls the kinetics of compound release, allowing the optimization of the blood concentration profile. Thirdly, it can also improve therapeutic indices of bioactive compounds like that described with micellar systems. This optimization diminishes the deleterious effects of bioactive compounds with short half lives. In addition, it permits a reduction in toxicity or synergize with the formulated drugs leading to a better treatment for the patient.

Many systems have been described to improve formulation of bioactive compounds. Among them are found colloidal drug delivery systems that are promising such as liposomes, microspheres, nanospheres and block copolymer micelles that increase the therapeutic index and improve the selectivity of various potent drugs (Gregoriadis G., (1995) TIBS, 13:527-537; Muller R.H., (1991) Colloidal Carriers for Controlled Drug Delivery and Targeting: Modification, Characterization and In vivo Distribution, CRC Press Inc., Florida; Kabanov A.V., Alakhov V. Y. (1997) " Micelles of Amphiphilic Block Copolymers as Vehicles for Drug Delivery" In Amphiphilic Block Copolymers: Self-Assembly and Applications edited by Alexamdris P., Lindman B., Elsevier, Netherlands; Kwon G. et al. (1997) J. Controlled Release, 48:195-201; La S.B. et al. (1996) Journal of Pharmaceutical Sciences, 85:85-90; Kataoka K. et al. (1992) J. Control. Release, 24:119-132).

These vehicles optimize the therapeutic efficacy of drugs by preventing their rapid elimination from the body, reducing their systemic toxicity, delaying their degradation and optimizing their metabolism (Muller R. H. (1991) *supra*; Kabanov A. V., Alakhov V. Y. (1997) *supra*). In addition, they also provide for effective delivery of drugs to specific target sites (Muller R. H., (1991) *supra*) and aid in overcoming both transport limitations and defense mechanisms associated with the multi-drug resistance phenotype.

Various approaches have been developed to provide continuous delivery of various biologically active agents, and, although these have overcome some of the problems of delivering the agents, numerous problems remain such as the linearity of release, bioavailability, absorption, biocompatibility of the materials used and loading capacity.

It would be highly desirable to be provided with a natural biopolymers forming micelles, being easily and inexpensively produced, that are enabling the delivery of an active molecule to a patient.

SUMMARY OF THE INVENTION

One aim of the invention is to use exopolysaccharides (EPS) micelles as a drug delivery system.

Another aim of the present invention is to describe a method of production of exopolysaccharides having micellar properties.

The active molecule may be lipophilic, hydrophilic, hydrophobic.

The micellar system of the present invention is also suitable to the cosmetic industry such as in the delivery of active agents in creams, toiletries, deodorants, skin and sunscreen preparation. The micellar system of the present invention is also useful in perfumes, by stabilizing the unstable components thereof and by controlling the release kinetics of the fragrance upon application.

In accordance with the present invention there is provided a delivery system for delivery of an active molecule to a patient, the delivery system

comprising a population of exopolysaccharide micelles, each micelle defining a core for containing the active molecule.

In a preferred embodiment of the present invention, the exopolysaccharide is produced by lactic acid bacteria, more preferably the bacteria is selected from the group consisting of *Lactobacillus* strain R2C2, *Lactobacillus* strain Inix, *Lactobacillus* strain Es1, *Lactobacillus* strain K2. Alternatively, the exopolysaccharide can be produced by a yeast like, but not limited to *Candida kefyr* or *Candida norvegensis*.

In accordance with a preferred embodiment of the present invention, the active molecule is selected from the group consisting of DNA, RNA, protein, peptide, peptidomimetic, virus, bacteria, neutraceutical product and pharmaceutical agent. Preferably, the pharmaceutical agent is selected from the group consisting of analgesic, anesthetic, antibiotic, anticancer, anti-inflammatory, and antiviral.

The anticancer agent is preferably selected from the group consisting of alkylating agents, alkyl sulfonates, aziridines, ethylenimines, methylamelamines, acetogenins, camptothecin, bryostatin, callystatin, CC-1065, cryptophycins, dolastatin; duocarmycin, eleutherobin, pancratistatin, sarcodictyin, spongistatin, nitrogen mustards, nitrosureas, antibiotics, anti-metabolites, folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate, purine analogs, pyrimidine analogs, androgens, anti-adrenals, folic acid replenisher, aceglatone, aldophosphamide glycoside, aminolevulinic acid, amsacrine, bestrabucil, bisantrene, edatraxate, defofamine, demecolcine, diaziquone, elformithine, elliptinium acetate, epothilone, etoglucid, gallium nitrate, hydroxyurea, lentinan, lonidamine, maytansinoids, mitoguazone, mitoxantrone, mopidamol, nitracrine, pentostatin, phenamet, pirarubicin, podophyllinic acid, 2-ethylhydrazide, procarbazine, PSK.RTM., razoxane, rhizoxin, sizofiran, spirogermanium, tenuazonic acid, triaziquone, 2, 2',2"-trichlorotriethylamine, trichothecenes, urethan, vindesine, dacarbazine, mannomustine, mitobronitol, mitolactol, pipobroman, gacytosine, arabinoside, thiotepa, taxanes, chlorambucil, gemcitabine, 6-thioguanine, mercaptopurine, methotrexate, platinum,

vinblastine, platinum, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, navelbin, novantrone, teniposide, daunomycin, aminopterin, xeloda, ibandronate, CPT-11, topoisomerase inhibitor RFS 2000, difluoromethylornithine, retinoic acid, capecitabine, bisphosphonates and anti-hormonal agents that act to regulate or inhibiting hormone action in hormonal dependent cancers.

The anti-hormonal agent is preferably an anti-estrogens or an anti-androgens selected from the group consisting of flutamide, nilutamide, bicalutamide, leuprolide, and goserelin, and pharmaceutically acceptable salts, acids or derivatives thereof.

The alkylating agents are preferably selected from the group consisting of thiotepa and cyclosphosphamide (CYTOXAN™).

The alkyl sulfonates are preferably selected from the group consisting of busulfan, improsulfan and piposulfan.

The aziridines are preferably selected from the group consisting of benzodopa, carboquone, meturedopa, and uredopa.

The methylamelamines are preferably selected from the group consisting of altretamine, triethylenemelamine,trietylenephosphoramide, triethylenethiophosphaor-amide and trimethylolomelamine.

The acetogenins are preferably selected from the group consisting of bullatacin and bullatacinone.

The camptothecin is preferably the synthetic analogue topotecan.

The CC-1065 is preferably selected from the group consisting of adozelesin, carzelesin and bizelesin synthetic analogues thereof.

The cryptophycins are preferably selected from the group consisting of cryptophycin 1 and cryptophycin 8.

The duocarmycin is preferably selected from the group consisting of KW-2189 and CBI-TMI.

The nitrogen mustards are preferably selected from the group consisting of chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide and uracil mustard.

The nitrosureas are preferably selected from the group consisting of carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine.

The anti-metabolites are preferably selected from methotrexate and 5-fluorouracil (5-FU).

The purine analogs are preferably selected from the group consisting of fludarabine, 6-mercaptopurine, thiamiprine and thioguanine.

The pyrimidine analogs are preferably selected from the group consisting of ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine and floxuridine.

The androgens are preferably selected from the group consisting of calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone.

The anti-adrenals are preferably selected from the group consisting of aminoglutethimide, mitotane and trilostane.

The antibiotics are preferably selected from the group consisting of enediyne antibiotics, aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, poffiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin.

The enediyne antibiotics are preferably selected from the group consisting of calicheamicin more preferably calicheamicin γ1I and calicheamicin θ1I, dynemicin more preferably dynemicin A, esperamicin, neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores.

In accordance with a preferred embodiment of the present invention, the micelles have a diameter varying from about 50 nanometers to about 700 nanometers.

In accordance with the present invention, there is provided a pharmaceutical composition comprising the delivery system of the present invention in association with a pharmaceutically acceptable carrier and method of use thereof.

In accordance with the present invention, there is provided an immunomodulator composition comprising an immunomodulating amount of the delivery system of the present invention in association with a pharmaceutically acceptable carrier and method of use thereof.

In accordance with the present invention, there is provided a method for producing the delivery system of the present invention, comprising the step of incubating exopolysaccharide in a suitable medium for a time sufficient to form micelle.

Administration of the delivery vehicle of the present invention can be performed by a route selected from the group consisting out local, parenteral, peritoneal, mucosal, dermal, epidermal, subcutaneous, transdermal, intramuscular, nasal, oral, topical, vaginal, rectal, intra-ocular, intravenous, intra-arterial and by inhalation.

For the purpose of the present invention the following terms are defined below.

The term "active molecule" is intended to mean, without limitations, nonpolar, lipophilic drugs, vitamins, polar molecules, immunosuppressants,

immunoactive agents, neutraceuticals, peptidomimetics mimicking growth factors and their antagonists and immunomodulator agents

All references herein are hereby incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates scans of pyrene dissolved in PBS, P85 and EPS;

Fig. 2 is an electronic microscopy image of pure EPS micelles; and

Fig. 3 is an electronic microscopy image of EPS micelles having 1% critical micellar concentration.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided a delivery system comprising exopolysaccharide micelles for delivering an active molecule to a patient.

Exopolysaccharides (EPS) produced by lactic acid bacteria (LAB) are known to act as viscosifying agents in fermented foods (Yang, Z. (2000) Antimicrobial and extracellular polysaccharides produced by lactic acid bacteria: Structures and properties, PhD thesis, University of Helsinki, Helsinki, 61 pp.) and as cytokine inducers *in vitro* (Chabot, S. et al, (2001) Lait, **81**:683-697). The rheological and immunomodulator properties of EPS are based on their monomeric composition and their assembling, conferring to those molecules a neutral to charged tri-dimensional structure that can actively interact with its environment. It was observed during the use of pyrene, an insoluble molecular probe in water, a modification of its fluorescent profile in the presence of EPS in comparison to the pluronic P85 (a tri-block copolymer known to form micelles in solution) profile in the same conditions. The temperature of formation is from room temperature to 37°C. The micelles formed rapidly and were left undisturbed for a period of 16 to 24 hours to allow equilibration before fluorescence reading. The estimated critical micellar concentration (0.007% (w/v)) and the presence of a significant excimer peak show that EPS is a potential high loading drug carrier, since the excimer state is attributable to molecular stacking of pyrene in an enclosed environment. An

electronic microscopy analysis has been processed on EPS samples, showing semi-spherical structures resembling monolayer liposomes. EPS represents an easy-to-produce, at a reasonable cost, bio-degradable polymer that is naturally derived from lactic acid bacteria (LAB) which are food-grade microorganisms with the GRAS status (Generally Recognized As Safe) (see Figs. 2 and 3). It is understood herein that any bacteria capable of producing a polymer is enclosed in the definition of a bacteria suitable to produce the EPS of the present invention. It is also understood that one having ordinary skills in the art would know what bacteria are suitable for pharmaceutical applications as described in the present application.

The micellar delivery system of the present invention is able to incorporate much higher concentrations of pyrene as compared to P85 (see Example 6) that will later be released slowly over time. This prevents at least some of the damaging effects that high doses induce.

The results obtained by the use of the exopolysaccharide micellar system of the present invention reveal that EPS is an effective delivery vehicle for the delivery of various active molecules including lipophilic drugs. The small size and high *in vitro* stability of the micelles render them useful for a wide variety of biomedical applications. Their loading capacity for the very hydrophobic pyrene compound shows that it allows incorporation of several different compounds with a high degree of hydrophobicity.

The degree of non-covalent incorporation of physical entrapment of a hydrophobic drug into a micelle is determined by the partition coefficient of the drug between the micellar core and the surrounding aqueous medium. The affinity of the exopolysaccharide micelles for lipophilic compounds was assayed by the determination of the partitioning coefficient (solubilization) for the hydrophobic model compound, pyrene, between the exopolysaccharide micelles and water. This method has been previously used to determine the partitioning coefficient of pyrene between several polymeric formulations forming micelles and water and the critical micellar concentration (CMC). This fluorescence method is based on pyrene's sensitivity to the hydrophobicity of its microenvironment. This is reflected in changes in the ratio of the I_3/I_1 bands

of its emission spectrum (see Fig. 1). The method requires the measurement of the I_3/I_1 ratio for pyrene in micelle solutions of various solvent mixtures. The CMC is calculated by plotting either the I_3/I_1(aqueous) peak fluorescence values over the concentrations of polymers or exopolysaccharide. The ratio is at first a measurement of the polarity of the solvent. This plot has a S-shape and the inflection point corresponds to the CMC. Futhermore, by plotting I_3 (test solution) on I_3(aqueous) for a range of concentration gives an idea of the capacity to solubilize the pyrene (partitioning coefficient). Finally, I_4 or I_e which corresponds to the peak of excimer (pile up of ground state and excited pyrene molecules) represents an accumulation of pyrene in an hydrophobic microdomain. By plotting I_4 or I_e (test solution) on I_4 or I_e (aqueous or control) for a range of concentration gives an indication about the magnitude of pyrene accumulation in hydrophobic pocket like micelles or micellar like structure. Also, the ratio I_4 or I_e (test solution) on I_3 (test solution) represents the degree of friction in the hydrophobic domains. A high ratio is a sign of high viscosity.

The *in vitro* toxicity of the exopolysaccaride micelles was tested by incubating the micelles with a wide range of cell lines for 4-hour, 8-hour, 72-hour and 96-hour periods. The XTT™ or Alamar Blue™ assays were used to quantify the survival rates in the presence of the exopolysaccharide micelles. The capability of the exopolysaccharide to deliver an active molecule was first tested *in vitro* using 5-Fluorouracil (5-Fu) as a model active molecule. The 4-hour and 8-hour exposure time were removed from the analysis due to the absence of reaction, 5-Fu being a low action drug and EPS showing no toxicities at those times. The 5-Fu/exopolysaccharide formulation was incubated with either B16F10 (murine skin melanoma), Caco-2 (human colon adenocarcinoma) or IEC-6 (rat epithelial small intestine) cells over 72-hour to 96-hour. Vital dyes (XTT or Alamar Blue) were added to supernatants for a period of 4 hours. Absorbance values were collected and plotted to evaluate the survival rate of the cells to the treatment. The results from XTT or Alamar Blue survival assays (Example 6) show a synergy between the EPS and the drug. It is also shown that combined use of 5-Fu and EPS has a higher cytotoxicity than the use of 5-Fu or EPS alone.

Beside the enhancement of *in vitro* toxicity of a drug which shows a synergy, EPS were used to formulate various molecules like fluorescin, rhodamine, 5-Fu, paclitaxel to demonstrate that EPS change the pharmacokinetic of those molecules (see Example 8) and cause an improvement of absorption, reduction of elimination, increase Cmax, and higher AUCs which leads to better therapeutic efficacy of 5-FU and paclitaxel (see Examples 9 and 10). A better efficacy translate into better overall survival or delay of death.

The exopolysaccharides are easy to use since there is no need for micelle preparation. The exopolysaccharides were dissolved in PBS pH 7.2 and incubated overnight at room temperature, which led to the spontaneous formation of the EPS micellar structure. Micellization process with exopolysaccharide is different than the conventional preparation of micelles (like P85) which is achieved by the addition of water in a dropwise fashion to a solution to form a micelle solution and the micelle solution did not need to be stirred overnight and then dialyzed against milli-Q™ distilled water using dialysis tubing with a change of water every hour for the first four hours and then every three hours for the next twelve hours like it is usually done.

The present invention describes the formation of micelles of different size composed of exopolysaccharide, preferably ranging from 50 to 700nm. The exopolysaccharide can be isolated from, but not limited to, *Lactobacillus* strain R2C2, *Lactobacillus* strain Inix, *Lactobacillus* strain Es1, Lactobacillus strain K2.

The exopolysaccharide not only can form micelles but can act as a biological response modifying agent on cells. As shown in Example 8, exopolysaccharide can activate genes in keratinocytes which shows that exopolysaccharides can be used not only as a drug delivery system but may have a synergistic effect with various drugs.

EXAMPLE 1

Extraction of EPS

EPS are extracted from biomass of a consortium of bacteria and yeast strains that include without limitation to the following ones: *Lactobacillus* strain R2C2, *Lactobacillus* strain Inix, *Lactobacillus* strain Es1, *Lactobacillus* strain K2, *Candida kefyr*, *Candida norvegensis*. EPS can also be produced from the purified bacterial strain mentioned above.

EXAMPLE 2

Crude preparation and production of EPS

Biomass (either consortium or purified bacterial stain) is added to hot water (0.5 to 5% (w/v)). The temperature of the solution is brought to 95°C and agitation is applied for a dissolution time of 3 hours. Dissolution of biomass is visually monitored and when no further dissolution occurs the incubation is pursued for an extra hour. A primary filtration is performed to remove aggregates and debris and the filtrate centrifuged. For large volumes (10L and over) a primary tangential filtration (0.45 μM) is performed to remove aggregates and debris. Filtrate is than concentrated by tangential filtration (3 kDaltons) to a minimal volume. Retentate is centrifuged at 7500g for 15 minutes to remove the precipitate. An equal volume of ice cold ethanol 100% (-70) is added to the supernatant to precipitate the exopolysaccharides for 16h. After centrifugation 7500g, 4°C for 15 minutes, the supernatant is discarded and the pellet resuspended in a minimal volume of water. Solution is centrifuged at 7500g for 15 minutes. The supernatant containing EPS is freeze-dried to yield the pure EPS.

EXAMPLE 3

Purified preparation of EPS

Biomass (either consortium or purified bacterial strain) is added to hot water (0.5 to 5% (w/v)). The temperature of the solution is brought to 95°C and agitation is applied for a dissolution time of 3 hours. Dissolution of biomass is visually monitored and when no further dissolution occurs the

incubation is pursued for an extra hour. A primary filtration is performed to remove aggregates and debris. For large volumes (10L and over) a primary tangential filtration (0.45 μM) is performed to remove aggregates and debris. Filtrate is concentrated by tangential filtration (3 kDaltons) to a minimal volume. Retentate is centrifuged at 7500g for 15 minutes to remove the precipitate. An equal volume of ice cold ethanol 100% (-70) is added to the supernatant to precipitate the exopolysaccharides for 16h. After centrifugation 7500g, 4°C for 15 minutes, the supernatant is discarded and the pellet resuspended in a minimal volume of water. Solution is centrifuged at 7500g for 15 minutes. The supernatant containing EPS is freeze-dried to yield the pure EPS.

EXAMPLE 4

Fractionation of EPS

Pure EPS are solubilized in water and fractionated by serial passages on membrane of different molecular weight cutoff. As example, when the membranes used are of 100 000, 50 000, 10 000 and 3 000 kDa, it yield fraction of EPS with the following range of molecular weight: EPS > 100 000 kDa, 50 000 kDA <EPS>100 000 kDa, 10 000 kDa <EPS>50 000 kDa, 3 000 kDa<EPS>10 000 kDa, EPS<3 000 kDa.

EXAMPLE 5

Measurement of critical micelle concentration (CMC) of EPS using Pyrene

A 1% solution of EPS (w/v) in PBS was prepared. The solution was vortexed for 2 minutes until EPS is well resuspended. Multiple dilutions were performed to obtain 2 ml of 0.1%, 0.01%, 0.001% EPS solution. In parallel, a series of borosilicate tubes were prepared to which 20 μl of a pyrene solution (50 μM solubilized in acetone (Sigma Aldrich cat. No. 18-551-5)) were added. The solutions were allowed to dry prior to the addition of the various EPS solutions. Once dried, 2 ml of the EPS solutions were transfered into the borosilicate tubes to be tested. This is called a redissolution test or solubilization or partitionating between water and test conditions. The control

tube (containing PBS only) was placed in the cell holder set at 37°C of an Varian Eclipse™ fluorometer set at Excitation: 340nm, Emission: 350-600nm. Samples were incubated over time and read to obtain the partitionating coefficient and CMC measurement. CMC is calculated according to the standard protocols as described previously. The data of Table 1 show the ratio of fluorescence intensity of peak I_3 over intensity of peak I_3 from PBS solution as a function of the concentration of EPS and P85. This data show that the EPS can solubilize the insoluble probe (pyrene) as much if not more than P85 from the range 0,001% to 0,1%. At higher concentration (1%), P85 seems to keep forming conventional micelles while the EPS start to decline which is explained by a shift in the spectrum profile. This shift is indicative of excimers formation.

Table 1

Ratio of fluorescence intensity of peak I_3 over intensity of peak I_3 from PBS solution in function of the concentration of EPS and P85

Concentration	P85	EPS lot 2
0,001%	1,107	1,125
0,01%	1,168	1,690
0,1%	2,564	3,989
1%	8,813	3,476

The data of Table 2 show the ratio of fluorescence intensity of peak I_e over intensity of peak I_e from PBS solution in function of the concentration of EPS and P85. This data show that EPS triggers a higher formation of excimer than P85.

Table 2

Ratio of fluorescence intensity of peak Ie over intensity of peak Ie from PBS solution in function of the concentration of EPS and P85

Concentration	P85	EPS lot 2
0,001%	0,9619	2,241
0,01%	1,233	19,51
0,1%	2,443	60,31
1%	4,829	11,97

The data of Table 3 show the ratio of fluorescence intensity of peak I_e over intensity of peak I_m as a function of the concentration of EPS and P85. This data show that the EPS are more viscous than P85.

Table 3

Ratio of fluorescence intensity of peak I_e over intensity of peak I_m as a function of the concentration of EPS and P85

Concentration	P85	EPS lot 2
0,001%	0,06239	0,1430
0,01%	0,07580	0,8293
0,1%	0,06841	1,086
1%	0,03935	0,2472

The data of Table 4 show the ratio of fluorescence intensity of peak I_3 over intensity of peak I_1 in function of the concentration of EPS and P85. This data show that both P85 and EPS are apolar media.

Table 4

Ratio of fluorescence intensity of peak I_3 over intensity of peak I_1 in function of the concentration of EPS and P85

Concentration	P85	EPS lot 2
0,001%	0,9271	0,9119
0,01%	0,9226	1,031
0,1%	0,9538	1,257
1%	0,9892	1,328

EXAMPLE 6

Formulation of 5FU with EPS and studies on B16, IEC-6 and Caco-2 cells

Caco-2, IEC-6 and melanocytes B16 cells were cultured in DMEM supplemented with 10% FBS. The cells were seeded at $2X10^3$ cells per well in a 96-well plate and left to rest 24 hours before EPS exposure at various concentrations and also with or without 5FU (0,001 μg/ml to 10 μg/ml). The cells were grown in a CO_2 incubator for 4 extra days. Cell survival was assessed with XTT™ according to manufacturer's recommendation. The data obtained show a synergistic effect between 5FU and the EPS while EPS show no or only modest toxicity. Furthermore, on differentiated Caco-2 cells, EPS from consortium show no toxicity neither alone nor in formulation unlike on proliferative Caco-2 cells where the synergy of EPS is evident. The data are as follows:

Table 5

Survival of IEC-6 rat normal small intestine epithelial cells in presence of different concentrations of 5-fluorouracil and exopolysaccharides

μg/ml	5-Fu alone	EPS 1% + 5-Fu	EPS 0.1% + 5-Fu	EPA 0.01% + 5-FU
0	100.1 ± 5.2	64.2 ± 14.8	86.0 ± 4.3	97.0 ± 11.1
5	16.9 ± 6.4	0.0 ± 2.1	0.0 ± 4.8	5.4 ± 1.5
50	28.0 ± 4.6	7.1 ± 3.5	9.3 ± 6.3	8.1 ± 5.1
250	35.4 ± 8.2	0.4 ± 3.1	18.2 ± 6.5	37.2 ± 11.7
500	33.4 ± 3.5	0.0 ± 5.3	22.1 ± 4.1	8.3 ± 4.6

Table 5 illustrates the results of three days exposure treatments run in quadruplicate and cell survival was revealed by absorbance using the vital dye Alamar Blue™ (Medicorp, Mtl). [NB073]. Percentages of EPS are expressed in (w/v).

Table 6

Survival of IEC-6 rat normal small intestine epithelial cells in presence of different concentrations of 5-Fluorouracil and exopolysaccharides

µg/ml	5-Fu alone	EPS 1% + 5-Fu	EPS 0.1% + 5-Fu	EPA 0.01% + 5-FU
0	100.0 ± 1.9	0.0 ± 6.7	91.8 ± 2.3	91.3 ± 2.1
1	78.0 ± 3.0	0.0 ± 4.3	76.7 ± 0.3	84.9 ± 1.5
10	75.4 ± 2.9	0.0 ± 4.7	76.4 ± 0.6	83.2 ± 3.1
50	81.8 ± 2.1	0.0 ± 4.4	71.7 ± 1.1	80.6 ± 2.2
100	80.2 ± 0.9	0.0 ± 1.2	67.0 ± 1.8	66.8 ± 2.7

Table 6 illustrates the results of three days exposure treatments run in quadruplicate and cell survival was revealed by absorbance using the vital dye Alamar Blue™ (Medicorp, Mtl). [NB073]. Percentages of EPS are expressed in (w/v).

Table 7

Survival of Caco-2 human colon adenocarcinoma cells in proliferation in presence of different concentrations of 5-Fluorouracil and exopolysaccharides

µg/ml	5-Fu alone	EPS 0.1% + 5-Fu (consortium)	EPS 0.1% + 5-Fu (pure strain)
0	100.0 ±1.2	98.0 ± 1.1	101.8 ± 2.3
1	74.1 ± 1.9	60.5 ± 3.4	119.7 ± 4.7
10	78.6 ± 2.4	34.2 ± 0.8	36.3 ± 0.5
50	80.3 ± 1.8	46.4 ± 1.4	52.2 ± 1.3
100	52.6 ± 4.8	47.4 ± 1.2	41.0 ± 1.1

Table 7 illustrates the results of four days exposure treatments were run is octoplicate and cell survival was revealed by absorbance using the vital

dye XTT™ (Sigma, St-Louis) [NB050]. Percentages of EPS are expressed in (w/v).

Table 8

Survival of Caco-2 human colon adenocarcinoma cells differentiated in presence of different concentrations of 5-Fluorouracil and exopolysaccharides

µg/ml	5-Fu alone	EPS 0.1% + 5-Fu (consortium)	EPS 0.1% + 5-Fu (pure strain)
0	100.0 ± 4.0	97.8 ± 1.9	105.7 ± 4.2
1	105.7 ± 4.2	100.9 ± 5.1	69.2 ± 0.3
10	85.6 ± 8.8	108.7 ± 4.4	75.4 ± 5.2
50	99.6 ± 6.8	98.3 ± 6.0	71.9 ± 2.8
100	78.1 ± 0.7	88.2 ± 3.6	77.2 ± 1.6

Table 8 illustrates the results of four days exposure treatments were run is octoplicate and cell survival was revealed by absorbance using the vital dye XTT™ (Sigma, St-Louis) [NB050]. Percentages of EPS are expressed in (w/v).

Table 9

Survival of B16F10 murine skin melanoma cells in presence of different concentrations of 5-Fluorouracil and expolysaccharides

µg/ml	5-Fu alone	EPS 0.1% + 5-Fu (consortium)	EPS 0.1% + 5-Fu (pure strain)
0	100.0 ± 4.1	95.8 ± 9.6	56.4 ± 4.5
1	62.6 ± 6.6	20.5 ± 1.4	21.2 ± 1.4
10	65.4 ± 10.4	35.4 ± 10.7	26.4 ± 3.4
50	54.9 ± 8.9	18.7 ± 2.7	28.2 ± 4.9
100	38.8 ± 8.5	20.9 ± 6.5	26.8 ± 2.6

Table 9 illustrates the results of four days exposure treatments were run is octoplicate and cell survival was revealed by absorbance using the vital dye XTT™ (Sigma, St-Louis) [NB050]. Percentages of EPS are expressed in (w/v).

EXAMPLE 7

Biological effects of EPS on keratinocytes

HEKa (human epithelial keratinocytes, adult, Cascade Biologics), grown in Medium 154 supplemented with human keraticocyte growth supplement (HKGS), were exposed to exopolysaccharide (10μg/ml) and the RNA extracted using Rneasy purification columns (Qiagen) as per manufacturers specifications. The RNA samples from control and treated cells was labeled with Cy3 and Cy5 respectively, and applied to human 1.7K microarray (Microarray Centre, University Health Network, Toronto) for competitive hybridization. Exopolysaccharides regulate the expression of a variety of genes that may be advantageous for cosmeceutical purposes but also shows that they are not inert and modulate genes that indicate the type of drugs that be formulated with EPS.

Table 10

Genes regulated by Exopolysaccharides

Down regulated genes	Up regulated genes
Tumor necrosis factor (TNF)	TGF-beta induced (TGFBi)
Cyclooxygenase 2 (COX)	Transglutaminase 2 (Tgase 2)
	Transgelin (TAGLN2)
	Thrombospondin 1 (THBS1)
	Keratin 7 (KRT7)
	Ferritin light polypeptide (FTL)

EXAMPLE 8

Change of pharmacokinetic of markers with EPS

Fluorescein (FL) pharmacolinetic on unfasten Wistar female rats of 200g and 8 weeks of age. Fluorescein concentration is 1 mg/kg. Exopolysaccharides (EPS) are diluted in water at 1% and 5% (w/v). Fluorescein is added to the latter solutions and vortexed. Formulations are fed to the animals with a canula. 200µl blood samples are collected through the jugular vein every 30 minutes for 2.5 hours. A final bleeding is made at 3.5h. Plasma are collected immediately after centrifugation (10 min @ 1300g). Plasma are frozen on dry ice until further analysis: 50µl of plasma are diluted in 550µl of saline 0.7% (w/v) and analyzed with a Varian Eclipse Spectrofluorometer (Excitation: 495nm Emission: 515nm). Concentrations were determined on a fluorescein standard curve (FL diluted in saline 0.7% and 50µl of normal serum as in the sample analysis).

Table 11

Fluorescein concentrations over time

Time (h)	FL-Water	Std Err	FL-EPS 1%	Std Err	FL-EPS 5%	Std Err
0.0	37.10	3.55	54.79	15.40	36.77	3.38
0.5	218.30	49.40	298.26	24.61	360.46	62.08
1.0	145.95	19.92	319.20	166.54	151.50	62.78
1.5	75.25	19.83	140.76	68.07	136.04	38.69
2.0	74.48	17.46	73.09	21.14	95.66	25.78
2.5	71.74	14.57	48.31	10.39	92.44	22.45
3.5	54.63	10.28	70.60	31.08	88.47	26.78

EPS used at 1% and 5% show a better absorption than non formulated fluorescein. Higher Cmax were obtained for both concentration around 1h after the gastric intubation. Overall, EPS 5% show a better

absorption of the drug up to 3.5h. The result of a better absorption is superior AUC for both concentration of EPS this translating to a better drug exposure and thus better treatments (see Example 10).

EXAMPLE 9

Formulation of B16 tumors in muscle following i.m. injection of B16 cells treated with a formulation of paclitaxel

Fifteen mice in group P1 (100x stock = 100 mM or 85,3 mg/ml, prepared from powdered stock in DMSO) and fourteen mice in group P2 (10X stock = 10mM in 1% EPS (in PBS), or 8.5 mg/ml, prepared from 100µl P1 in 900µl 1% EPS (in PBSP)). Cells (50000 cells per 50µl injections) for a concentration of 10^6 cells per ml. The remaining of the test was performed as follows:

- Cells from $1XT_{75}$ (80% confluence) are trypsinized with 2ml trypsin, diluted in 12 ml media, spun down, rinsed once in 5ml PBS, and resuspended in 2ml PBS. Cells are then counted and concentration adjusted to 10^6 cells per ml.
- P1 treatment: Add 20µl P1 stock and 180µl PBS of cell suspension (10^6 cells) and incubate at room temperature 3 hrs. Final Paclitaxel = 1mM, DMSO = 1%.
- P2 treatment: Add 200µl P2 stock to 1.8ml of cell suspension (10^6 cells) and incubate at room temperature 3 hrs. Final Paclitaxel = 0.1mM, DMSO = 1%, EPS = 0.1%.
- 1ml of cells from P1 and P2 treatment groups were replated following a rinse in PBS. No growth was observed from either group after 2 weeks in culture.

Table 12

Tumor-free mice (%)

Formulation	P1	P2
Growth in tissue culture	negative	negative
Intramuscular tumor formation	12/15	1/14

Cells treated with Paclitaxel do not have the capacity to recover from this treatment in tissue culture, however, in an environment rich in nutrients and growth factors, such as *in vivo* within the muscle, the cells can recover from the drug and form intramuscular tumors. However, as shown herein, the presence of EPS potentiates the effect of Paclitaxel in this model.

EXAMPLE 10

Treatment with a 5FU/EPS formulation of mice grafted with B16 cells injected i.p.

Tumor cells were trypsinized and washed 3 times with PBS. Cells (1x10E6) were injected i.p. to Balb/c mice to induce a cancer in the peritoneal cavity. After 4 days of implantation, treatments of 5FU alone (100mg/kg) or EPS alone (0.1% w/v) or a combination of both were given to the mice. Survival was observed for 44 days from day 0 of injection. An additive effect was observed between EPS and 5FU from day 29 leading to a better survival than the group treated with 5FU alone. EPS alone show efficacy until day 29 compared with other treatments but protects the animals better overall. This highlighting again that EPS are not inert and may contribute to the therapeutic index.

Table 13

Tumor-free mice over time (%)

Days	Saline (Placebo)	5-FU	EPS 0.1% (w/v)	EPS 0.1% (w/v) + 5-FU
0	100	100	100	100
15	12.5	62.5	37.5	62.5
29	12.5	12.5	37.5	37.5
44	12.5	12.5	37.5	25

EXAMPLE 11

Modification of the pharmacokinetic of Rhodamine formulated with EPS

The pharmacokinetic of Rhodamine 123 (R123) was performed on unfasten Wistar female rats of 200g and 8 weeks of age. Rhodamine concentration is 5 mg/kg. Exopolysaccharides (EPS) were diluted in water at 1% and 3% (w/v). Rhodamine is added to the latter solutions and vortexed. Formulations are fed to the animals with a canula and 200µl blood samples are collected through the jugular vein every hour for 4 hours (plus 1 sample at 0.5h). Plasma are collected immediately after centrifugation (10 min @ 13000g). Plasma are frozen on dry ice until further analysis: 50µl of plasma are diluted in 550µl of saline 0.7% (w/v) and analyzed with a Varian Eclipse™ Spectrofluorometer (Excitation: 505nm Emission: 540nm). Concentrations were determined on a Rhodamine 123 standard curve (R123 diluted in saline 0.7% and 50µl of normal serum as in the sample analysis). The data are as follows:

Table 14

Rhodamine 123's concentration over time

Time (h)	R123 – Water (ng/ml)	R123 – EPS 1% (ng/ml)	R123 – EPS 3% (ng/ml)
0.0	2.2 ± 2.2	0.2 ± 0.1	1.3 ± 0.7
0.5	20.4 ± 2.2	10.1 ± 2.2	21.7 ± 4.2
1.0	20.9 ± 2.6	11.6 ± 3.0	24.3 ± 3.5
2.0	15.4 ± 2.0	9.1 ± 2.9	20.2 ± 4.8
3.0	10.9 ± 1.4	6.0 ± 1.2	20.3 ± 4.6
4.0	11.2 ± 1.8	3.0 ± 0.7	15.7 ± 3.6

Rhodamine formulated with EPS used at 3% show approximately the same absorption as in water except that the AUC (area under the curve) is increased. Absorption, distribution and elimination seem to be closely linked between those two groups showing no influence of the EPS in the blood circulation. EPS 1%, on their side, show a peculiar profile. The absorption is slower and lower. EPS 1% might retain the R123, preventing it from penetrating in the blood stream or EPS 1% could stay with R123 and block its fluorescence.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A delivery system for delivery of an active molecule to a patient, said delivery system comprising a population of exopolysaccharide micelles, each said micelle defining a core for containing said active molecule.

2. The delivery system of claim 1, wherein said exopolysaccharide is produced by lactic acid bacteria.

3. The delivery system of claim 2, wherein said bacteria is selected from the group consisting of *Lactobacillus* strain R2C2, *Lactobacillus* strain Inix, *Lactobacillus* strain Es1, *Lactobacillus* strain K2, *Candida kefyr* and *Candida norvegensis*.

4. The delivery system of claim 1, wherein said active molecule is selected from the group consisting of DNA, RNA, protein, peptide, peptidomimetic, virus, bacteria, neutraceutical product and pharmaceutical agent.

5. The delivery system of claim 4, wherein said pharmaceutical agent is selected from the group consisting of analgesic, anesthetic, antibiotic, anticancer, anti-inflammatory, and antiviral.

6. The delivery system of claim 5, wherein said anticancer agent is selected from the group consisting of alkylating agents, alkyl sulfonates, aziridines, ethylenimines, methylamelamines, acetogenins, camptothecin, bryostatin, callystatin, CC-1065, cryptophycins, dolastatin; duocarmycin, eleutherobin, pancratistatin, sarcodictyin, spongistatin, nitrogen mustards, nitrosureas, antibiotics, anti-metabolites, folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate, purine analogs, pyrimidine analogs, androgens, anti-adrenals, folic acid replenisher, aceglatone, aldophosphamide glycoside, aminolevulinic acid, amsacrine, bestrabucil, bisantrene, edatraxate, defofamine, demecolcine, diaziquone, elformithine, elliptinium acetate, epothilone, etoglucid, gallium nitrate, hydroxyurea, lentinan, lonidamine, maytansinoids, mitoguazone, mitoxantrone, mopidamol, nitracrine, pentostatin, phenamet, pirarubicin, podophyllinic acid, 2-ethylhydrazide, procarbazine, PSK.RTM., razoxane, rhizoxin, sizofiran, spirogermanium, tenuazonic acid, triaziquone, 2, 2',2''-trichlorotriethylamine, trichothecenes, urethan, vindesine, dacarbazine, mannomustine, mitobronitol, mitolactol, pipobroman, gacytosine,

arabinoside, thiotepa, taxanes, chlorambucil, gemcitabine, 6-thioguanine, mercaptopurine, methotrexate, platinum, vinblastine, platinum, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, navelbin, novantrone, teniposide, daunomycin, aminopterin, xeloda, ibandronate, CPT-11, topoisomerase inhibitor RFS 2000, difluoromethylornithine, retinoic acid, capecitabine, anti-hormonal agents that act to regulate or inhibiting hormone action in hormonal dependent cancers.

7. The delivery system of claim 6, wherein said anti-hormonal agent is an anti-estrogens or an anti-androgens selected from the group consisting of flutamide, nilutamide, bicalutamide, leuprolide, and goserelin, and pharmaceutically acceptable salts, acids or derivatives thereof.

8. The delivery system of claim 6, wherein said alkylating agents is selected from the group consisting of thiotepa and cyclosphosphamide (CYTOXAN™).

9. The delivery system of claim 6, wherein said alkyl sulfonates is selected from the group consisting of busulfan, improsulfan and piposulfan.

10. The delivery system of claim 6, wherein said aziridines is selected from the group consisting of benzodopa, carboquone, meturedopa, and uredopa.

11. The delivery system of claim 6, wherein said methylamelamines is selected from the group consisting of altretamine, triethylenemelamine,trietylenephosphoramide, triethylenethiophosphaor-amide and trimethylolomelamine.

12. The delivery system of claim 6, wherein said acetogenins is selected from the group consisting of bullatacin and bullatacinone.

13. The delivery system of claim 6, wherein said camptothecin is the synthetic analogue topotecan.

14. The delivery system of claim 6, wherein said CC-1065 is selected from the group consisting of adozelesin, carzelesin and bizelesin synthetic analogues thereof.

15. The delivery system of claim 6, wherein said cryptophycins is selected from the group consisting of cryptophycin 1 and cryptophycin 8.

16. The delivery system of claim 6, wherein said duocarmycin is selected from the group consisting of KW-2189 and CBI-TMI.

17. The delivery system of claim 6, wherein said nitrogen mustards is selected from the group consisting of chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide and uracil mustard.

18. The delivery system of claim 6, wherein said nitrosureas is selected from the group consisting of carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine.

19. The delivery system of claim 6, wherein said anti-metabolites is selected from methotrexate and 5-fluorouracil (5-FU).

20. The delivery system of claim 6, wherein said purine analogs is selected from the group consisting of fludarabine, 6-mercaptopurine, thiamiprine and thioguanine.

21. The delivery system of claim 6, wherein said pyrimidine analogs is selected from the group consisting of ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine and floxuridine.

22. The delivery system of claim 6, wherein said androgens is selected from the group consisting of calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone.

23. The delivery system of claim 6, wherein said anti-adrenals is selected from the group consisting of aminoglutethimide, mitotane and trilostane.

24. The delivery system of claim 5, wherein said antibiotics is selected from the group consisting of enediyne antibiotics, aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, poffiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin.

25. The delivery system of claim 24, wherein said enediyne antibiotics is selected from the group consisting of calicheamicin, dynemicin, esperamicin, neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromomophores.

26. The delivery system of claim 25, wherein said callicheamicin is selected from the group consisting of calicheamicin γ_1^I and calicheamicin θ_{1I}.

27. The delivery system of claim 25, wherein said dynemicin is dynemicin A.

28. The delivery system of claim 1, wherein said micelles are having a diameter varying from about 50 nanometers to about 700 nanometers.

29. A pharmaceutical composition comprising the delivery system of any one of claims 1 to 28 in association with a pharmaceutically acceptable carrier.

30. An immunomodulator composition comprising an immunomodulating amount of the delivery system of any one of claims 1 to 28 in association with a pharmaceutically acceptable carrier.

31. A method for delivering an active molecule to a patient comprising the step of administering the composition of claim 29 to said patient.

32. The method of claim 31, wherein said administering can be from a route selected from the group consisting out local, parenteral, peritoneal, mucosal, dermal, epidermal, subcutaneous, transdermal, intramuscular, nasal, oral, topical, vaginal, rectal, intra-ocular, intravenous, intra-arterial and by inhalation.

33. A method for inducing immunomodulation in a patient comprising the step of administering the composition of claim 30 to said patient.

34. The method of claim 33, wherein said administering can be from a route selected from the group consisting out local, parenteral, peritoneal, mucosal, dermal, epidermal, subcutaneous, transdermal, intramuscular, nasal, oral, topical, vaginal, rectal, intra-ocular, intravenous, intra-arterial and by inhalation.

35. Use of the composition of claim 29 for delivering an active molecule to a patient.

36. The use as claimed in claim 35, wherein said delivering can be from a route selected from the group consisting out local, parenteral, peritoneal, mucosal, dermal, epidermal, subcutaneous, transdermal, intramuscular, nasal, oral, topical, vaginal, rectal, intra-ocular, intravenous, intra-arterial and by inhalation.

37. Use of the composition of claim 30 for inducing immunomodulation in a patient.

38. The use as claimed in claim 37, wherein said delivering can be from a route selected from the group consisting out local, parenteral, peritoneal, mucosal, dermal, epidermal, subcutaneous, transdermal, intramuscular, nasal, oral, topical, vaginal, rectal, intra-ocular, intravenous, intra-arterial and by inhalation.

39. A method for producing the delivery system of claim 1, comprising the step of incubating exopolysaccharide in a suitable medium for a time sufficient to form micelle.

9.2 Brevet en phase internationale

Simard, E. Goyette, P. Lemieux, P. 2002. International 2003. Chemotherapeutic agents as anti-cancer vaccine adjuvants and therapeutic methods thereof. PCT/CA2003/000434. 29 pages.

Ce brevet fut déposé le 25 mars 2002 en dépôt provisoire au État-Unis. Le dépôt international fut effectué le 25 mars 2003 pour les Etats-Unis, l'Europe, le Canada.

J'ai effectué la rédaction du brevet et j'ai eu une contribution majeure à la revue des brevets existants et à la revue de littérature.

CHEMOTHERAPEUTIC AGENTS AS ANTI-CANCER VACCINE ADJUVANTS AND THERAPEUTIC METHODS THEREOF

ABSTRACT

The present invention relates to an anti-cancer vaccine composition comprising an antigen in association with an effective amount of at least one immunomodulator chemotherapeutic adjuvant eliciting an immune response in a patient and a pharmaceutically acceptable carrier. It also relates to method of preventing tumor growth and reducing tumor growth using the anti-cancer vaccine composition of the present invention.

CHEMOTHERAPEUTIC AGENTS AS ANTI-CANCER VACCINE ADJUVANTS AND THERAPEUTIC METHODS THEREOF

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The present invention relates essentially to novel anti-cancer vaccine adjuvant composed of immunomodulator chemotherapeutic agents and uses thereof.

(b) Description of Prior Art

It is well accepted that vaccines have contributed to improve quality of life of mankind and livestock and currently many different vaccines are sill being developed to treat, cure, or prevent disease that can benefit from an immune response raised against an unwanted antigenic expression. Diseases like infectious diseases, autoimmune diseases, allergies, and cancers can benefit from the use of an efficient vaccine. For instance, as a tumor grows in size, it typically becomes more refractory to most chemotherapies. Accordingly, many tumor eradication procedures include radiotherapy or a surgical debulking step to decrease the mass of the tumor prior to the administration of anti-neoplastic agents. However, radiotherapy and debulking do not always result in tumor eradication, even when combined with powerful chemotherapeutic agents. Accordingly, there is a need in the art for new treatments that target both proliferating and non-proliferating cancer cells for the treatment of malignancies. Cancer vaccines have shown promise so far and with the examples given in this invention those may reach another level of efficacy with the use of chemotherapeutic agent used as adjuvants. Recent advances in the understanding of immune function with regard to the generation of a potent antitumor response have resulted in a renewed interest in cancer vaccines and point to a role for immunotherapy in the treatment of cancer.

Cancer vaccines are on the threshold of taking their place alongside the more traditional and conventional cancer treatment modalities of surgery, radiation therapy and chemotherapy. The toxicology and immunopharmacology of therapeutic cancer vaccines, particularly those that secrete granulocyte macrophage colony stimulating factor (GM-CSF), are currently under active clinical investigation (Cell Genesys). GM-CSF is used as an adjuvant to help function of antigen presentation. This kind of vaccine however requires multiple steps like expansion of cancer cell in culture, transfection with GM-CSF, inactivation with irradiation before the reinoculation. With this kind of vaccine, a priming step of the patient may be enhancing the therapeutic effect. Interestingly, drugs traditionally used for tumor cytoreduction can have both positive and negative effects on host immunity. Exploration of the potential pharmacodynamic interactions of antineoplastic drugs administered systemically long with GM-CSF-secreting vaccines has revealed that low doses of some chemotherapeutics can augment the antitumor immunity induced by GM-CSF-secreting vaccines.

Chemotherapeutics are the mainstay of the majority of antitumor treatment strategies. These agents are usually administered at or near maximum tolerated doses resulting in frequent dramatic toxicities that compromise the quality of life and the immune response towards microbial pathogens. A number of observations suggest that low-dose treatment with chemotherapeutics is sometimes equal or even superior to high-dose chemotherapy. The efficacy of low-dose chemotherapy can be at least partly explained by the regulation of the antitumor immune response. The immunomodulatory effects of some chemotherapeutics might be further potentiated by combinations with selected biological response modifiers such as recombinant cytokines (IL-2, TNF, IL-12 and others). The effectiveness of such treatment combinations have already proven effective in preclinical animal models. However, the efficacy in humans is still to be demonstrated in rationally designed clinical trials (Zagozdzon et al. *Int J Oncol* 2001 Feb;18(2):417-24).

For instance, it was observed that cyclophosphamide, paclitaxel, and doxorubicin, when given in a defined sequence either before or after the whole-cell vaccine and by a different route of administration with a GM-CSF-secreting, neu-expressing whole-cell vaccine, enhanced the vaccine's potential to delay tumor growth in neu transgenic mice. In addition, it was showed that these drugs mediate their effects by enhancing the efficacy of the vaccine rather than via a direct cytolytic effect on cancer cells. Furthermore, paclitaxel and cyclophosphamide appear to amplify the T helper 1 neu-specific T-cell response. These findings suggest that the combined treatment with immune-modulating doses of chemotherapy and the GM-CSF-secreting neu vaccine can overcome immune tolerance and induce an antigen-specific antitumor immune response (Machiels et al. *Cancer Res* 2001 May 1;61(9):3689-97). The approach described above is unfortunately complex and requires a timing allowing the best performance possible of the whole-cell vaccine. Thus, there is a need to simplify and to increase the efficacy of such approach. Chemotherapeutic agents have been shown to stimulate immune responses against antigens (Bystryn US patent 6,338,853). For instance, in some clinical trials it was found that antibody and/or cellular immune responses to melanoma were induced more frequently in Stage II (69% of 36 patients) than in Stage III (53% of 19 patients) disease. The ability of different immunization schedules, alum or pretreatment with low dose cyclophosphamide, to potentiate immunogenicity was compared after 2 months of immunization. Bi-weekly immunization with a fixed intermediate dose of vaccine was more immunogenic than weekly immunization with escalating vaccine doses. Alum increased slightly the intensity of cellular responses while pretreatment with cyclophosphamide augmented slightly both the incidence and intensity of cellular immune responses. There was a reciprocal relationship between the induction of humoral and cellular immune responses. The most effective immunization schedule consisted of pretreatment with cyclophosphamide which augmented antibody and/or cellular immune responses to melanoma in 83% of patients. In addition, recently, paclitaxel, another well known and widely used chemotherapeutic agent was found to trigger the same signal transduction pathways than LPS,

and to be also a strong inducer of multiple immune responses (Byrd-Leifer et al. *Eur J Immunol* 2001 Aug;31(8):2448-57).

These observations are of interest but schedules and the timing of treatment are complicated since the chemotherapetic agents have to be injected either before or after the antigens. Furthermore, chemotherapeutic agents have to be administered at the appropriate concentration in order to promote the stimulation of the immune system rather than a systemic immunosuppression normally associated with the use of chemotherapeutic agents.

Despite the development of Monophosphoryl lipid A (MPL), the latter has shown limited efficacy in clinical trials on cancer vaccines and other types of vaccines. Thus, there is still a need for novel and more efficacious adjuvants that may enhance further immunogenicity of conventional vaccines but especially of new-generation vaccines (including recombinant subunit, peptide and protein-based vaccines, DNA vaccines, whole-cell vaccine, mucosal vaccines) that are less immunogenic. GM-CSF is being used as a molecular adjuvant (Cell Genesys) to enhance immunogenicity of whole-cell vaccines against cancer. The GM-CSF gene is transfected into the cells before being expanded in culture and be inoculated.

Currently, aluminum salts and MF59 are the only vaccine adjuvants approved for human use. Aluminum salts used as adjuvants are now suspected to be the culprit of the Gulf war syndrome which translates to a chronic fatigue due to an accumulation of aluminum crystals in the muscle leading to a constant immunostimulation and eventually to depletion of competent immune cells. With the development of new-generation vaccines (including recombinant subunit, DNA vaccines, whole-cell vaccine, mucosal vaccines) that are less immunogenic, the search for more potent vaccine adjuvants has intensified. Of the novel compounds recently evaluated in human trials, immunostimulatory molecules such as the lipopolysaccharide derived MPL (Corixa) and the saponin derivative QS21 appear most promising, although doubts have been raised as to their safety in humans. Preclinical work with particulate adjuvants, such as the MF59 microemulsion

and lipid-particle immune-stimulating complexes (Iscoms), suggest that these molecules are potent elicitors of humoral and cellular immune responses. In addition, preclinical data on CpG oligonucleotides appear to be encouraging, particularly with respect to their ability to selectively manipulate immune responses. While all these adjuvants show promise, further development of more potent adjuvants may allow vaccines to be used as therapeutic, rather than prophylactic agents.

It would be highly desirable to be provided with an anti-cancer vaccine composition comprising immunomodulator chemotherapeutic agents as adjuvant to elicit an immune response against cancer in a patient.

SUMMARY OF THE INVENTION

In accordance with the present invention there is provided an anti-cancer vaccine composition comprising an antigen in association with an effective amount of at least one immunomodulator chemotherapeutic adjuvant eliciting an immune response in a patient and a pharmaceutically acceptable carrier.

The anti-cancer vaccine in accordance with a preferred embodiment of the present invention, wherein the vaccine is selected from the group consisting of whole-cell vaccine, DNA vaccine, peptide-based vaccine, protein-based vaccine and attenuated organism vaccine.

The anti-cancer vaccine in accordance with a preferred embodiment of the present invention, wherein the antigen is inactivated tumor cells more preferably wherein the tumor cells being inactivated by the chemotherapeutic agent or wherein said tumor cells are inactivated by radiotherapy and/or chemotherapy.

The anti-cancer vaccine in accordance with another embodiment of the present invention, wherein the tumor cells are inactivated by radiotherapy.

The anti-cancer vaccine of in accordance with a preferred embodiment of the present invention, wherein said vaccine is eliciting an immune response against a cancer selected from the group consisting of

basal cell carcinoma, bladder cancer, bone cancer, brain cancer, CNS cancer, breast cancer, cervical cancer, colon cancer, rectum cancer, connective tissue cancer, esophageal cancer, eye cancer, kidney cancer, larynx cancer, liver cancer, lung cancer, Hodgkin's lymphoma, non-Hodgkin's lymphoma, melanoma, myeloma, leukemia, oral cavity cancer, ovarian cancer, pancreatic cancer, prostate cancer, rhabdomyosarcoma, skin cancer, stomach cancer, testicular cancer, neoplasia and uterine cancer.

The anti-cancer vaccine of a preferred embodiment of the present invention, wherein the vaccine is eliciting an immune response against a cancer selected from the group consisting of bladder cancer, prostate cancer, melanoma, breast cancer, colon cancer, lung cancer, liver cancer, lymphoma, myelona, leukemia and ovarian cancer.

In the anti-cancer vaccine of a preferred embodiment of the present invention, wherein the chemotherapeutic adjuvant is a taxane.

In the anti-cancer vaccine of a preferred embodiment of the present invention, the chemotherapeutic adjuvant is selected from the group consisting of Cyclophosphamide, Doxorubicin, Cisplatin, Paclitaxel and 5-Fluorouracil.

The anti-cancer vaccine in accordance with another embodiment of the present invention, further comprising a therapeutically effective amount of a therapeutic agent.

In the anti-cancer vaccine in accordance with another embodiment of the present invention, the therapeutic agent is selected from the group consisting of cytokine, antibody, systemic chemotherapeutic agent, biological response modifier and hormones.

In the anti-cancer vaccine in accordance with another embodiment of the present invention, the antibody is a monoclonal antibody.

In the anti-cancer vaccine in accordance with another embodiment of the present invention, the biological response modifier is selected from the group consisting of interferon and lymphokine.

In the anti-cancer vaccine in accordance with another embodiment of the present invention, the lymphokine is IL-2.

The anti-cancer vaccine in accordance with another embodiment of the present invention, further comprising a non-chemotherapeutic adjuvant selected from the group consisting of polysaccharides, CpG nucleic acids, MF59, SAF, MPL and QS21.

The anti-cancer vaccine in accordance with another embodiment of the present invention, further comprising a non-chemotherapeutic adjuvant selected from the group consisting of adjuvants that create a local reservoir of drug, adjuvants that stimulate the immune system, mucosal adjuvants, phosphazene and *Leishmania* elongation factor.

In accordance with the present invention, there is provided a method for eliciting an immune response against cancer in a patient, the method comprising administering a therapeutically effective quantity of the anti-cancer vaccine of the present invention to the patient.

The method in accordance with a preferred embodiment of the present invention, wherein the vaccine is administered by a route selected from the group consisting of locally, parenterally, peritoneally, mucosaly, dermaly, epidermaly, subcutaneously, transdermally, intramuscularly, nasaly, orally, topically, vaginally, rectally, intra-ocularly, intravenously, intra-arterially and by inhalation.

The method in accordance with a preferred embodiment of the present invention, wherein the vaccine is administered locally intramuscularly or subcuteanously.

In accordance with the present invention, there is provided a method for reducing tumor growth in a patient comprising administering a therapeutically effective quantity of the anti-cancer vaccine of the present invention to the patient to elicit an immune response in the patient, thereby reducing tumor growth.

The method in accordance with another embodiment of the present invention, further comprising administering at least one of radiotherapy, surgery and chemotherapy to the patient prior to vaccination.

The method in accordance with another embodiment of the present invention, further comprising administering at least one of radiotherapy, surgery and chemotherapy to the patient after vaccination.

The method in accordance with another embodiment of the present invention, further comprising administering a further adjuvant prior to vaccination.

The method in accordance with another embodiment of the present invention, further comprising administering a further adjuvant after vaccination.

In accordance with the present invention, there is provided a method for preventing tumor growth in a patient, comprising administering a prophylactic effective amount of the anti-cancer agent of the present invention to the patient to elicit an immune response in the patient, thereby preventing tumor growth.

For the purpose of the present invention the following terms are defined below.

The term "antigen" is intended to mean any molecule or cellular organism able to trigger an immune response from a patient and includes, without limitation, inactivated tumor cells.

The term "vaccine" is intended to mean a recombinant subunit, peptide vaccine, protein-based vaccines, DNA vaccines, whole-cell vaccine, mucosal vaccines, attenuated and live organism vaccines.

The term "adjuvant" is intended to mean a substance or substances that help eliciting an immune response against antigen by a patient.

The term "immunomodulator chemotherapeutic adjuvant" is intended to mean any chemotherapeutic agent capable of acting as an adjuvant as previously defined, in that they can elicit an immune response

probably due to their structural effect on the cell's cytoskeleton and/or microtubules.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B illustrate a comparison between a whole-cell vaccine of the prior art and the whole-cell vaccine as described in one embodiment of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided an anti-cancer vaccine composition comprising chemotherapeutic agents as adjuvant to elicit an improved immune response in a patient.

There is also provided a method to induce immune response against cancer in a patient and a method for preventing and/or reducing tumor growth in a patient.

What is being described in this application is the use of immunomodulator chemotherapeutic agents as adjuvants for anti-cancer vaccine to elicit immune response to a patient. It was found that it is possible to enhance immunogenicity of a vaccine by combining directly the immunomodulator chemotherapeutic agents with the vaccine in one single administration. The intramuscular route of administration is preferred but administration can also be done mucosally, orally, intranasally, intratracheally by inhalation, ocularlly, vaginally, intravenously, intra-arterially and rectally.

As shown in Example I, paclitaxel triggers the induction of MCP-1, a chemokine known to recruit dendritic cells (APC) at the injection site, a critical event for the induction of immune responses. Furthermore, Examples II and IV show that local injection of high concentration of paclitaxel is possible and do not provoke local toxicity that could be leading to immunosuppression. The fact that antibodies could be raised against an antigen in presence of paclitaxel is a sign that paclitaxel does not impede with the development of a normal immune reaction. In fact, it even helped to raise the immune response against a protein that always needs to be adjuvanted in order to be

immunogenic. This is unexpected since chemotherapeutic agents are associated with immunosupression due to death of immune cell. In fact, the collateral presentation of the whole-cell vaccine helps to break tolerance against cancer cells as shown in Examples III and V.

This unexpected observation lead to the immunological rationale for testing immune-modulating doses of chemotherapy in combination with an antigen. As described in Example II, paclitaxel increased the humoral response against ovalbumin. The testing of paclitaxel as improving a whole-cell vaccine in mice was performed as shown in Examples III and V. This principle is illustrated in Fig. 1. Sterilizing cancer cells with chemotherapeutic agents before immunization of the mice with a composition of cancer cells undergoing apoptosis and/or necrosis and chemotherapeutic agents protected the animals against a lethal tumor challenge. This was unexpected since chemotherapeutic agents are known to be toxic and to trigger systemic immunosuppression. Also, to have in one single composition a chemotherapeutic agent(s) and an antigen administered simultaneously simplifies the use of chemotherapeutic agents as potential vaccine adjuvants. The presence of the chemotherapeutic agent in the anti-cancer vaccine composition also renders moot or diminish the need to proceed to the inactivation by irradiation of the tumoral cells forming the anti-cancer vaccine before reinoculation. The drugs, especially paclitaxel, helps to raise an immune response by inducing a variety of genes known to be antigenic. In the case of melanoma cells, for which it is difficult to treat because they are known to be resistant to chemotherapy, these antibenic genes are usually differentiation genes known to play a role in the production of pigments in the skin. Melanine levels in B16 cells pretreated with paclitaxel increases and seems to be correlated with immunogenicity of the cells. Furhtermore, we noticed that doses of paclitaxel used to sterilize B16 cells *in vitro* do not correlated with the same doses used to immunized. Time of exposure and doses of paclitaxel needed to assure the sterilization *in vivo* are usually higher than what observed *in vitro*. This has some implications to avoid the reinoculation of cells in a distant injection site of a patient. If not properly

sterilized with the drugs, cancer cells may grow in a second site which is not desired.

Based on the Examples provided in this application, more particularly Examples V and VI, it is shown that chemotherapeutic agents can act as adjuvant in virtually any kind of antigenic compositions.

The chemotherapeutic agents that are intended to be included in the present application are any chemotherapeutic agents having an immunomodulator effect and/or eliciting an immune response from a patient, possibly due to their structural effect on the cell's cytoskeleton and/or microtubules. Examples of these chemotherapeutic agents include, but are not limited to, taxanes, Paclitaxel (Taxol™), cisplatine, doxorubicin, cyclophosphamide and 5-fluorouracil.

The compositions of the invention also comprise the use of various drug delivery systems or formulations. These could further improve the adjuvant property or a depot of the chemotherapeutic agents described above. The chemotherapeutic agents could be formulated in a form selected but not limited to the group consisting of a liquid solution, a powder, a polymer system, a biopolymer and natural polymer, a microparticle, a bioadhesive polymer, needleless delivery system, a scarification delivery system, and a tyne delivery system or formulated with any known and newly developed drug delivery systems (see Example IX)

Beside whole-cell vaccine, the type of vaccines that can be potentially be improved and benefit from chemotherapeutic agents-based adjuvants are DNA vaccine, conventional vaccines (protein and peptide-based), attenuated and live viruses or with already existing selected vaccine from the group consisting of but not limited to, EGF, Anti-idiotypic cancer vaccines, Gp75 antigen, GMK melanoma vaccine, MGV ganglioside conjugate vaccine, Her2/neu, Ovarex, M-Vax, O-Vax, L-Vax, STn-KHL theratope, BLP25 (MUC-1), liposomal idiotypic vaccine, Melacine, peptide antigen vaccines, toxin/antigen vaccines, MVA-based vaccine, PACIS, BCG vacine, TA-HPV, TA-CIN, DISC-virus and ImmuCyst/TheraCys. Rituxan, IDEC-C2B8, anti-

CD20 Mab, Panorex, 3622W94, anti-EGP40 (17-1A) pancarcinoma antigen on adenocarcinomas Herceptin, anti-Her2, Anti-EGFr, BEC2, anti-idiotypic-GD.sub.3 epitope, Ovarex, B43.13, anti-idiotypic CA125, 4B5, Anti-VEGF, RhuMAb, MDX-210, anti-HER-2, MDX-22, MDX-220, MDX-447, MDX-260, anti-GD-2, Quadramet, CYT-424, IDEC-Y2B8, Oncolym, Lym-1, SMART M195, ATRAGEN, LDP-03, anti-CAMPATH, ior t6, anti CD6, MDX-11, OV103, Zenapax, Anti-Tac, anti-IL-2 receptor, MELIMMUNE-2, MELIMMUNE-1, CEACIDE, Pretarget, NovoMAb-G2, TNT, anti-histone, Gliomab-H, GNI-250, EMD-72000, LymphoCide, CMA 676, Monopharm-C, ior egf/r3, ior c5, anti-FLK-2, SMART 1D10, SMART ABL 364, and ImmuRAIT-CEA.

Preferably the vaccine formulations will contain the adjuvant compositions of the present invention and an antigen or antigenic composition capable of eliciting an immune response against a human or animal pathogen. Antigen or antigenic compositions known in the art can be used in the compositions of the invention, including polysaccharide antigens, antigen or antigenic compositions derived from HIV-1, (such as gp120 or gp160), any of Feline Immunodeficiency virus, human or animal herpes viruses, such as gD or derivatives thereof or Immediate Early protein such as ICP27 from HSV1 or HSV2, cytomegalovirus (especially human) (such as gB or derivatives thereof), Varicella Zoster Virus (such as gpI, II or III), or from a hepatitis virus such as hepatitis B virus for example Hepatitis B Surface antigen or a derivative thereof, hepatitis A virus, hepatitis C virus and hepatitis E virus, or from other viral pathogens, such as Respiratory Syncytial virus (for example HSRV F and G proteins or immunogenic fragments thereof disclosed in U.S. Pat. No. 5,149,650 or chimeric polypeptides containing immunogenic fragments from HSRV proteins F and G, eg FG glycoprotein disclosed in U.S. Pat. No. 5,194,595), antigens derived from meningitis strains such as meningitis A, B and C, *Streptoccoccus Pneumonia*, human papilloma virus, *Influenza* virus, *Haemophilus Influenza B* (Hib), Epstein Barr Virus (EBV), or derived from bacterial pathogens such as *Salmonella*, *Neisseria*, *Borrelia* (for example OspA or OspB or derivatives thereof), or *Chlamydia*, or *Bordetella* for

example P.69, PT and FHA, or derived from parasites such as plasmodium or toxoplasma.)

In order to further improve the efficacy of the type of vaccines and compositions described above (vaccines adjuvanted with chemotherapeutic agents) can be combined before or after their administration with other therapeutic agents like 1) cytokines, 2) antibodies either monoclonal or polyclonal, 3) systemic chemotherapeutic agents (Example VII), 4) biological response modifiers including but not limited to interferon, and lymphokines such as IL-2, 5) hormone replacement therapy including but not limited to tamoxifen alone or in combination with progesterone. In addition, in order to further improve the efficacy of the type of vaccines and compositions described above (vaccines adjuvanted with chemotherapeutic agents) can be combined before or after their administration with other treatment modalities like 1) radiotherapy and 2) debulking surgery.

Other known adjuvants may be used before, after, simultaneously with the anti-cancer vaccine composition of the present invention which includes chemotherapeutic adjuvants (Example IX). The adjuvants are selected from, but not limited to, the group consisting of CpG nucleic acids, MF59, SAF, MPL, BCG and QS21 or selected from the group consisting of adjuvants that create a depot effect, adjuvants that stimulate the immune system, and adjuvants that create a depot effect and stimulate the immune system and mucosal adjuvants (alum; emulsion-based formulations including mineral oil, non-mineral oil, water-in-oil or oil-in-water-in oil emulsion, oil-in-water emulsions such as Seppic ISA series of Montanide adjuvants; PROVAX, saponins purified from the bark of the Q. saponaria tree; poly[di(carboxylatophenoxy)phosph- azene; derivatives of lipopolysaccharides, muramyl dipeptide and threonyl-muramyl dipeptide; OM-174; and *Leishmania* elongation factor, ISCOMs; SB-AS2; SB-AS4; non-ionic block copolymers that form micelles such as CRL 1005; and Syntex Adjuvant Formulation. Mucosal adjuvants are selected from the group consisting of CpG nucleic acids, Bacterial toxins, Cholera toxin, CT derivatives, CT B subunit; CTD53; CTK97; CTK104; CTD53/K63; CTH54; CTN107; CTE114; CTE112K; CTS61F;

CTS106; and CTK63, Zonula occludens toxin, zot, *Escherichia coli* heat-labile enterotoxin, Labile Toxin, LT derivatives, LT B subunit; LT7K; LT61F; LT112K; LT118E; LT146E; LT192G; LTK63; and LTR72, Pertussis toxin, PT-9K/129G; Toxin derivatives; Lipid A derivatives, MDP derivatives; Bacterial outer membrane proteins, outer surface protein A (OspA) lipoprotein of *Borrelia burgdorferi*, outer membrane protein of *Neisseria meningitidis*; Oil-in-water emulsions, Aluminum salts; and Saponins, ISCOMs, the Seppic ISA series of Montanide adjuvants, Montanide ISA 720; PROVAX; Syntext Adjuvant Formulation; poly[di(carboxylatophenoxy) phosphazene and *Leishmania* elongation factor.

One category of subjects intended for treatment according to the methods of the invention and the compositions include those having a cancer or are at risk of developing a cancer selected from the group consisting of basal cell carcinoma, bladder cancer, bone cancer, brain and CNS cancer, breast cancer, cervical cancer, colon and rectum cancer, connective tissue cancer, esophageal cancer, eye cancer, kidney cancer, larynx cancer, liver cancer, lung cancer, Hodgkin's lymphoma, Non-Hodgkin's lymphoma, melanoma, myeloma, leukemia, oral cavity cancer (e.g., lip, tongue, mouth, and pharynx), ovarian cancer, pancreatic cancer, prostate cancer, rhabdomyosarcoma, skin cancer, stomach cancer, testicular cancer, and uterine cancer. In preferred embodiments, the cancer to be treated may be selected from the group consisting of esophageal cancer, eye cancer, larynx cancer, oral cavity cancer (e.g., lip, tongue, mouth, and pharynx), skin cancer, cervical cancer, colon and rectum cancer, melanoma, stomach cancer, and uterine cancer.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

Example I

Paclitaxel induces MCP-1 in muscle tissue

Mice are injected intramuscularly (bilaterally) with varying doses of paclitaxel (100μM to 0.01 μM). Doses of paclitaxel are prepared by 10-fold serial dilutions, in saline, of a 10mM DMSO stock. The muscles are harvested, on dry ice, 6 hours following injection, and frozen at –80 °C. RNA is isolated using TRIZOL reagent (Gibco) as per manufacturers specifications. Ten micrograms of total RNA is reverse transcribed using Superscript RT (Gibco), 500ng oligodT primers (Gibco), and 250ng Random Hexamer primers (Gibco) for 20 minutes at room temperature, followed by 2 hours at 42°C.

MCP-1 is amplified from 2μl of reverse transcription reaction using Titanium PCR kit (Clontech). Amplification primers are as follows:

MCP-1 forward 93-114	AGGTCCCTGTCATGCTTCTGGG	SEQ ID NO:1
MCP-1 reverse 490-467	GGTTGTGGAAAAGGTAGTGGATGC	SEQ ID NO:2

The PCR reaction is performed for 30 cycles using 100pmole for each primer, with an annealing temperature of 68 degrees. The PCR products are analyzed on a 1.5% agarose gel, and have an expected size of 397bps.

Table 1

RT-PCR results

Conditions	Fold-induction over GAPDH
Paclitaxel (0.01, 1, 100 μM per injection)	2, 3, 6
Plasmid DNA (50 μg per injection)	4
LPS (10μg per injection)	3

The data are showing that paclitaxel can induce MCP-1 production to the same extent than CpG (plasmid DNA and LPS) and therefore showing that local inflammation is taking place in the muscle.

Example II

Paclitaxel increase humoral response against ovalbumin

The animals are injected intramuscularly into *tibialis anterior* muscles with 50 µl containing either 1 µg of ovalbumin formulated with or without paclitaxel at $10E^{-2}$ molar. Mice are sacrificed at various time points to perform the ELISA (2 weeks post-immunization). Ovalbumin-specific antibody responses are quantified by enzyme-linked immunosorbent assay (ELISA). Microtiter plates are coated overnight with 1 µg per well of ovalbumin. Plates are then washed once with PBS-Tween 0.01% and blocked for 2 hours at 37°C with PBS+1%BSA. The plates are then incubated with serial dilutions of mouse sera for 1 hour at 37°C. Plates are washed twice and subsequently incubated with peroxidase-conjugated goat anti-mouse IgG heavy and light chain antiserum (1:1000 in PBS-BSA 1%, Sigma #A-8924). Bound antibody is detected by incubation with ABTS substrate (Sigma #A-9941), followed by determination of absorbance at 405 nm. Anti-ovalbumine antibody levels are defined as the limiting dilution titer of serum producing an A405 greater than 0.2. The data are expressed as the percentage of responding mice and as the mean titers of responding mice.

The data shows the following titers after 2 weeks:

Table 2

Groups	% seroconversion (titers)
Ovalbumin	20% (1:20)
Ovalbumin + paclitaxel ($1X10^{-2}$ M)	90% (1:1500)
Ovalbumin + doxorubicin ($1X10^{-6}$ M)	25% (1:100)
Ovalbumin + cisplatin ($1X10^{-4}$ M)	40% (1:150)
Ovalbumin + cyclophosphamide ($1X10^{-5}$M)	50% (1:800)

Example III

Paclitaxel stimulates immune response and antitumor response against cancer cells.

Lewis Lung carcinoma cells (3LL) are cultured in RPMI supplemented with 10% FBS and penicillin and streptomycin in 5% CO_2. Parental 3LL cells are used in tumor challenge experiments. The cells for the challenge are administered by either the intravenous (i.v.) or subcutaneous (s.c.) route on both flanks, at a dose of $5x10^5$ cells/mouse. Survival is monitored for mice challenged by the i.v. route. C57Bl/6 (6-8-week-old females) are used throughout this study. The animals are kept in groups of 4 and fed *ad libidum*. Before each intramuscular injection, the animals are anesthetized with a mixed solution of ketamine/xylazine (10:1 w:w) at a concentration of 110 mg/Kg. The animals are injected intramuscularly into *tibialis anterior* muscles with 50 μl containing either $10E^5$ cells with or without paclitaxel ($10E^{-3}$ M) dissolved in DMSO. Mice are immunized on the same day than the tumor challenge. Mice are sacrificed when the tumors are getting close to a non-humane size (Institutional guidelines are followed). The results show that there is a tumor protection (40% survival) following a lethal tumor challenge only in the groups immunized with 3LL cells sterilized with paclitaxel while the animals immunized with 3LL alone all developed considerable tumors (0% survival).

Example IV

Histology on muscles injected with chemotherapeutic agents

Paclitaxel (PTX) (Bristol-Myers Squibb, Princeton, NJ), Doxorubicin (DOX) (Gensia, Irvine, CA), and Cisplatin (CIS) (Bristol-Myers Squibb) are diluted before injection. Cyclophosphamide (CTX) (Bristol-Myers Squibb) is diluted in sterile water before injection. PTX, CTX, Dox and CIS are injected intramuscularly (i.m.). (50μl containing various concentrations of chemotherapeutic agents). Muscles are harvested and sectioned to perform histological analysis to assess tissue damage. After 7 days, no sign of tissue damage is observed, only mild to moderate infiltration of lymphocytes is

observed which is in correlation with normal inflammation caused by the needle track.

Example V

PTX, CTX, Dox and CIS improve whole-cell vaccine.

PTX (Bristol-Myers Squibb, Princeton, NJ), DOX (Gensia, Irvine, CA), and CIS (Bristol-Myers Squibb) are diluted before injection. CTX (Bristol-Myers Squibb) is diluted in sterile water before injection. PTX, CTX, Dox and CIS are injected i.m. in combination with the whole-cell vaccine (B16 cells). On the day of vaccination, vaccine cells grown *in vitro* are trypsinized, washed three times in HBSS (pH 7.4; Life Technologies, Inc.), and counted. The cells are resuspended in HBSS at 107 cells/ml and sterilized with PTX, CTX, DOX and CIS. Eight-week-old C57Bl/6 mice are given three simultaneous 100-µl s.c. injections (right and left hind limbs and left arm) using a 1-ml tuberculin syringe with a 27-gauge needle. The mice in the vaccine group receive three simultaneous injections of 106 B16 cells. Tumor occurrence (shown as the tumor-free probability) or changes in tumor growth are monitored twice a week. Changes in tumor growth (mm^2) are determined by multiplying the two perpendicular diameters.

Table 3

Groups immunized i.m.	**Average time for the tumor to reach 1 cm^3 (Days)**
Non-treated	7
B16 + Gamma-irradiated (75 Gray)	11
B16 + paclitaxel ($1X10^{-5}M$)	20
B16 + doxorubin ($1x10^{-5}M$)	6
B16 + cyclophosphamide ($1X10^{-5}M$)	15
B16 + cisplatin ($1X10^{-5}M$)	13

The experiment shows that B16 cells killed with PTX, CTX, DOX, and CIS generate a significant delay of tumor growth as opposed to B16 cells sterilized by standard irradiation protocols. Compared to whole-cell vaccines killed by standard irradiation, the delay of tumor growth has doubled in mice immunized with B16 cells killed by chemotherapeutic agents. This data show that chemotherapeutic agent-based vaccines are better than irradiated whole-cell vaccines.

Example VI

Using PTX as adjuvants to improve DNA-based tumor vaccination

Melanoma is generally resistant to chemotherapy and radiation therapy. Its unique immunological properties give support for developing innovative new therapies via manipulation of the patient's own immune system. These new therapies use of whole-cell-based tumour vaccines, including autologous, whole-cell allogeneic and cytokine gene-modified vaccines, as well as tumour lysate vaccines, for active specific immunotherapy of melanoma. The stimulatory effect of CpG sequences in the plasmid DNA promotes the Th1 (cellular) immune response observed in DNA vaccines. It is possible to combine this effect with the described immune stimulatory effect of PTX in an anti-tumor DNA vaccination protocol. Briefly, plasmid encoding MAGE or β-galactosidase (negative control), 50μg in 50 μl saline is injected intramuscularly into 6-8 week-old female C57/Bl mice alone or with varying doses of PTX (1μM). Injections are performed twice at 2-week intervals, the initial injection at Day 0 and then at Day 14 alternatively into the anterior tibialis of the left and right legs. The vaccination protocol is tested in *in vivo* tumor models: for survival following i.v. challenge with B16 melanoma cells. In the survival assay, $2x10^5$ B16 melanoma tumor cells are injected intravenously to mice vaccinated with either MAGE plasmid or β-galactosidase plasmid (as above), Day 14 after immunizations. Each group consisted in 10 mice. This protocol is performed to evaluate survival of vaccinated animals (MAGE) versus negative control (β-galactosidase) animals. Survival is scored as percent surviving mice over time (days).

Table 4

Groups	Survival at day 40 (%)
untreated	0
Immunized with Bgal	10
Immunized with MAGE + paclitaxel (100 μM)	50
Immunized with MAGE alone	30

The experiment showed that the mice immunized with the plasmid encoding MAGE formulated with chemotherapeutic agents survived the longest as compared to untreated mice and mice treated with non formulated naked plasmid DNA or treated with a non-relevant antigen like β-galactosidase. The factor of improvement is at least 2- to 3-fold better.

Example VII

Use of other adjuvant or drug delivery system to improve further chemotherapeutic agent-based vaccine

The experiment described in Example III is repeated using exopolysaccharide-formulated paclitaxel (Profilactis™). The formulation is used to deliver paclitaxel and to sterilize.. The microspheres are generated by spray-drying and used to deliver paclitaxel and to sterilize the cancer cells before immunization. Compared to DMSO, exopolysaccharide-formulated paclitaxel is more efficient to enhance survival of mice challenged with cancer cells. All mice survived a lethal tumor challenged.

Example VIII

Evaluation of the paclitaxel toxicity on C2C12 cells

C1C12 cells were plated in a 12-wells plate at $7X10^4$ cells/ml/well in complete DMEM (10 FBS, 1% Strepto-pen, 1% Hepes) and incubated O/N at 37°C under 5% CO2 atmosphere. Twenty-four hours later, normal DMEM was changed for DMEM containing 2% Horse serum, 1% Strepto-pen and 1% Hepes. The medium was then changed everyday with the complete DMEM 2% Horse serum. For an extra 7 days, complete DMEM 10% Horse serum was added to the cells. After about 1 week, all cells were differentiated and treated with various concentrations of paclitaxel. Even at a high concentration of paclitaxel, differentiated cells now forming elongated muscular fibers, were not affected by any treatment probably due to the non-dividing state of the cells. This observation support the fact that drugs can be injected in the muscle without necessarily causing damages or cell death.

Example X

Prophylactic immunization with cancer cells pretreated with paclitaxel or γ-irradiated

Tumor cells were trypsinized and washed 3 times with PBS. Cells were gamma-irradiated with 75 Gy using a Cs^{137} source and injected i.m. or kept in culture for further in vitro analysis. For induction of apoptosis (cell death), tumor cells were exposed to Profilactis™ (paclitaxel 100μM + 0.1% exopolysaccharides) for 4 hours before being used for immunization. Complete cell death was confirmed by trypan blue staining before injection and subsequent cell culturing. Ten mice were vaccinated twice (days 0 and 14) with B16 cells pretreated with Profilactis™ and with γ-irradiated cells. After 3 weeks, mice were challenged with $1X10^6$ viable B16 tumor cells. Mice were monitored for survival and presence of tumors over time.

Table 5

Tumor-free mice over time (%)

Days	$1x10^4$ cells pretreated with Paclitaxel	$1X10^5$ cells treated with Paclitaxel	$1X10^4$ γ-irradiated cells	$1X10^5$ γ-irradiated cells
20	100	100	100	100
40	100	100	60	80
80	100	100	20	40
160	80	100	0	0

Example XI

Therapeutic immunization with cancer cells pretreated with paclitaxel or γ-irradiated

Tumor cells were trypsinized and washed 3 times with PBS. Cells were gamma-irradiated with 75 Gy using a Cs^{137} source and injected i.m. or kept in culture for further in vitro analysis. For induction of apoptosis (cell death), tumor cells were exposed to Profilactis™ (paclitaxel 100μM + 0.1% exopolysaccharides) for 4 hours before being used for immunization. Complete cell death was confirmed by trypan blue staining before injection and subsequent cell culturing. Ten mice were challenged (i.v.) with $1x10^6$ viable B16 tumor cells on day 0 and treated on day 7 with B16 cells pretreated with Profilactis™ and with γ-irradiated cells. Mice were monitored for survival and presence of tumors over time.

Table 6

Tumor-free mice over time (%)

Days	$1x10^4$ cells pretreated with Paclitaxel	$1X10^5$ cells treated with Paclitaxel	$1X10^4$ γ-irradiated cells	$1X10^5$ γ-irradiated cells
20	100	100	70	100
40	100	100	0	50
80	70	100	0	0
160	60	70	0	0

Example XII

Prophylactic immunization with cancer cells pretreated with paclitaxel or γ-irradiated in cyclophosphamide-immunocompromised mice

Tumor cells were trypsinized and washed 3 times with PBS. Cells were gamma-irradiated with 75 Gy using a Cs^{137} source and injected i.m. or kept in culture for further in vitro analysis. For induction of apoptosis (cell death), tumor cells were exposed to Profilactis™ (paclitaxel 100μM + 0.1% exopolysaccharides) for 4 hours before being used for immunization. Complete cell death was confirmed by trypan blue staining before injection and subsequent cell culturing. In one group, cyclophosphamide was used at 100μg/ml injected 3 times a week by the I.P. route to induce immunosuppression as measured by an hematocrit evaluation. Ten mice were vaccinated twice (days 0 and 14) with B16 cells pretreated with Profilactis™, with γ-irradiated cells and with γ-irradiated cells formulated with MPL. After 3 weeks, mice were challenged with $1x10^6$ viable B16 tumor cells. Mice were monitored for survival and presence of tumors over time.

Table 7

Tumor-free mice over time (%)

Days	$1x10^5$ cells pretreated with Profilactis™	$1X10^5$ cells pretreated with Profilactis™ but in cyclophosphamide-immunocompromised mice	$1X10^5$ γ-irradiated cells	$1X10^5$ γ-irradiated cells and formulated with MPL
20	100	100	100	100
40	100	100	70	60
80	100	70	30	20
160	80	50	0	0

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. An anti-cancer vaccine composition comprising an antigen in association with an effective amount of at least one immunomodulator chemotherapeutic adjuvant eliciting an immune response in a patient and a pharmaceutically acceptable carrier.

2. The anti-cancer vaccine of claim 1, wherein said vaccine is selected from the group consisting of whole-cell vaccine, DNA vaccine, protein-based vaccine, peptide-based vaccine and attenuated organism vaccine.

3. The anti-cancer vaccine of claim 1, wherein said antigen is inactivated tumor cells.

4. The anti-cancer vaccine of claim 1, wherein said antigen is tumor cells inactivated by said chemotherapeutic agent.

5. The anti-cancer vaccine of claim 3, wherein said tumor cells are inactivated by radiotherapy and/or chemotherapy.

6. The anti-cancer vaccine of claim 3, wherein said tumor cells are inactivated by radiotherapy.

7. The anti-cancer vaccine of any one of claims 1-6, wherein said vaccine is eliciting an immunoprotective response against a cancer selected from the group consisting of basal cell carcinoma, bladder cancer, bone cancer, brain cancer, CNS cancer, breast cancer, cervical cancer, colon cancer, rectum cancer, connective tissue cancer, esophageal cancer, eye cancer, kidney cancer, larynx cancer, liver cancer, lung cancer, Hodgkin's lymphoma, non-Hodgkin's lymphoma, melanoma, myeloma, leukemia, oral cavity cancer, ovarian cancer, pancreatic cancer, prostate cancer, rhabdomyosarcoma, skin cancer, stomach cancer, testicular cancer, neoplasia and uterine cancer.

8. The anti-cancer vaccine of any one of claims 1-6, wherein said vaccine is eliciting an immune response against a cancer selected from the group consisting of bladder cancer, prostate cancer, melanoma, breast cancer, colon cancer, lung cancer, liver cancer, lymphoma, myelona, leukemia and ovarian cancer.

9. The anti-cancer vaccine of claim 1, wherein said chemotherapeutic adjuvant is a taxane.

10. The anti-cancer vaccine of claim 1, wherein said chemotherapeutic adjuvant is selected from the group consisting of Cyclophosphamide, Doxorubicin, Cisplatin, Paclitaxel and 5-fluorouracil.

11. The anti-cancer vaccine of any one of claim 1-10, further comprising a therapeutically effective amount of a therapeutic agent.

12. The anti-cancer vaccine of claim 11, wherein said therapeutic agent is selected from the group consisting of cytokine, antibody, systemic chemotherapeutic agent, biological response modifier and hormones.

13. The anti-cancer vaccine of claim 12, wherein said antibody is a monoclonal antibody.

14. The anti-cancer vaccine of claim 12, wherein said biological response modifier is selected from the group consisting of interferon and lymphokine.

15. The anti-cancer vaccine of claim 14, wherein said lymphokine is IL-2.

16. The anti-cancer vaccine of any one of claims 1-15, further comprising a non-chemotherapeutic adjuvant selected from the group consisting of polysaccharides, CpG nucleic acids, MF59, SAF, MPL and QS21.

17. The anti-cancer vaccine of any one of claims 1-16, further comprising a non-chemotherapeutic adjuvant selected from the group consisting of adjuvants that create a local reservoir of drug, adjuvants that stimulate the immune system, mucosal adjuvants, phosphazene and *Leishmania* elongation factor.

18. A method for eliciting an immune response against cancer in a patient, said method comprising administering a therapeutically effective quantity of the anti-cancer vaccine of any one of claims 1 to 17 to said patient.

19. The method of claim 18, wherein said vaccine is administered by a route selected from the group consisting of locally, parenterally, peritoneally, mucosaly, dermaly, epidermaly, subcutaneously, transdermally, intramuscularly, nasaly, orally, topically, vaginally, rectally, intra-ocularly, intravenously, intra-arterially and by inhalation.

20. The method of claim 18, wherein said vaccine is administered intramuscularly or subcutaneously.

21. A method for reducing tumor growth in a patient comprising administering a therapeutically effective quantity of the anti-cancer vaccine of any one of claims 1 to 17 to said patient to elicit an immune response in said patient, thereby reducing tumor growth.

22. The method of claim 21, wherein said vaccine is administered by a route selected from the group consisting of locally, parenterally, peritoneally, mucosaly, dermaly, epidermaly, subcutaneously, transdermally, intramuscularly, nasaly, orally, topically, vaginally, rectally, intra-ocularly, intravenously, intra-arterially and by inhalation.

23. The method of claim 21, wherein said vaccine is administered intramuscularly or subcutaneously.

24. The method of claim 21, further comprising administering at least one of radiotherapy, surgery and chemotherapy to said patient prior to vaccination.

25. The method of claim 21, further comprising administering at least one of radiotherapy, surgery and chemotherapy to said patient after vaccination.

26. The method of claim 21, further comprising administering a further adjuvant prior to vaccination.

27. The method of claim 21, further comprising administering a further adjuvant after vaccination.

28. The method of any one of claim 26 and 27, wherein said further adjuvant is selected from the group consisting of CpG, nucleic acids, MF59, SAF, MPL and QS21.

29. The method of any one of claim 26 and 27, wherein said further adjuvant is selected from the group consisting of adjuvants that create a depot effect, adjuvants that stimulate the immune system, mucosal adjuvants, phosphazene and *Leishmania* elongation factor.

30. A method for preventing tumor growth in a patient, comprising administering a prophylactic effective amount of the anti-cancer vaccine of any one of claims 1 to 17 to said patient to elicit an immune response in said patient, thereby preventing tumor growth.

31. The method of claim 30, wherein said vaccine is administered by a route selected from the group consisting of locally, parenterally, peritoneally,

mucosaly, dermaly, epidermaly, subcutaneously, transdermally, intramuscularly, nasaly, orally, topically, vaginally, rectally, intra-ocularly, intravenously, intra-arterially and by inhalation.

32. The method of claim 30, wherein said vaccine is administered intramuscularly or subcutaneously.

33. The method of claim 30, further comprising administering at least one of radiotherapy, surgery and chemotherapy to said patient prior to vaccination.

34. The method of claim 30, further comprising administering at least one of radiotherapy, surgery and chemotherapy to said patient after vaccination.

35. The method of claim 30, further comprising administering a further adjuvant prior to vaccination.

36. The method of claim 30, further comprising administering a further adjuvant after vaccination.

37. The method of any one of claim 35 and 36, wherein said further adjuvant is selected from the group consisting of CpG, nucleic acids, MF59, SAF, MPL and QS21.

38. The method of any one of claim 35 and 36, wherein said further adjuvant is selected from the group consisting of adjuvants that create a depot effect, adjuvants that stimulate the immune system, mucosal adjuvants, phosphazene and *Leishmania* elongation factor.

Chapitre 10 : Applications cosméceutiques des MPM.

Contenu du chapitre : manuscrit déposé en révision.

Lemieux, P., Boutin, Y., Roy, M., Goyette, P. Simard, E. 2005. A novel whey protein-based ingredient as a natural and topical cosmeceutical to nourish skin and protect epidermal homeostasis. Letter to Editor. Journal of Dermatological Science. 7 pages.

Le 10 janvier 2005, la secrétaire de l'éditeur en chef nous informait que le manuscrit contenait trop de mots pour sa publication sous cette forme. Il fut modifié pour être soumis de nouveau.

J'ai eu une contribution majeure à la revue de littérature, une contribution moyenne à la rédaction et j'ai effectué la révision des brevets existants pour la prise du brevet des MPM présenté au chapitre 4 (l'utilisation des MPM pour des applications cosmétiques et cosméceutiques y est protégée).

10.1 Manuscrit depose en révision

Lemieux, P., Boutin, Y., Roy, M., Goyette, P. Simard, E. 2005. A novel whey protein-based ingredient as a natural and topical cosmeceutical to nourish skin and protect epidermal homeostasis. Letter to Editor. Journal of Dermatological Science. 7 pages.

Letter to the editor

A novel whey protein-based ingredient as a natural and topical cosmeceutical to nourish skin and protect epidermal homeostasis.

The novel cosmeceutical ingredient described herein originates from a fermentation process of whey proteins using a proprietary friendly bacteria from the *Lactobacillus* species (patent pending). The resulting product, called commercially *Cosmelactis*™, is 100% natural and technically described as a Malleable Protein Matrix (MPM). MPM has the appearance of a beige colored cream with a sweet odor exhibiting a fatty acid-like texture that has a high content of moisture (80% w:v). The texture comes from aggregated whey proteins/peptides, calcium, lactic acid bacteria and its corresponding exopolysaccharides. Following a detailed biochemical analysis, we concluded that MPM was comprising several ingredients recognized and known to exert biological activities on skin. We thus sought to tabulate its potential topic activity solely based on its composition and test its topical activity in experimental systems. The origin of its biological activity are likely to come from its 3 major components (water essential for moisturizing, whey proteins/peptides and bacterial components (bacteria and exopolysaccharide) but also from a synergistic/additive effect of several minor components. Whey proteins/peptides have been used by several companies in cosmectic product such as Immunotec, Biosana and Estee Lauder for their immunomodulatory, antioxidative, anti-aging and collagen booster properties (1-3). In MPM, these properties associated with whey proteins/peptides might be potentiated further by the presence of lactic acid bacteria used for the fermentation. By themselves, those friendly bacteria and their capsular exopolysaccharides have been reported to benefit individuals suffering from skin problems such as psoriasis, atopic dermatitis and eczema (4-6). Thus, Table I A summarizes in more details the cosmeceutical ingredients and nutrients contained in MPM either generated during the fermentation (endogenous) or added to make MPM (exogenous) along with their corresponding topical activity on skin.

In Table IB, we are showing the most important observations obtained following microarray genomic profiling assays (*Galactis*™ system) used to assess the effect of the MPM on keratinocytes. Among the most interesting observation, we found that MPM induced several genes in the signalization pathways of TGFβ. This suggest that MPM may exhibit skin repair and maintain topical homeostasis (7). Among them, 7 genes were activated by the MPM; keratins, collagens, integrins, transglutaminase 2, furine, thrombospondin and and TGFβ-i (keratoepithelin). It is important to note that furine and thrombospondin are both needed to convert TGFβ in its active biological form. This suggests that latent TGFβ which is the most abundant growth factor in liquid whey, was activated by lowering the pH, rising the temperature and by a protease activity, conditions all encountered during the process of fermentation leading to the production of MPM. Furthermore, we found that genes associated with an immune modulation were also activated such as Defensin 2, TNFα, and IL-6 as mentioned in Table IB. This analysis suggests that proteins, polypeptides and peptides generated and recovered in MPM are still biologically active. Furthermore, they exert their activity differently than the isolated components also present in MPM such as calcium and microbial exopolysaccharides (bacteria) since both components did not triggered in the same fashion Defensin 2, TNFα and IL-6 (Figure 1A). It is important to

note that TNFα and IL-6 are cytokines described to promote wound healing.

Before proceeding to the evaluation of MPM on human skins, we ascertained that MPM showed no signs of irritancy and allergenicity as tested in a Human Repeat Insult Patch Test monitored by a certified dermatologist. Fifty volunteers were tested and none reported discomfort or skin rashes. Topical activity of MPM was thereafter monitored by means of sensitive and meaningful biomarkers of skin integrity such as prostaglandin E2 (PGE2) and cyclooxygenase 2 (Cox-2), both guardians of the degree of epithelial homeostasis (8). MPM was compared to a nonsteroidal anti-inflammatory drug (Ibuprofen), a non selective inhibitor of Cox-2 and to an expensive commercial product with a popular brand name, Regenerist of Olay. The goal was to monitor the effect of MPM on Cox-2 expression and also basal and induced levels of PGE2 following a solar and environmental ultraviolet (UVB)-induced insult. In these experiments MPM was used prophylactically or therapeutically on human skin. In all experimental conditions tested, MPM showed a significant inhibitory effect on both biomarkers of integrity. The expression of Cox-2 was reduced following an MPM exposure as shown in Figure 1A. Following this observation, we sought to explain the inhibition of Cox-2 by exploring the genes that could differentiate and explain this activity and rule out a potential negative affect. We found that, in addition to reducing expression of Cox-2, that the expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), a prostaglandin-degrading enzyme that physiologically and naturally antagonizes Cox-2 was enhanced (data not shown). It was recently reported by others that epithelial cancers further target the prostaglandin biogenesis pathway by ubiquitously abrogating expression of 15-PGDH. In interrogating the mechanism for 15-PGDH expression loss in epithelial cancers, Yan *et al.* reported that 15-PGDH expression is directly controlled and strongly induced by activation of the TGFβ tumor suppressor pathway (9). These findings thus delineate an enzymatic pathway that induces epithelial cancer suppression, a pathway that is probably activated by TGFβ and mediated by MPM as mentioned above. To push further this observation, we measured the consequence of Cox-2 reduction on the biosynthesis of PGE2 in human keratinocytes and human skin exposed to MPM. We found that MPM was reducing basal levels of PGE2 by about 75% after a 24-hour exposure in absence of external insult (Figure 1B). In a situation where UVB was used as an environmental insult, MPM prevented the induction of PGE2 suggesting a protective role of MPM (Figure 1C). Finally, when used either before or after the UVB exposure, MPM exhibited the same protective activity and even a therapeutic activity as demonstrated when MPM was applied after the UVB exposure (Figure 1D). It is also important to note that Regenerist was less efficacious than MPM even used non-diluted. MPM could not be tested in a non diluted state in this experimental setting but we believe that its topical activity would even greater be increased if used non-diluted. Taken together, these data suggest that MPM exhibit an interesting biological functionality on human skin.

In terms of technological functionality, MPM reduce preparation and formulation time since it is available hydrated as bulk material. MPM can be lyophilized to give a beige powder that can rehydrated to form and allow stable emulsions enabling flexibility for cosmetologists. MPM turns out to be versatile and might become a valuable product that could be viewed from 2 perspectives: 1) as a

synergistic or additive biovehicle of a variety of actives principles like vitamins, growth factor and drugs having dermatological activity since it allows for the incorporation of both hydrophilic and hydrophilic molecules and 2) as a cosmeceutical ingredient exhibiting biological properties for skin care and maintenance, repair, skin regeneration, and anti-aging in products like skin care, sunscreens, which include baby creams, emollient creams, cold creams, conditioning creams, protective creams, sunscreen lotion, lip balm, lipsticks, eyeshadows and bar soaps.

In conclusion, this product may answer a new trend which is that cosmetic and skin care companies both in the U.S. and abroad are beginning to address the older consumer's desire to marry cosmeceutical and nutraceuticals to smooth and improve skin. Primarily composed of ingredients that are natural ie. Food-grade ingredients Generally Recognized As Safe, MPM may address and offer the possibility to develop an ingestible cosmetic or a dietery supplement for skin care (to be continued). As demonstrated by its ability to induce the expression of several genes of biological significance involved in immunomodulation, structure, and repair of epithelial cells leading to a protective topical activity, MPM might become a valuable cosmeceutical ingredient to nourish skin and protect epidermal homeostasis. Novel and natural products are in demand to provide with anti-aging and help the skin's ability to withstand pollution and environment factors. Cleopatra would be happy to learn that there is now a possible alternative to rejuvenating goat milk baths.

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References

1. Bounous G. Whey protein concentrate (WPC) and glutathione modulation in cancer treatment. Anticancer Res 2000 Nov-Dec;20(6C):4785-92.

2. Robinson DS, Larche M, Durham SR. J Clin Invest. 2004 Nov;114(10):1389-97. Tregs and allergic disease.

3. Rayner TE, Cowin AJ, Robertson JG, Cooter RD, Harries RC, Regester GO, Smithers GW, Goddard C, Belford DA. Mitogenic whey extract stimulates wound repair activity in vitro and promotes healing of rat incisional wounds. Am J Physiol Regul Integr Comp Physiol. 2000 Jun;278(6):R1651-60.

4. Perdigon G, Fuller R, Raya R. Lactic acid bacteria and their effect on the immune system. Curr Issues Intest Microbiol 2001 Mar;2(1):27-42.

5. Tzianabos AO. Polysaccharide immunomodulators as therapeutic agents: structural aspects and biologic function. Clin Microbiol Rev 2000 Oct;13(4):523-33.

6. Perdigon G, Alvarez S, Rachid M, Aguero G, Gobbato N. Immune system stimulation by probiotics. J Dairy Sci 1995 Jul;78(7):1597-606.

7. Beanes SR, Dang C, Soo C, Ting K. Skin repair and scar formation: the central role of TGFb. Expert Rev Mol Med. 2003 Mar 21;2003:1-22.

8. Lee L. J. *et al.* Cyclooxygenases in the skin: pharmacological and toxicological implications. Tox. Applied Pharmacology. 192 (2003) 294-306.

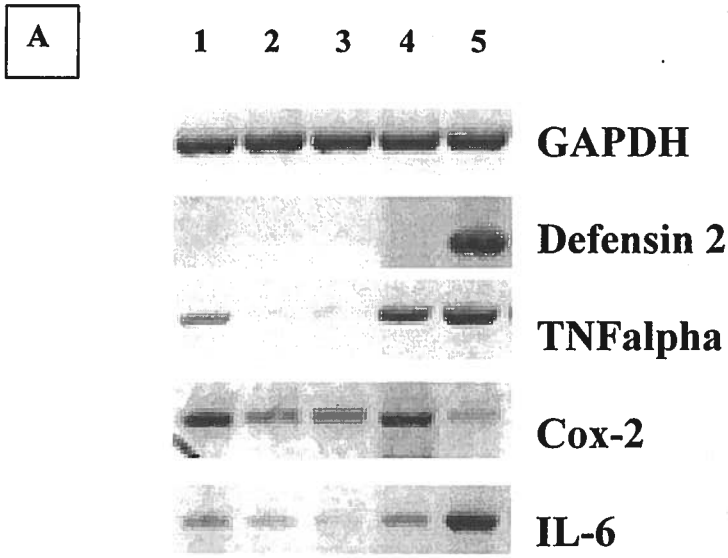
9. Yan M, Rerko RM, Platzer P, Dawson D, Willis J, Tong M, Lawrence E, Lutterbaugh J, Lu S, Willson JK, Luo G, Hensold J, Tai HH, Wilson K, Markowitz SD. 15-Hydroxyprostaglandin dehydrogenase, a COX-2 oncogene antagonist, is a TGF-beta-induced suppressor of human gastrointestinal cancers. Proc Natl Acad Sci U S A. 2004 Dec 14;101(50):17468-73.

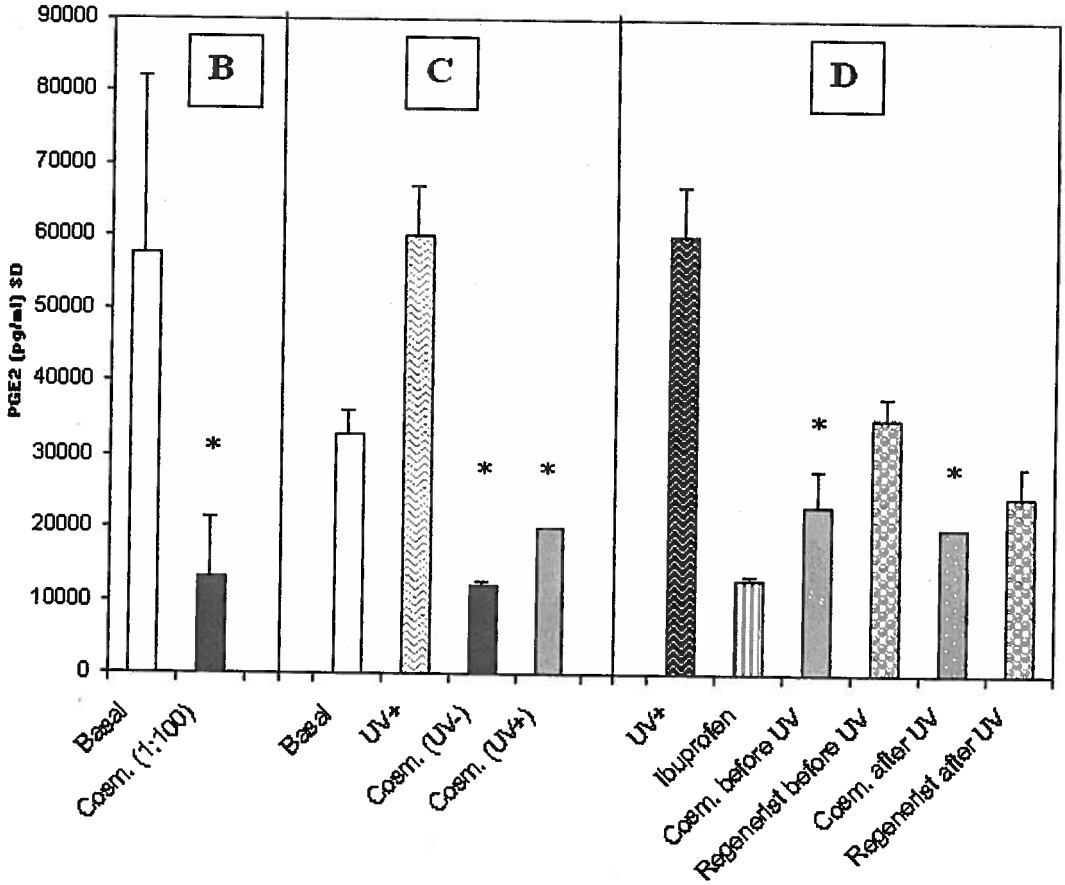
10. Lansdown AB. Calcium: a potential central regulator in wound healing in the skin. Wound Repair Regen. 2002 Sep-Oct;10(5):271-85.

Table I: Cosmeceutical ingredients, nutrients and measurable effects of MPM.

A - Cosmeceutical ingredients and nutrients present in MPM	
Name	**Roles**
Alpha hydroxy acids • Lactic acid	• Enhances epidermal shedding, decreases signs of aging, smoother skin, increases collagen.
Growth factors and Hormones • IGF1 • TGFβ or TGFβ-like activity	• Promotes wound healing. • Stimulates skin growth and repair, promotes wound healing.
Antioxidants and vitamins • GSH-like activity, Cysteine, -SH groups • Niacin (Vitamin B3)	• Protects against oxidative stress and UV-induced immunosuppression. • Increases epidermal turnover and exfoliation, suppresses of UV-B photocarcinogenesis.
Antimicrobial and anti-inflammatory agents • Lactoferrin • Lactoperoxidase	• Possesses bactericidal activity, suppresses free radical-mediated damage, influences cutaneous immune and inflammatory processes.
Minerals • Calcium (10)	• Essential for proper skin formation and homeostasis.
Phospholipids	• Moisturizer, skin conditioning agent.
B - Genes induced by MPM in human keratinocytes (from microarray gene profiling analyses – Galactis™ system)	
Gene	**Roles**
Antimicrobial agents • Defensin 2	• Broad spectrum antimicrobial peptide, cutaneous immunity.
Cytokines and growth factor • IL-6, TNFα, TGFβi (keratoepithelin)	• Role in wound healing.
Structural proteins • Integrins, thrombospondin • Keratins, collagens • Transglutaminase 2, Furine	• Structure and strength. • Skin structure and strength. • Formation of the stratum corneum and wound healing

Figure 1: Gene expression and production of PGE2 in human skin following different conditions. **A)** Gene expression following a 16-hour exposure with the following, Lane 1, Control; lane 2, Lipopolysaccharide (10 ug/ml); lane 3, Exopolysaccharide (10ug/ml); lane 4, Calcium (1.5mM); lane 5, MPM diluted in PBS 1:1000 on sub-conlfuent human keratinocytes (HEKA – Cascade Biologics), **B)** PGE2 production measured by ELISA (Cayman Cat 514010) after 24 hours in the EPI-200 model (Mattek) composed mainly of non- and differentiated keratinocytes, **C)** PGE2 levels at 6 hours in the 3-dimensional EpiDermFT model (Mattek) following a 15-minute UVB exposure. MPM was used at a dilution of 1:10 and applied before the UVB exposure, **D)** PGE2 levels at 6 hours in the 3-dimensional EpiDermFT model (Mattek) following a 15-minute UVB exposure. MPM was used at a dilution of 1:10 and applied before and after the UVB exposure. Ibuprofen was used at 50uM and applied before the UVB exposure while Regenerist of Olay was used non-diluted. * $p<0.05$ compared to control conditions.

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Regeneriat after UV

Annexe 2 : Note explicative sur la protection d'une invention

Une invention peut consister en une nouvelle application d'une molécule existante, une nouvelle méthode de production de cette molécule ou encore, une nouvelle molécule produite pour les même applications. Ainsi, un brevet peut couvrir un ou plusieurs aspects de l'invention comme la méthode de production, la description du produit ou de ses applications. Cela permet généralement un retour sur l'investissement effectué en recherche et développement. De plus, pour être brevetables, les inventions doivent : être nouvelles, impliquer une activité inventive et être susceptibles d'applications industrielles. Le critère de nouveauté s'apprécie par rapport à un état de la technique constitué notamment par tout ce qui a été rendu accessible au public avant la date de dépôt de la demande de brevet (principalement les articles scientifiques et brevets), y compris les divulgations antérieures faites par le breveté. Une invention est considérée comme impliquant une activité inventive si, pour un homme de métier, elle ne découle pas d'une manière évidente de l'état de la technique (ce que l'on appel le caractère non-évident de l'invention). Une invention est susceptible d'applications industrielles si son objet peut être fabriqué ou utilisé dans tout genre d'industrie, y compris l'agriculture. Ce critère exclut en particulier les méthodes de traitement chirurgical thérapeutique ou de diagnostic appliquées au corps humain ou animal.

Le brevet a pour but de protéger l'utilisation commerciale de l'invention durant une période de temps définie. La protection du brevet permet entre autre :

- d'interdire à un tiers de fabriquer ou de commercialiser, dans le ou les pays où l'invention est brevetée, le produit défini dans le brevet,
- d'organiser l'exploitation de l'innovation brevetée par des partenaires, dans le cadre de licences,
- de capitaliser le fruit des travaux de recherche, en vue d'une valorisation,
- d'identifier le patrimoine intellectuel de la société, et de déterminer le territoire technologique de l'entreprise par rapport à celui de ces concurrents.

En résumé, la protection par brevet implique que l'invention ne peut être réalisée, utilisée, distribuée ou vendue sans le consentement du titulaire du brevet. Le non-respect des droits des brevets est sanctionné par une action devant les tribunaux. Un brevet ne produit des effets juridiques que dans le pays où il est déposé, ou dans les pays où des extensions internationales ont été déposées.

En vue d'obtenir un brevet, l'inventeur ou son ayant droit doit effectuer le dépôt d'une demande de brevet dans laquelle l'invention doit être décrite de façon suffisamment claire et complète pour qu'un homme du métier puisse l'exécuter. Cette description est accompagnée de revendications qui définissent l'objet de la protection. Ces revendications doivent être claires et concises et se fonder sur la description. La revue de littérature effectuée doit couvrir toutes les facettes possibles de l'invention de façon à s'assurer qu'en aucun point l'invention ne contrevient à des inventions existantes ou encore, que certaines de ces facettes sont du domaine public. Cette revue de littérature exhaustive n'est pas décrite en entier à l'intérieur du brevet. Seulement les articles et brevets portant sur des sujets très apparentés seront cités afin de décrire comment l'invention s'en distincte.

Dans un délai de 12 mois à compter de la requête, le bureau des brevets établit un rapport de recherche préliminaire. Si ce rapport de recherche cite des antériorités pertinentes, le demandeur doit, sous peine de rejet, expliquer les différences et la pertinence de son invention par rapport aux antériorités soulevées, déposer des nouvelles revendications ou présenter des observations à l'appui des revendications maintenues. Le dépôt international du brevet atteste que cette étape fut franchie avec succès. Au terme d'un délai de 18 mois à compter de sa date de dépôt ou de sa date de priorité, le bureau des brevets assure la publication de la demande de brevet et met à la disposition du public le dossier de la demande. Les brevets en phase internationale fournis dans le présent document sont les formes publiées de ces demandes.