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Criblage, évaluation et modélisation mathématique de l'effet antimicrobien de composés d'origine naturelle pour le contrôle de *Listeria monocytogenes in situ*

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Thèse présentée pour l'obtention du grade de
Philosophiae doctor (Ph.D.) en biologie

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Résumé

Dans un contexte d'une population sans cesse grandissante et de la diminution des terres arables, la production alimentaire doit être de plus en plus efficace pour assurer un meilleur rendement. Avec la mondialisation de l'alimentation, les distances parcourues par les denrées sont augmentées, ce qui accroît les risques de contamination et de dégradation. Pour pallier à cette problématique, il est primordial de trouver de nouvelles technologies afin d'accroître la salubrité alimentaire. De plus, une meilleure éducation des consommateurs les ont poussé à s'intéresser à la nourriture qu'ils mangent. De ce fait, ils demandent maintenant des aliments sans additif et aussi ils aiment reconnaître le nom des matières premières sur la liste des ingrédients. Ces nouvelles demandes de la part des consommateurs amènent de nouveaux défis pour les industriels produisant ces aliments. De ce fait, le but de cet ouvrage était de développer de nouvelles solutions antimicrobiennes d'origines naturelles afin d'améliorer la sécurité des aliments prêts à manger.

Les résultats obtenus lors de ce travail ont permis de mettre en évidence les propriétés antimicrobiennes des huiles essentielles et des métabolites bactériens tels les acides organiques et les bactériocines. En premier lieu, un criblage *in vitro* a permis de sélectionner les meilleures molécules. Par la suite, la mise au point d'un modèle alimentaire de charcuteries représentant une contamination par la bactérie *Listeria monocytogenes* a permis de faire un second criblage *in situ*. Toutefois, avec l'augmentation de la popularité des aliments prêts à manger, il est possible que ces solutions ne s'appliquent pas à toutes les situations. De ce fait, la modélisation mathématique est un des moyens pouvant être utilisé pour prédire la sécurité des aliments.

Ainsi, la réalisation d'un modèle mathématique prédisant la croissance de *L. monocytogenes* dans les charcuteries prêtes à manger répondait à cette problématique. Ce modèle est un outil disponible pour les industriels leur permettant de minimiser le nombre de formulations alimentaires à tester. De plus, ce modèle a permis de mettre en évidence des interactions entre les molécules antimicrobiennes et certains paramètres intrinsèques de l'aliment.

Étudiant	Directrice de recherche

Remerciements

Tout d'abord, j'aimerais remercier ma directrice de recherche, Prof. Monique Lacroix, pour m'avoir donné ma chance et m'avoir permis de découvrir ma passion pour la recherche. Merci à la compagnie BSA et à ses membres (Nathalie, Jocelyn et Claude) qui ont mis temps et argents dans l'élaboration de ces recherches. Merci à tous les membres, passés et présents, du Laboratoire RESALA. Mathieu, Stéphane, les employés de la compagnie Nordion, tous mes amis, Tom, ma mère Carole et ainsi que ma copine Isabelle. Enfin, sans l'appuie financier accordé par le CRSNG, le FQRNT et le CRIBIQ à ce projet cette recherche n'aurait pu voir le jour.

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Liste des abréviations

ADA	*Agar diffusion assay*
ATCC	American type culture collection
ATP	Adénosine triphosphate
BNF	β-naphthoflavone
CFR	*Code federal regulation*
CFU	*Colony forming unit*
CMC	Carboxy methyle cellulose
CMI	Concentration minimale inhibitrice
CMT	Concentration minimale tolérée
EO	*Essential oil*
FD	*Fermented dextrose*
FDA	Food and drug administration
FQRNT	Fond québecois de recherche nature et technologies
GFSE	*Grapefruit seed extract*
GRAS	*Generally reconized as safe*
GTE	*Green tea extract*
HACCP	*Hazard analysis and critical control points*
HE	Huile essentielle
HPB	*Health product branch*
HPLC	*High performance liquid chromatography*
INT	Iodonitrotetrazolium
IU	*International unit*
kDA	kiloDalton
kGy	kilogray
LAB	*Lactic acid bacteria*
LSPQ	Laboratoire santé publique du Québec
MAP	*Modified atmosphere packaging*
MAPAQ	Ministère de l'agriculture, des pêcheries et de l'alimentation du Québec
ME	*Methanol extract*
M-H	*Mueller Hinton*
MIC	*Minimum inhibitory concentration*
MTC	*Minimum tolerated concentration*
NMR	*Nuclear magnetic resonance*
NSERC	*Natural Sciences and Engineering Research Council*
OR	Oléorésine (*Oleoresin*)
PAM	Prêt-à-manger
PC	*Pure compound*
RESALA	Recherche en sciences appliquées à l'alimentation de Laval
RSM	*Response surface methodology*

RTE	*Ready to eat*
SPI	*Soy protein isolate*
TSB	*Trypsic soy broth*
UFC	Unité formatrice de colonie
UV	Ultraviolet

Note

Les termes écrits en italique sont exprimés en anglais.

Introduction

Déjà 10 000 ans avant notre ère, la conservation des aliments était une préoccupation importante pour la race humaine. Les moyens de conservation privilégiés étaient le froid, la chaleur, le séchage et la salaison. La plupart des techniques utilisées furent découvertes grâce au mode de recherche essais et erreurs. Suite au développement des méthodes scientifiques et de la découverte des agents responsables de la détérioration des aliments, des actions réfléchies ont permis d'élaborer des nouvelles technologies augmentant les temps de conservation et la sécurité des aliments. De plus, dans le contexte où une population sans cesse grandissante et une diminution des terres arables sont observées, la production alimentaire doit être de plus en plus efficace pour assurer un meilleur rendement. Le phénomène de mondialisation de production alimentaire augmente les distances parcourues par les denrées ce qui accroît les risques de contamination et de dégradation des aliments. L'organisation mondiale de la santé estime que 25% des denrées alimentaires produites sont perdues avant la vente aux consommateurs. Aux États-Unis, 76 millions de maladies sont d'origine alimentaire et causent 325 000 hospitalisations et plus de 5000 décès chaque année. Au banc des accusés nous retrouvons des genres bactériens pathogènes tels que *Campylobacter*, *Salmonella*, *Clostridium* et *Listeria*. Pour pallier à cette problématique, il est primordial de trouver de nouvelles technologies afin d'assurer la salubrité alimentaire. La problématique associée aux contaminations microbiennes des mets prêts à manger est d'autant plus importante puisque ce type d'aliment n'est habituellement pas soumis à un traitement thermique suffisant pour détruire les microorganismes avant d'être consommé.

L'année 2008 fut déterminante pour le secteur de l'industrie agroalimentaire canadien. Un épisode de contamination sans précédent vu le jour. Le décès de 23 personnes causé par une contamination par la bactérie *Listeria monocytogenes* de viande prête à manger amena des changements dans la mentalité des consommateurs et aussi des industrielles produisant la nourriture consommée au Canada. De plus, d'importantes modifications à la règlementation canadienne ont été apportées afin d'améliorer la sécurité des aliments. Ainsi depuis ce jour, la sécurité alimentaire est devenue une des préoccupations les plus importantes pour les Canadiens.

Une autre tendance a aussi vu le jour au début des années 2000. Les consommateurs ont développé un intérêt pour la nourriture qu'ils consomment et de ce fait ils regardent plus que jamais la composition des aliments qu'ils mangent. En inspectant les listes d'ingrédients, les acheteurs sont à la recherche de noms qu'ils connaissent. La tendance est à s'éloigner de l'utilisation d'ingrédients que les clients ne retrouvent pas dans leur cuisine. Ainsi, suite à ce nouveau penchant, de nouveaux défis sont apparus pour les producteurs d'aliments. Alors que par le passé un ingrédient ayant un nom se rapportant à une molécule de synthèse étaient adéquat pour pallier à une problématique spécifique (ex : le nitrite de sodium pour empêcher la croissance des pathogènes) il est maintenant nécessaire de développer de nouvelles méthodes se rapportant à des items ayant une origine plus naturelle (ex : vinaigre, jus de citron, etc.).

Depuis l'apparition de la vie sur terre, les organismes vivants sont en constante compétition pour leur survie. Afin de résister à l'envahissement, les espèces vivantes ont développées des moyens de défense. Par exemple, les végétaux synthétisent des molécules pour empêcher d'être détruits par des insectes, des champignons ou des bactéries. Il est possible d'extraire et concentrer ces molécules lors d'une distillation du matériel végétal afin de produire une huile essentielle. Cette huile essentielle pourrait être utilisée afin d'éliminer les microorganismes pathogènes dans les aliments.

Les bactéries lactiques sont aussi bien connues pour leurs défenses contre les envahisseurs. Par exemple, elles produiront des acides organiques pour acidifier leur environnement. Les bactéries lactiques possèdent une résistance accrue à un environnement acide donc elles diminueront les compétiteurs potentiels. Les bactéries lactiques possèdent aussi d'autres mécanismes plus spécifiques. Par exemple, elles peuvent produire des peptides antimicrobiens, appelés bactériocines, qui seront spécifiques à certaines familles de bactéries.

La combinaison de la demande pour des aliments plus naturels et une sécurité accrue amène les scientifiques en sciences alimentaires à mieux acractériser l'efficacité antimicrobienne des aliments de base utilisés depuis des générations. Les nouvelles connaissances sur les microorganismes qui causent les maladies d'origines alimentaires les plus fréquentes permettent de mieux contrôler cette problématique. Une recherche axée sur la combinaison de traitements pour améliorer l'efficacité antimicrobienne

permettra de mettre en évidence des synergies entre des molécules spécifiques et répondra cette double problématique.

Revue de la littérature

Les maladies d'origine alimentaire

Les aliments sont une source importante de maladies de toutes sortes (prions, virus, bactéries, parasites, insectes, métaux lourds, etc.). La contamination des aliments peut causer des gastroentérites et mener à des conséquences sévères telles que les maux d'estomac, les méningites, des défaillances d'organes et même la mort. Un des problèmes majeurs reliés aux maladies alimentaires est que la majorité des infections ne sont pas signalées aux autorités gouvernementales vu la faible intensité des malaises que la plupart des infections causeront. Ainsi, la majorité des infections ne nécessiteront pas de traitements médicaux et se résorberont en quelques jours. De ce fait, l'évaluation de la problématique de la contamination des aliments est d'autant plus difficile à évaluer. En 1999, aux États-Unis, il était estimé que la consommation humaine d'aliments était responsable de 76 millions d'épisodes de maladie, 325 000 hospitalisations et 5000 morts chaque année. Les microorganismes connus causaient 14 millions d'infections, 60 000 hospitalisations et 1800 morts chaque année. Les trois plus grands groupes responsables de ces infections étaient les virus (60%), les bactéries (30%) et les parasites (3%). La distribution des infections par des bactéries pathogènes en 1999 est illustrée dans la Figure 1. Les bactéries les plus fréquemment responsables des infections étaient *Campylobacter* spp. (47 %), *Salmonella* non-Typhi (32 %), *Clostridium perfringens* (6 %), *Staphylococcus* spp. (4 %), *Escherichia coli* O157:H7 (2 %), *Yersinia enterocolitica* (2 %), *Shigella* spp. (2 %), *Bacillus cereus* (1 %), *Streptococcus* spp. (1 %) and *Escherichia coli* non-O157:H7 (1 %) (Mead et al., 1999).

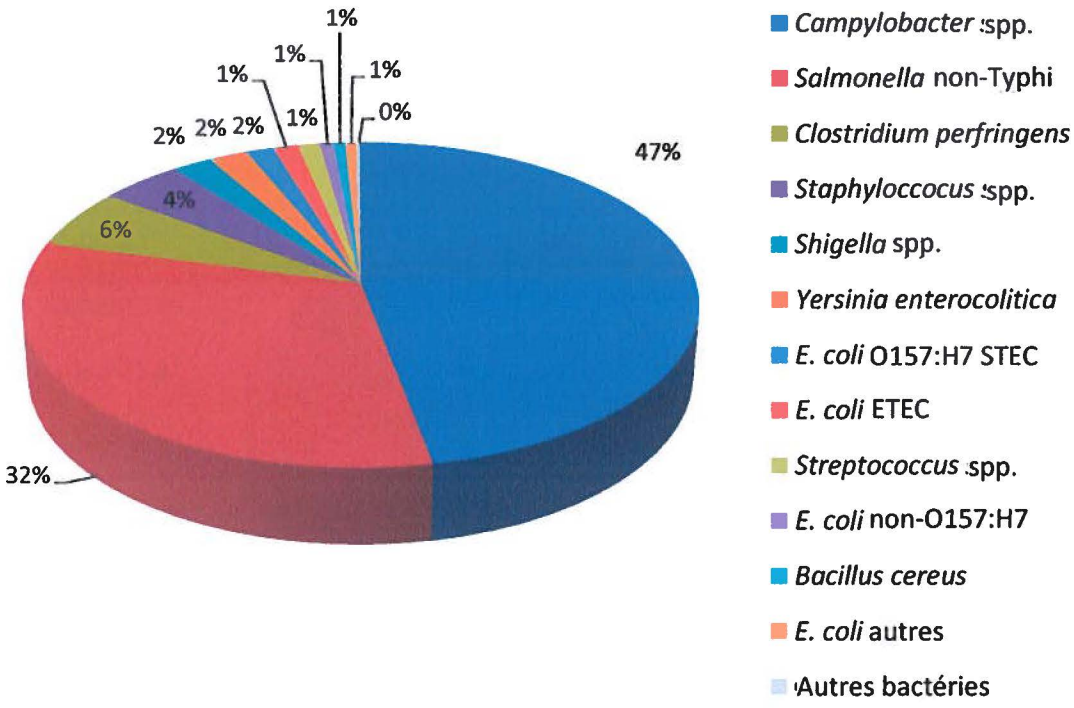


Figure 1: Distribution des maladies causées par des bactéries pathogènes connues aux États-Unis avant 1999 (Mead et al., 1999).

Une nouvelle étude, parue en 2011, a évalué l'impact des maladies d'origine alimentaire (Scallan, Griffin, Angulo, Tauxe, & Hoekstra, 2011; Scallan, Hoekstra, et al., 2011). Aux États-Unis, il était estimé que la consommation humaine d'aliments contaminés causait 48 millions de maladies, 128 000 hospitalisations et 3000 morts. De ces nombres alarmants, 9.4 millions de maladies, 56 000 hospitalisations et 1350 morts sont causés par les virus (59%), bactéries (39%) et les parasites (3%). La distribution des infections par les agents pathogènes en 2011 est illustrée dans la Figure 2. Les bactéries les plus fréquemment responsables des infections sont *Salmonella* non-Typhi (28 %), *Clostridium perfringens* (27 %), *Campylobacter* spp. (23 %), *Staphylococcus* spp. (7 %), *Shigella* spp. (4 %), *Escherichia coli* non-O157:H7 (3 %), *Yersinia enterocolitica* (3 %), *Bacillus cereus* (2 %) and *Escherichia coli* O157:H7 (2 %) and *Vibrio parahaemolyticus* (1 %) (Scallan, Hoekstra, et al., 2011). Une comparaison entre l'étude de 1999 et 2011 permet de mettre en évidence une augmentation importante des infections par *Clostridium* (6 contre 28%) et une diminution des infections par *Campylobacter* (47 contre 23%).

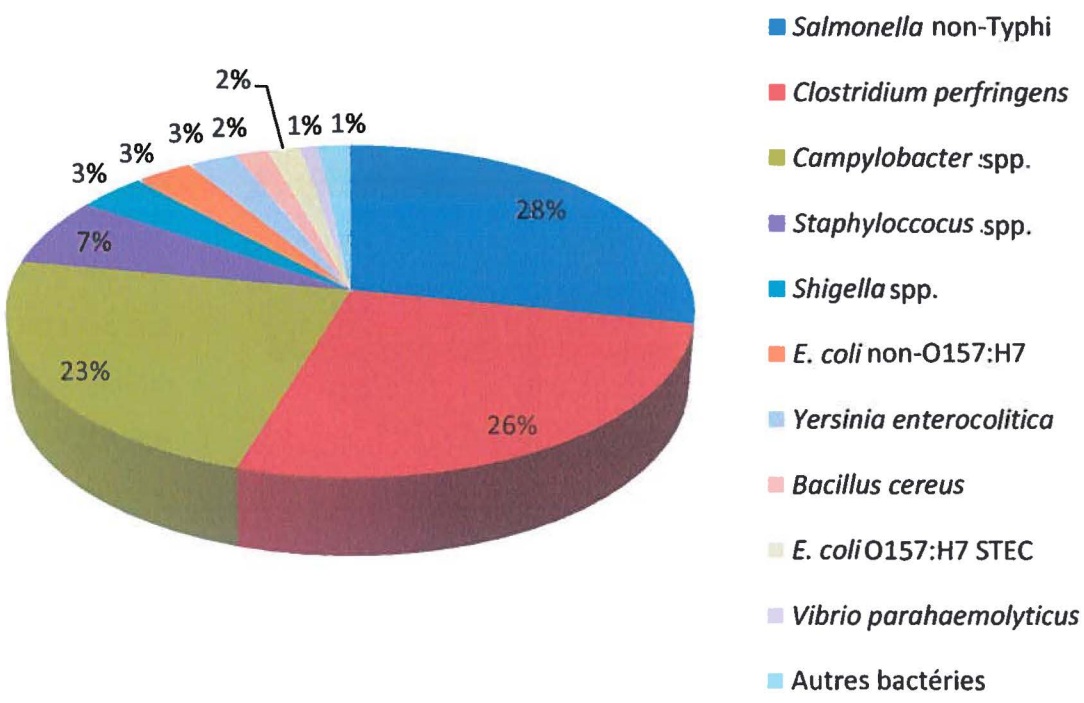


Figure 2: Distribution des maladies causées par des bactéries pathogènes connus aux États-Unis avant 2011 (Scallan, Hoekstra, et al., 2011).

Aux États-Unis, il a été évalué que les maladies d'origine alimentaire coûtent en moyenne 77 milliards de dollars par années (Scharff, 2012). Au Canada, les maladies sévères gastrointestinales ont un coût estimé de 3.7 milliards de dollars par année (Thomas, Majowicz, Pollari, & Sockett, 2008).

Habituellement, les maladies alimentaires causent des symptômes plus sévères chez les individus ayant un système immunitaire plus faible. Les jeunes enfants, les personnes âgées et les patients immunosupprimés (SIDA, cancer et receveur d'organe) sont plus susceptibles de succomber aux complications d'une infection par un microorganisme pathogène. Toutefois, les microorganismes possèdent un degré de virulence variable et peuvent surmonter la défense immunitaire d'un humain en santé et peuvent ultimement mener à la mort de son hôte.

Listeria monocytogenes

L. monocytogenes est une bactérie Gram positive en forme de bâtonnet, mobile (sous certaines conditions), avec un métabolisme anaérobie facultatif et est responsable de maladies alimentaires nommée listériose. Il a été démontré que le pH intracellulaire de *L. monocytogenes* ne variera pas (pH_{in}=8) lorsque que le milieu extracellulaire varie entre 5 et 8 (Budde & Jakobsen, 2000). Cette maladie se décrie comme une infection systémique des personnes immunosupprimées et des personnes âgées. Cette bactérie peut aussi traverser la barrière placentaire et causer la mort du fœtus. Aux États-Unis, ce pathogène est responsable chaque année de 1591 épisodes de maladies, 1455 hospitalisations et 255 morts (Scallan, Hoekstra, et al., 2011). Le taux de mortalité d'une personne infectée par ce microorganisme est parmi les plus élevés des pathogènes alimentaires et se situe au alentour de 20%. Au Canada, la moyenne varie entre 1.8 et 3.4 cas par million d'habitants par année pour les années 1995 à 2004. Dans cette même période, les sérotypes de *L. monocytogenes* les plus fréquemment isolés lors d'épisodes de contamination sont 1/2a (49 %), 4b (33%) and 1/2b (13%) (Clark et al., 2010).

Cette bactérie peut éviter la réponse immunitaire du corps humain en voyageant entre les cellules sans s'exposer au milieu extracellulaire. *L. monocytogenes* est une bactérie ubiquitaire car il est possible de la retrouver dans le sol, l'eau, les végétaux, le tractus intestinal d'animaux et même chez certains humains qui sont en bonne santé (porteurs sains). Entre 5 à 10% des adultes en bonne santé pourraient être porteur de ce microorganisme. Son caractère ubiquitaire la rend très difficile à éliminer et elle peut se multiplier dans un large éventail de température (0 à 40 °C). Il est d'autant plus difficile de relier les cas de contamination à un aliment spécifique puisque la période d'incubation avant de développer les symptômes de la listériose peut s'étendre jusqu'à 70 jours. (Salyers & Whitt, 2002). Toutefois, la bactérie *L. monocytogenes* ne forme pas de spores et est facilement éliminée par les traitements de chaleur utilisés lors des étapes de cuisson de la plupart des aliments (70 à 75 °C). L'augmentation de la popularité des mets prêt à manger (PAM) est une opportunité parfaite pour la multiplication des cas de contamination par cette bactérie.

En 2008, une éclosion majeure des cas de contamination de *L. monocytogenes* a été découverte. La contamination provenait d'une usine de transformation de la viande située en Ontario et détenue par la compagnie Maple Leaf. Les produits étaient distribués dans sept provinces canadiennes. De cet épisode, 57 cas de listériose ont été confirmés et de ce nombre 23 personnes ont perdu la vie. La contamination a coûté plus de 45 millions de dollars à la compagnie Maple Leaf. Cette crise a mené à des changements majeurs à la politique agroalimentaire canadienne afin de prévenir les contaminations futures (Farber, Kozak, & Duquette, 2011).

La composition de la viande

Le mot viande est le terme général qui décrit la partie comestible de la chair du muscle d'un animal. Cet aliment est une excellente source de nutriments tels que les protéines, vitamines et minéraux. De façon générale, la viande se compose principalement d'eau (75%), protéines (20%), gras (5%), sucres (1%) et vitamines (1%). Ces pourcentages variront en fonction de l'animal et du muscle choisis. La protéine majoritaire dans la viande est la myosine. La myosine possède un point isoélectrique (pI) de 5.4. Lorsque le pH de la viande est de 5.4, la charge globale de la protéine sera neutre et ainsi les charges positives et négatives s'attireront. Lorsque le pH est supérieur au pI, les protéines possèdent les mêmes charges et auront tendance à se repousser et ainsi pourront du même fait emmagasiner une quantité supérieure d'eau entre les protéines de la viande. Les concentrations élevées d'humidité et de nutriments de la viande sont des éléments qui supportent la croissance de plusieurs microorganismes. À l'origine, la viande est stérile avant l'abatage des animaux. Lors de la découpe, la brèche de la peau expose la couche externe des pièces de viande et permet la contamination potentielle des aliments par les microorganismes présents dans l'environnement. Les bonnes pratiques de fabrication limiteront les contaminations. Un niveau plus faible de bactéries totales après le découpage permettra une conservation plus longue. Les bactéries lactiques sont principalement responsables de la dégradation de la viande fraîche et cuite en agissant par l'acidification de celle-ci (Huff-Lonergan & Lonergan, 2005).

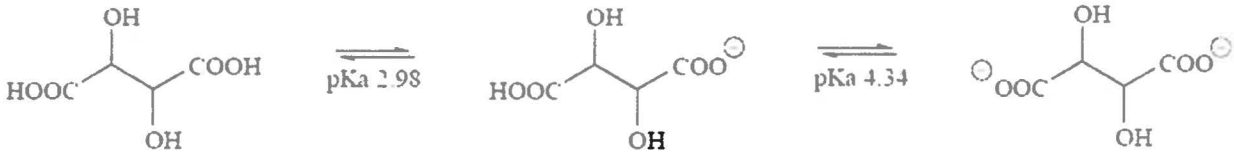
Les acides organiques

Les acides organiques (AO) sont des composés organiques possédant des propriétés acidifiantes. Le groupe des AO le plus répandu est le groupe des acides carboxyliques qui se décrivent par la présence d'un ou plusieurs groupes carboxyliques (-COOH) sur la molécule. Les acides carboxyliques les plus fréquemment retrouvés sont décrits dans le Tableau 1. Plusieurs AO sont utilisés dans l'industrie alimentaire pour leurs propriétés antimicrobiennes et antioxydantes. Au Canada, la majorité des acides organiques sont permis dans les aliments. Toutefois, certaines restrictions quant à leur utilisation s'appliquent sur le type d'aliment ainsi qu'à la concentration permise. Il est possible de consulter le règlement sur les aliments et drogues (C.R.C, ch 870) section B.16.001 pour les limites spécifiques de chacuns des composés. La plupart des AO utilisés industriellement sont d'origine synthétique. Il est possible de faire l'extraction des AO des végétaux (ex: l'acide ascorbique extrait des agrumes) et des produits de fermentation des microorganismes (ex : acide lactique des bactéries lactiques) (Smid & Corris, 2007).

Tableau 1: Acides organiques les plus fréquemment retrouvés dans l'industrie alimentaire (adapté de Van Immerseel et al., 2006).

Acide	Formule chimique	Poids moléculaire (g L^{-1})	pKa
Formique	HCOOH	46.03	3.75
Acétique	CH_3COOH	60.05	4.76
Propionique	CH_3CH_2COOH	74.08	4.88
Butyrique	$CH_3CH_2CH_2COOH$	88.12	4.82
Lactique	$CH_3CH(OH)COOH$	90.08	3.83
Sorbique	$CH_3CH:CHCH:CHCOOH$	112.14	4.76
Fumarique	COOHCH:CHCOOH	116.07	3.02
Benzoique	C_6H_5COOH	122.12	4.19
Methyl Butanoique	$CH_3SCH_3CH_2CH(OH)COOH$	149.00	3.86
Malique	$COOHCH_2CH(OH)COOH$	134.09	3.40, 5.13
Tartarique	$COOHCH(OH)CH(OH)COOH$	150.09	2.98, 4.34
Ascorbique	$C_6H_8O_6$	176.12	4.10
Érythorbique	$C_6H_8O_6$	176.12	4.10
Citrique	$COOHCH_2C(OH)(COOH)CH_2COOH$	192.14	3.13, 4.77, 6.40

La production de viande PAM autre que les produits fermentés requière un pH légèrement acide qui varie entre 6.0 et 6.4. Tel que décrit précédemment, outre l'effet sur la multiplication des microorganismes, le pH aura un effet principalement sur l'absorption de l'eau par l'aliment. Pour cette raison, il est impossible d'ajouter les AO sous leur forme acide. Il est donc possible de neutraliser les OA avec une base forte (ex : NaOH) afin de produire le sel de l'acide organique (SAO). Par exemple, en combinant l'acide lactique avec le NaOH, il y a production du lactate qui est une molécule d'acide lactique avec un atome de sodium remplaçant l'hydrogène sur le groupe carboxylique (RCOOH → RCOONa). Quand l'AO est en solution aqueuse, il y a un équilibre entre la forme conjuguée (RCOOH) et la forme dissociée ($RCOO^-$ $H+$) de l'acide. Il y a 50% de chaque forme quand le pH du milieu est égal au pKa de l'acide. Si le pH est supérieur au pKa il y aura plus de la forme dissociée et si le pH est plus faible que le pKa il y aura plus de la forme conjuguée. Certains AO possèdent plus d'un pKa car ils possèdent plusieurs groupements carboxyliques (Horton, Moran, Ochs, Rawn, & Scrimgeour, 2002).



Acide tartarique | Hydrogène tartarate | Tartarate

Figure 3: Effet du pH sur la forme majoritaire des acides organiques en solution aqueuse

L'équation de Henderson-Hasselbach

La proportion d'acides conjugués se calcule selon l'équation de Henderson-Hasselbach : pH = pKa + (log([A-]/[HA]) où le pH est celui du milieu, pKa est la constante de dissociation de l'acide et [A-]/[HA] est le ratio des concentrations d'acides conjugués par rapport aux acides dissociés. Lorsque le SAO est ajouté en solution il se dissociera et retrouvera son équilibre (conjugué/dissocié) selon le pH du milieu et le pKa de l'acide. Les AO agissent sur les bactéries en pénétrant à travers la membrane cellulaire sous leur forme conjuguée seulement et en se dissociant par la suite à l'intérieur du

cytoplasme. La dissociation dans le cytoplasme aura pour effet d'acidifier le milieu et interfèrera avec la machinerie cellulaire de l'organisme (Figure 4). Les microorganismes possèdent des pompes à protons régulant l'acidité du cytoplasme mais requièrent de l'énergie (ATP) pour les faire fonctionner. Ainsi, l'énergie dépensée à la régulation le l'homéostasie ne pourra être utilisée pour se multiplier. Pour cette raison, les acides organiques en solution légèrement acide possèdent principalement un effet bactériostatique et non bactéricide (Jamilah, Abbas, & Rahman, 2008).

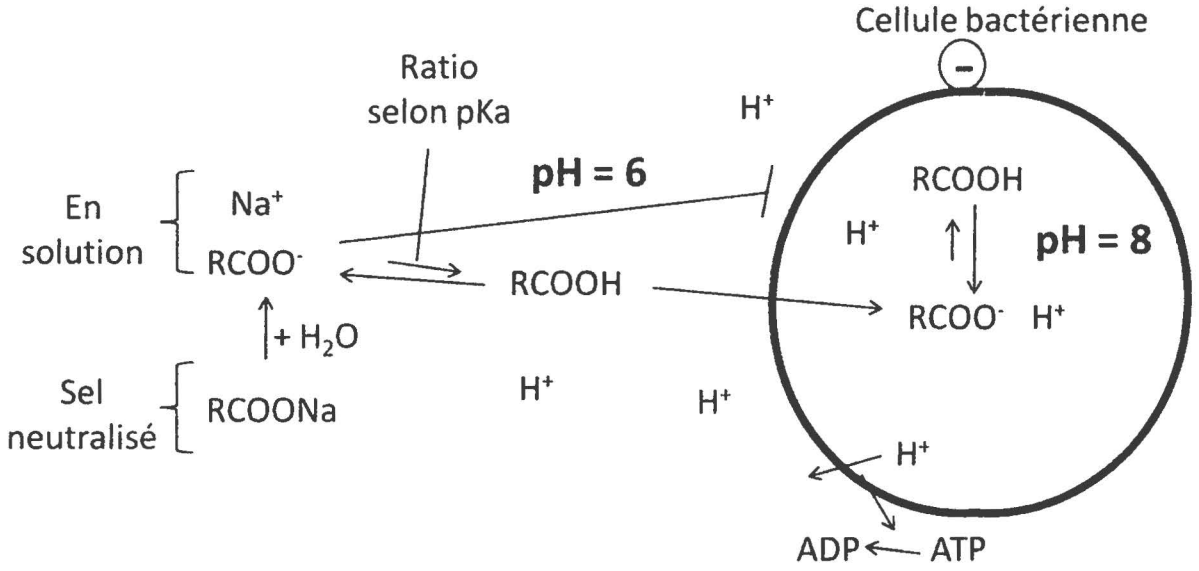


Figure 4: Réaction des acides organiques et de leurs sels lorsque mis en solution et ainsi que leurs effets sur les bactéries.

Les acides organiques dans les aliments

Aux États-Unis, plusieurs AO sont considérés comme « GRAS » par le FDA (http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS) . Au Canada, les AO sont permis dans les aliments par Santé Canada et l'Agence de l'Inspection des Aliments (ACIA). Toutefois, l'utilisation des AO spécifiquement comme antimicrobiens est encadrée par des limites au niveau de chaque catégorie de produits dans lesquels ils peuvent être incorporés et par une concentration maximale pouvant être ajouté. Vu leur caractère sécuritaire pour la consommation humaine et leur propriétés antimicrobiennes, plusieurs recherches utilisent les acides organiques pour contrôler la multiplication des populations bactériennes dans les aliments.

Analyse des acides organiques

La réalisation des études de validation requiert que les antimicrobiens présents dans les aliments puissent être mesurés de façon quantitative. La méthode habituellement sélectionnée sépare les acides organiques par HPLC et les quantifie à l'aide d'un détecteur UV. Plus précisément, la méthode d'exclusion d'ion est utilisée pour la séparation de molécules faiblement ionisables comme les AO. Le copolymère à l'intérieur des colonnes de HPLC est un polystyrène-divinylbenzène sulfoné. Les molécules chargées négativement sont repoussées du polymère et les molécules neutres peuvent pénétrer dans les billes de polymère. La phase mobile sélectionée avec ce type de colonne est acide (5 mM acide sulfurique). Normalement les AO seront élués selon leurs pKa, les valeurs élevés seront détectées les premières. La détection se fera à l'aide de l'absorbance de l'éluant par un détecteur UV à 210 nm où le lien carboxylique produira une absorbance caractéristique (Pescuma, Hebert, Mozzi, & Font de Valdez, 2008).

La combinaison lactate et diacétate

Certains acides organiques, tels l'acide lactique et acétique, ont déjà été étudiés par le passé pour leur efficacité antimicrobienne. L'acide lactique est une molécule impliquée dans différents processus biochimiques chez les être vivants. Plus particulièrement, l'acide lactique est aussi un métabolite produit par les bactéries lactiques. Cet acide se présente sous deux formes d'énantiomères D-(−)-acide lactique et L(+)-acide lactique. La forme L est produite chez les organismes vivants. Il est à noter que D-(−)-acide lactique est faiblement plus antimicrobien que L(+)-acide lactique (Gravesen, Diao, Voss, Budde, & Knochel, 2004). Le lactate est le sel neutralisé de l'acide lactique. L'acide acétique peut être produit de façon synthétique ou par la fermentation de l'alcool par des bactéries appartenant au genre *Acetobacter*. L'acide acétique est la composante caractérisant le vinaigre (5%). Le diacétate est le sel partiellement neutralisé de l'acide acétique. Le ratio d'acide acétique et acétate est de 1 : 1 dans le diacétate. Plusieurs articles ont rapporté l'utilisation du lactate (de sodium (LS) ou de potassium (LP)) et du diacétate (de sodium (DS) pour l'inhibition de la croissance de *L. monocytogenes* dans les aliments PAM (Barmpalia et al., 2005; Mbandi & Shelef, 2002; Stekelenburg, 2003). Il est à noter que le LS et LP utilisés lors des études est

habituellement une solution ou « sirop » à 60%. À 4°C, la combinaison de LS (1.8%) et de DS (0.25%) ont empêché la croissance de *L. monocytogenes* (avec une concentration initiale de 10^3 bactéries par cm^2) dans le jambon de Bologne pendant 90 jours (Barmpalia et al., 2005). La combinaison de LS (1.8%) et de DS (0.125%) n'a pas empêché la croissance de *L. monocytogenes* complètement mais a diminué le taux de croissance de 46%. Toutefois, lorsque la température est augmentée à 10°C au lieu de 4°C la combinaison LS (1.8%) et de DS (0.25%) ne peut inhiber complètement la croissance de *Listeria* mais peut toutefois réduire son taux de croissance. L'effet bactériostatique du SL, PL et SD n'est pas spécifique pour *L. monocytogenes* seulement car ces antibactériens peuvent inhiber la croissance des bactéries lactiques ainsi que les bactéries qui composent la flore aérobique mésophile totale (Barmpalia et al., 2005).

L'acétate

L'acétate est le sel neutralisé de l'acide acétique. L'utilisation de l'acétate (de sodium, de potassium et de calcium) comme antimicrobiens a été confirmée plusieurs fois dans la littérature. Une concentration de 0.9% d'acide lactique était capable de réduire de 4 log UFC/g de *L. monocytogenes* dans le milieu de culture infusion de cerveau et de cœur (*brain heart infusion*, BHI) à pH 3.5, 28°C en 66 jours comparativement au contrôle acidifié nécessitant 172 jours (Golden, Buchanan, & Whiting, 1995). Il est à noter que l'évaluation *in vitro* de l'efficacité des acides organiques est plus complexe puisque l'effet des acides organiques est pH dépendant tel qu'expliqué précédemment. La plupart des milieux de culture pour les bactéries possèdent un pH neutre ce qui diminue l'efficacité des acides organiques. Pour cette raison, les analyses *in vitro* nécessiteront des concentrations plus fortes qu'*in situ* si les méthodes d'analyses habituelles (méthode de dilution en bouillon) ne sont pas modifiées. Une autre expérience démontra qu'un trempage dans une solution d'acétate de sodium à 2% pour 30 minutes en combinaison avec un emballage sous une atmosphère modifiée (70% N_2 et 30% CO_2) à permis de réduire le compte des bactéries psychrophiles et mésophiles durant la conservation du poisson (Manju, Jose, Gopal, Ravishankar, & Lalitha, 2007). La combinaison de l'acide acétique ou du diacétate de sodium avec la nisine a aussi démontrée une réduction du taux de croissance de *L. monocytogenes* dans les aliments (Samelis et al., 2005).

Le propionate

L'acide propionique peut être produit de façon synthétique ou par la fermentation anaérobique des bactéries appartenant au genre *Propionibacterium*. L'acide propionique est utilisé actuellement principalement dans le domaine de la boulangerie pour inhiber la croissance des moisissures. Il n'est présentement pas permis d'utiliser l'acide propionique comme antimicrobien dans les charcuteries au Canada. Le propionate est le sel neutralisé de l'acide propionique. Dans le BHI à pH 3.5, une concentration de 0.9% d'acide propionique est capable de réduire de 4 log UFC/g *L. monocytogenes* en 65 jours comparativement au témoins acidifié prenant 172 jours (Golden et al., 1995). Dans des pommes fraîchement coupées, une solution de trempage contenant du propionate de calcium a réussi à réduire la croissance des levures, moisissures et *E. coli* pendant l'entreposage (Guan & Fan, 2010). Dans la dinde cuite et le jambon, le propionate a permis l'inhibition la croissance de *L. monocytogenes* lors de la conservation à 4°C pendant 12 semaines (K. A. Glass, McDonnell, Rassel, & Zierke, 2007). Dans le jambon de Bologne, l'acide propionique en combinaison avec le benzoate a permis d'inhiber la croissance de *L. monocytogenes* pendant 13 semaines (K. Glass, Preston, & Veesenmeyer, 2007).

L'hypothèse voulant que la forme dissociée du lactate interfère avec le cycle du pyruvate

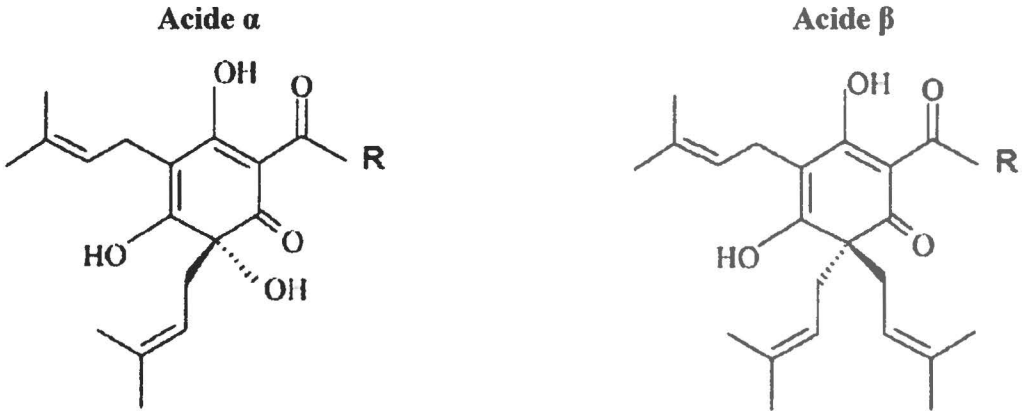
Ce paragraphe vise à faire une mise au point sur une hypothèse véhiculée par certains auteurs mais n'ayant jamais été démontrée. Cette hypothèse suppose que l'ion lactate ($RCOO^-$) pourrait interférer avec le cycle du pyruvate de la bactérie et ralentir le métabolisme de celle-ci, ce qui expliquerait l'effet bactériostatique du lactate. Dans l'article de revue de (Houtsma, Kusters, Dewit, Rombouts, & Zwietering, 1994) cette idée est présentée et référée de l'article (Papadopoulos, Miller, Acuff, Vanderzant, & Cross, 1991). Ce dernier, présente cette idée en référant (Maas, Glass, & Doyle, 1989) qui avait émis comme hypothèse que le lactate pourrait réduire la production de toxine chez *Clostridium botulinum*. Toutefois, aucune expérimentation n'a été effectuée pour vérifier

l'hypothèse. Ainsi, les explications fournies dans les sections précédentes restent les seules ayant été démontrées expérimentalement.

Le houblon

La bière est la troisième boisson la plus consommée au monde après l'eau et le thé. La bière traditionnelle est faite d'eau, d'orge malté et de houblon qui sont fermentés par une levure. Le houblon (*Humulus lupulus* L.) est une source importante de composés phénoliques qui se retrouvent dans la bière. Les cônes de houblons séchés contiennent entre 4 et 14 % de composés phénoliques comprenant les acides phénoliques, chalcones, flavonoïdes, catéchines et proanthocyanidines (Gerhauser, 2005a).

Les acides amers (5-20% du poids du houblon) sont des dérivés phloroglucinol habituellement appelés acides α et acides β. Chaque groupe contient 3, 4, 5 ou 6 chaînes carbonées oxo-alkyles. Les acides α et β sont structurellement différents pour un ou plusieurs groupes prényles (Figure 5). Les acides amers sont présents dans le houblon dans un mélange variable de chaque forme. L'acide α majoritaire est l'humulone (35-70 % des acides α totaux), cohumulone (20-65 %) et adhumulone (10-15 %). L'acide β majoritaire est le lupulone (30–55% des acides β totaux), colupulone et adlupulone (Zanoli & Zavatti, 2008).



R= $CH_2CH(CH_3)_2$	α (Humulone)	β (Lupulone)
R= $CH(CH_3)_2$	α (Cohumulone)	β (Colupulone)
R= $CH(CH_3)CH_2CH_3$	α (Adhumulone)	β (Adlupulone)

Figure 5: Structure chimique des acides amers du houblon

L'activité antimicrobienne, principalement contre des bactéries Gram positives, a été documentée pour l'humulone, le lupulone et leurs dérivés (acides α et β). L'activité antimicrobienne agit en créant des pertes de contenu cellulaire qui sont causées par les interactions hydrophobiques entre les acides et la membrane bactérienne (Zanoli & Zavatti, 2008). Une revue de la littérature sur les composés du houblon, décrivent aussi le xanthohumol, une autre molécule présente dans le houblon comme ayant de bonnes propriétés antimicrobiennes contre les bactéries, virus, moisissures et parasites (Gerhauser, 2005b). L'utilisation des acides amers du houblon est approuvée par le FDA pour l'ajout de ceux-ci dans certaines applications alimentaires spécifiques (GRAS Notice No. GRN 000063, http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/GRASListings/ucm153973.htm).

Les modèles prévisionnels pour la croissance microbienne dans les aliments

La prédiction de la croissance microbienne dans les aliments est un domaine qui combine les sciences alimentaires, la microbiologie, les mathématiques et les statistiques. Une revue étendue de la littérature est disponible pour la modélisation de la croissance des microorganismes pour la fermentation industrielle en réacteur puisque l'observation des différents paramètres et le contrôle de ceux-ci est définitivement plus facile que pour les modèles alimentaires. Toutefois, des recherches ont produit des données sur la modélisation de la croissance bactérienne dans les aliments (Whiting, 1995). Les modèles prévisionnels peuvent avoir plusieurs utilités comme la prédiction du risque, le contrôle de la qualité, la recherche et le développement et l'éducation (McDonald & Sun, 1999). Les modèles prévisionnels sont divisés en trois groupes: primaire, secondaire et tertiaire (Whiting & Buchanan, 1993).

Le modèle primaire sert à quantifier la biomasse microbienne (UFC/g) dans une condition environnementale spécifique. Un des premiers modèles adopté et encore utilisé aujourd'hui pour décrire la cinétique de croissance bactérienne est celui de Monod :

$N=N_0 e^{kt}$ ou le k défini le taux de croissance de la bactérie (Monod, 1949). L'équation de Gompertz (Bhaduri et al., 1991; Gibson, Bratchell, & Roberts, 1987) et celle de Baranyi (Baranyi & Roberts, 1994) sont d'autres exemples d'équations pouvant être utilisées pour décrire la cinétique bactérienne dans les aliments. L'équation de Monod est une équation exponentielle ne prenant pas en compte la phase de latence avant la phase exponentielle. L'équation de Gompertz et Baranyi est une équation exponentielle de type sigmoïdale. Ces deux dernières équations prendront en compte la phase de latence. Toutefois, pour la modélisation du comportement de bactéries dans un modèle alimentaire sur la durée de vie de celui-ci, la détection de la phase de latence est plus difficile à déterminer vu l'écart qui est plus grand entre les prélèvements des données. Pour cette raison, l'utilisation du modèle simple de Monod est souvent privilégiée. Les modèles secondaires décrivent la réponse des microorganismes par rapport à différents paramètres environnementaux tels que la température, le pH, l'humidité, les nutriments et les antimicrobiens. Les modèles tertiaires sont habituellement des logiciels d'utilisation de routine qui convertissent les modèles primaires et secondaires en outils faciles d'utilisation pour des utilisateurs ne connaissant pas nécessairement la mécanique derrière la modélisation de la croissance des microorganismes mais qui sont plutôt soucieux du résultat sur le produit fini (Whiting, 1995). Plusieurs logiciels différents existent tels que : SSSP, Purac, DMRI, μfit, pathogen modelling program, foodmicro, growth predictor et sym'previous.

Les modèles décrivant la croissance de Listeria monocytogenes

Un des premiers modèles secondaires *in situ* à être publié sur le pathogène *L. monocytogenes* était celui de Seman, Borger, Meyer, Hall, & Milkowski, 2002. Ce modèle décrit la croissance de *L. monocytogenes* dans les produits de charcuteries prêts-à-manger. Les paramètres évalués de ce modèle sont le taux d'humidité, le pourcentage de chlorure de sodium, de lactate de potassium et de diacétate de sodium. Il est à noter que la compagnie américaine Purac a créé à l'aide de ce modèle un logiciel (modèle tertiaire) afin de prévoir la croissance du pathogène dans les charcuteries. Purac est un leader mondial dans la fabrication de dérivés d'acide lactique pour différents marchés. Une autre équation a été créée par Ole Mejlholm & Dalgaard, 2007 pour prédire la croissance de *L. monocytogenes* dans les fruits de mer en évaluant la température, le chlorure de sodium,

le pH, l'activité de l'eau (a_w), les phénols, le CO_2, le lactate et le diacétate. Par la suite, le modèle a été mis à jour pour inclure l'acide acétique, l'acide benzoïque, l'acide citrique et l'acide ascorbique (O. Mejlholm & Dalgaard, 2009). Afin de valider leur modèle, une étude comparative avec d'autres modèles publiés précédemment a été effectuée (O. Mejlholm et al., 2010). Dans cette étude, les modèles suivant ont été comparés : (Delignette-Muller, Cornu, Pouillot, & Denis, 2006), (Augustin, Zuliani, Cornu, & Guillier, 2005), (Zuliani et al., 2007), Purac (2007), Listeria model du Danish Meat Research Institute (DMRI; http://1.test.dezone.dk/ (username: matmodel and password: listeria) et (O. Mejlholm & Dalgaard, 2009). Un modèle tertiaire est maintenant disponible (Seafood Spoilage and Safety Predictor (SSSP) software v. 3.1) et qui est base sur les recherches de (O. Mejlholm & Dalgaard, 2009). À ce jour, aucun modèle pour prédire la croissance de *L. monocytogenes* dans les aliments prend en compte l'utilisation du propionate, de la nisine et du houblon.

Chapitre de livre 1: Essential Oils in Edible Films and Coatings

Chapitre publié:

D. Dussault and M. Lacroix, 2012, Essential Oils in Edible Films and Coatings, In: Essential Oils as Natural Food Additives (eds. Luca Valgimigli), Nova Science Publishers, Inc., NY, NY, pp. 413-426.

Contribution des auteurs

J'ai écris en collaboration avec ma directrice de recherche Prof. Monique Lacroix ce chapitre de livre. La partie décrivant les différents polymères a été écrite par la Prof. Monique Lacroix. J'ai fait la revue de la littérature et l'écriture de la section sur les huiles essentielles. J'ai aussi réalisé la mise en forme de la publication.

Résumé en français

En dépit des nouvelles technologies et des concepts de sécurité modernes tel que le HACCP (*Hazard Analysis and Critical Control Points*, Analyse des dangers et points critiques pour leur maîtrise), le nombre de maladies d'origine alimentaire est toujours croissant. Selon Santé Canada, le coût annuel pour traiter les maladies d'origine alimentaire est estimé à 1 milliard de dollars au Canada et entre 5 et 86 milliard aux États-Unis. Les huiles essentielles servent depuis longtemps comme additifs de saveurs dans les aliments et breuvages. Vu leur composition variée en agents antimicrobiens et antioxydants, ces extraits ont un potentiel pour devenir des agents de conservation naturels pour les aliments. Les composés actifs des huiles essentielles peuvent être ajoutés à l'emballage des aliments pour augmenter le temps de conservation, conserver la couleur et augmenter les qualités nutritionnelles. L'utilisation de films comestibles offre la possibilité d'augmenter l'efficacité des composés antimicrobiens et de prolonger la durée de conservation en assurant un relargage contrôlé des molécules actives dans l'aliment.

Abstract

In spite of modern technologies and safety concepts, such as HACCP, the reported numbers of food-borne illnesses and intoxications are still increasing. According to Health and Welfare Canada, the annual costs to treat foodborne illness are estimated at $1 billion in Canada and from $5 to $86 billion in the United States. Essential oils have long served as flavouring agents in food and beverages, and due to their versatile content of antimicrobial and antioxidant compounds; they possess potential as natural agents for food preservation. Active compounds can be added to food packaging in order to extend the shelf life, preserve the color and improve the nutritional value of foods. The use of edible films offers the possibility to improve the efficiency of the antimicrobial compounds and prolong the shelf life of foods by assuring a controled release of the active compounds.

Key words: edible films and coating, essential oils, antimicrobial, food safety, crosslinking.

Introduction

In spite of modern technologies and safety concepts, such as HACCP, the reported numbers of food-borne illnesses and intoxications are still increasing [1]. According to the United Nations, more than 30% of the mortality rate world-wide is caused by alimentary diseases. According to Health and Welfare Canada, the annual costs to treat foodborne illness are estimated at $1 billion in Canada and from $5 to $86 billion in the United States [2]. Still, in the United-States it was estimated that 76 million illnesses, 325 00 hospitalizations and 5000 death occur every year. Known pathogenic organisms are causing 38 million infections per year (50 % of the total cases) and the three most important groups are virus, bacteria and parasites responsible for 67 %, 30 % and 3 % respectively [3]. *Campylobacter*, *Listeria*, *Shigella*, *Escherichia coli* and *Salmonella* are the most important bacteria responsible for foodborne illness in Canada. *Salmonella*, *Campylobacter jejuni*, *E. coli* 0157:H7, *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium botulinum* can contaminate meat, poultry, eggs, seafood and dairy products. The desire of most countries to make food safer for consumption requires better food preservation and production techniques. In this regard, the use of natural antimicrobial compounds is an interesting alternative to be considered [2].

The principal role of food packaging is to protect food products from outside influences and damage, to contain the food and to provide consumers with ingredient and nutritional information [4]. Food packaging can also maintain the quality and the safety of the food and provide protection against chemical, biological and physical external influences [5]. The use of plastics in food packaging is very popular due in part for the low cost of materials and functional advantages [6]. Despite, environmental concerns are opening the way to biodegradable material for food packaging. Biodegradable films are derived from replenishable agricultural feedstock, animal sources, marine food processing industry wastes, or microbial sources [5]. The inclusion of essential oils and bacteriocins in food packaging should lead to beneficial effects such as improved safety, quality and flavour; and potential in the biopreservation of food. Some compounds are not effective alone, however, when combined together, a synergistic effect could be found [7].

Edible Films and Coatings

Edible films are freestanding structures, first formed, then applied to foods and eaten with the food without removing it. Edible films typically contain three major components: proteins, polysaccharides and lipids. These composite films may be of heterogenic nature and be formed via a mixture of protein/polysaccharide/plasticizer and lipid [8]. This approach allows a better exploitation of the functional properties of each of the film's components. They are formed by casting and drying film-forming solutions on a levelled surface, drying a film-forming solution on a drum drier or using traditional plastic processing techniques, such as extrusion. Edible coatings are generally applied by spraying or dipping. Edible coatings can potentially extend the shelf life and improve the quality of food system by the control of mass transfer, moisture and oil diffusion, gas permeability (O_2, CO_2), and flavour and aroma losses. Coating formulations could be used to serve as adhesives for seasoning or to improve the appearance of foods. For example, edible coating can be applied to the surface of snack foods and crackers to serve as a foundation or adhesive for seasoning [9]. Active compounds (antimicrobials, antioxidants and nutrients) can be added to food coatings in order to extend the shelf life, preserve the color and improve the nutritional value of foods. Edible coatings also have the potential for maintaining the quality of food after the packaging is opened by protecting against moisture change, oxygen uptake and aroma loss [10].

The film and coating requirements for food are complex. Unlike inert packaged commodities, foods are often dynamic systems with limited shelf-life and very specific packaging needs. In addition, since foods are consumed to sustain life, the need to guarantee safety is a critical dimension of their packaging requirements. While the issue of food quality and safety is first and foremost in the mind of the food scientist, a range of other issues surrounding the development of any food package must be considered before a particular packaging system becomes a reality. Secondary packaging is often used for physical protection of the product. It may be a box surrounding a food packaged in a flexible plastic bag. It could also be a corrugated box containing a number of primary packages in order to ease handling during storage and distribution, improve stackability, or protect the primary packages from mechanical damage during storage and distribution. Due to the barrier properties of edible films secondary packaging may not require high-

barrier packaging materials. Therefore, the entire packaging structure can be simplified, while satisfying the barrier requirement. The barrier functions concern oxygen, moisture and aroma blocks, as well as physical damage prevention. Edible film and coating must meet the criteria that apply to conventional packaging materials associated with foods. These relate to barrier properties (water, gases, light, aroma), optical properties (e.g. transparency), strength, welding and moulding properties, marking and printing properties, migration/scalping requirements, chemical and temperature resistance properties, disposal requirements, antistatic properties as well as issues such as the user-friendly nature of the material and whether the material is price-competitive. Edible film materials must also comply with food and packaging legislation, and interactions between the food and packaging material must not compromise food quality or safety. In addition, intrinsic characteristics of edible film materials, for example whether or not they are biodegradable or edible can place constraints on their use for foods [11].

Antimicrobial Properties of Edible films and Coating Containing Essential Oils

Contamination by pathogens is a great concern in food sciences. Most of the class I recalls result from postprocessing contamination during subsequent handling and packaging [12]. Organic acids, essential oils, salts, lipids, spices and bacteriocins (nisin) have been largely studied for their efficiency to control microbial growth and pathogenic bacteria in foods [1, 13-16].

Edible films and coating containing natural antimicrobials may provide protection for perishable foods but also for the packaging itself during storage. In active packaging systems, the choice of incorporative components is often limited by the incompatibility of the component with the packaging material or by the heat liability of the component with the packaging material [17]. It has been demonstrated that whey protein isolate or soya protein – based films have great potential for acting as an excellent carrier for bioactive compounds in active food packaging systems [18, 19]. Disulfide crosslinking induced by thermal denaturation is an essential step in these investigations [19, 20]. The use of γ-irradiation in order to produce cross-linking between the protein chains has permitted a significant increase of the mechanical properties of the films [21, 22, 23]. Moreover,

glycerol was found to play a double role in enhancing the formation of cross-links within casein chains [24]. The development of new technologies (functionalization, cross-linking, immobilization etc.) to improve the film properties (control release of active molecules, bioactivities protection, resistance to water, etc.) of edible films and coatings represents a need for the future. One strategy to reduce the rate of diffusion is to entrap the antimicrobial in the polymeric matrix [12]. Essential oils from spices or herbs can be entrapped into films to modify the flavour but also to introduce antimicrobial and antioxidant properties. The use of these compounds are however limited to their strong flavour. The selection of extracts active at low concentrations represents an interest for the food industry.

In vitro Assay

The most popular assay used to verify the antimicrobial activity of edible films is the agar diffusion assay (ADA) (Figure 1). This assay consists of placing a circular piece of film, usually around 5 to 10 mm in diameter, on the center of an agar plate already inoculated with the indicating microorganisms. After an incubation period to allow the microorganisms to replicate on the agar plate, the results can then be reported as the diameter of the growth inhibition of the bacterium around the film. Since, the size and the thickness of the films, the bacterial strain and the agar medium used are not standardized in between each experiments, it is hard to compare results between the different publications. Although, it is still a good screening experiment to select the best formulation for further studies *in situ*.

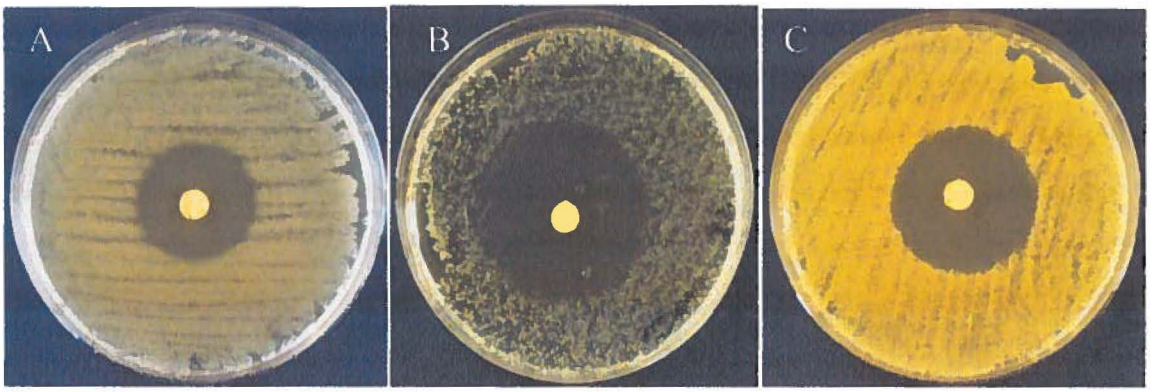


Figure 1: Agar diffusion assay of edible films containing cinnamon essential oils against A) *E. coli* O157:H7, B) *S.* Typhimurium and C) *S. aureus* (unpublished data Dussault and Lacroix).

Alginate

Alginate, a polysaccharide derived from brown seaweed known as *Phaeophyceae*, is comprised of (1-> 4) linked polyuronic containing three types of block structure: β-D-mannuronic acid (M block) and poly α-L-guluronic acid (G block) residues and MG block containing both polyuronic acids. Alginates produce uniform, transparent and water-soluble film. Divalent cations are used as gelling gents in alginate film formation to induce ionic interactions followed by hydrogen bonding [25]. Alginates possess good film forming properties but tend to be quite brittle when dry but may plasticize by the inclusion of glycerol. Alginate based films are impervious to oils and fats but are poor moisture barriers. However, alginate gel coatings can significantly reduce moisture loss from foods by acting sacrificially. Moisture is lost from the coating before the food dehydrates significantly. Also, alginate coatings are good oxygen barriers and can retard lipid oxidation in foods [11]. A film-forming solution made from apple puree and alginate containing oregano, lemongrass or cinnamon essential oils was found to be bactericidal against *E. coli* O157:H7 when assayed *in vitro*. When the film-forming solution was casted into a free standing film, these films still had antimicrobial activity when tested in the ADA [7, 26, 27].

Agar

Agar, a gum derived from a variety of red seaweeds and like carrageenan, is a galactose polymer. It forms strong gels characterized by melting points (85°C) far above the initial gelation (40°C) temperature [28]. Edible films made from algal extracts (*Gelidium corneum*) containing 0.4 to 1 % carvacrol showed antimicrobial activity on the ADA against *E. coli* O157:H7 and *L. monocytogenes* [29].

Carboxymethyl Cellulose

Edible coatings made from carboxymethyl cellulose (CMC) have been applied to a variety of food to provide a moisture, oxygen or oil barrier and to improve batter adhesion. CMC can be solubilised in aqueous or aqueous-ethanol solutions and the films obtained present good film-forming properties but are soluble in water. These cellulose ether films are generally transparent, flexible, odourless, tasteless, water-soluble and resistant to oils and fat [11]. Film-forming solution of chitosan or carboxymethyl cellulose containing 1 % olive, rosemary or capsicum oleoresin showed antimicrobial activity in the ADA against *L. monocytogenes* and native microflora of butternut squash [30].

Chitosan

Chitosan is a polysaccharide derived from chitin and found in abundance in the shells of crustaceans. Chitosan is mainly composed of 2-amino-2-deoxy-β-D-glucopyranose repeating units but still retaining a small amount of 2-acetamido-2-deoxy-β-D-glucopyranose residues. Chitosan with a high amino content is water-soluble in aqueous acids [31]. Chitin and chitosan are natural antimicrobial compounds against different groups of microorganisms such as bacteria, yeast and fungi [32].

Edible films made from chitosan containing Mexican oregano (*Lippia berlandieri* Schauer) and bergamot essential oils could inhibit the growth of moulds like *Aspergillus niger* and *Penicillium* spp. at 0.5 % concentration using the ADA [4, 33, 34]. Also chitosan-based films containing cinnamon essential oil at levels of 0.4 to 2 % showed antimicrobial activity in the ADA against *L. monocytogenes, L. plantarum, L. sakei, P. fluorescens* and *E. coli* [35].

Gelatin

Collagen is a constituent of skin, tendon and connective tissues. It is a fibrous protein and represents about 30% of the total mass of the body. Hydrolysis of collagen results in gelatin. Gelatin has been known to form clear, flexible, strong and oxygen-impermeable films when cast from aquous solutions in presence of plasticizers [8]. Edible films made with fish-skin gelatin incorporated with chitosan and clove essential oil showed growth inhibition of *Shewanella putrifaciens*, *Photobacterium phosphoreum*, *L. acidophilus*, *P. fluorescens*, *L. innocua* and *E. coli* on the ADA [7,36 ,37].

Pectin

Pectin, is a complex anionic polysaccharide composed of β-1, 4-linked D-galacturonic acid residues, wherein the uronic acid carboxyls are either fully (high methoxy pectin) or partially (low methoxy pectin) methyl esterified. These films are solution cast by air-drying at ambient temperature [11]. In order to detect an antimicrobial property in the ADA against *E. coli* O157:H7 for edible films made from tomato puree and pectin a minimum concentration of 0.75% of carvacrol should be added. It was also noticed that carvacrol concentrations and antimicrobial properties were stable for up to 98 days at 5 and 25 °C in film made from tomatoes [38]. Films from tomato puree and pectin containing allspice, garlic and oregano essential oils from 0.5 to 3% showed antimicrobial efficiency with the ADA and vapor diffusion assay against *Salmonella enterica, E. coli* O157:H7 and *L. monocytogenes*. Garlic essential oils containing films were only inhibitory to *L. monocytogenes* as compared to oregano and allspice that also inhibited Gram negative bacteria [39].

Edible film prepared from apple puree and pectin containing 1% carvacrol showed antimicrobial properties against *E. coli* O157:H7 in the ADA. Antimicrobial properties and carvacrol concentrations were stable for up to 7 weeks in edible film made from apple puree and pectin [40]. Films from apple puree and pectin containing 0.5 to 3 % of cinnamon, allspice and clove bud essential oils showed antimicrobial efficiency with the ADA and vapor diffusion test against *S. enterica, E. coli* O157:H7 and *L. monocytogenes*.

These films showed a greater antimicrobial activity towards *L. monocytogenes* than *Salmonella enterica, E. coli* O157:H7 [41].

Soy Protein

Soy protein films are clear in appearance and contain insoluble particles. Antibacterial activity of soy protein edible films incorporated with 1 to 5 % oregano or thyme essential oils showed growth inhibition activity on the ADA against *E. coli*, *E. coli* O157:H7 and *S. aureus*, although *Lactobacillus plantarum* and *Pseudomonas aeruginosa* seemed to be more resistant to the film than the previous microorganisms [42].

Starch

Biodegradable films made from cellulose and starches have been the most common cellulose-based polymer. Starch-based polymers will swell and deform when exposed to moisture. Edible films made from starch containing 0.5% Mexican oregano (*Lippia berlandieri* Schauer) could inhibit the growth of two types of moulds: *Aspergilus niger* and *Penicillium* spp. when tested in the ADA[33]. Starch-chitosan films containing 0.1 to 1 % oregano essential oils showed antimicrobial activity in the ADA against *Bacillus cereus*, *E. coli*, *S. enteritidis* and *S. aureus* [43].

Whey Protein Isolate

Milk proteins, such as whey and caseinate proteins were extensively studied, owing to their excellent nutritional value and their numerous functional properties, which are important for the formation of edible films. Caseinates can easily form films from aqueous solutions due to their random-coil nature and ability to form extensive intermolecular hydrogen, electrostatic and hydrophobic bonds, resulting in an increase of the interchain cohesion. Moreover, edible films based on milk proteins were reported to be flavourless, tasteless and flexible, and depending on the formulation, they varied from transparent to translucent. Whey and caseins are the main milk protein fractions. Caseins and whey proteins represent respectively 80% and 20% of the total composition of cow milk proteins with a combined concentration of 3% in milk [8]. Films prepared with whey protein isolate are flexible, transparent, and flavourless and have excellent barrier

properties for oxygen, oils and aromas. Whey protein isolates film containing 1 to 4 % oregano, garlic and *Zataria multiflora* Boiss. essential oils showed antimicrobial activity against *L. innocua*, *S. aureus* and *S. enteritidis E. coli* O157:H7, *L. monocytogenes* and *L. plantarum* in the ADA [44-46].

Zein

Zein, the major storage protein of corn (*Zea mays* L.), has been extensively used to produce biodegradable films. Zein can form tough, glossy, hydrophobic, greaseproof films that are resistant to microbial attack, with excellent flexibility and compressibility [47]. Zein based-films were used to immobilize thymol and these films were evaluated against *Bacillus cereus*, *Pseudomonas* sp., *Candida lusitaniae* and *Streptococcus thermophilus*. *In vitro* results showed that the films were effective in inhibiting the growth of the investigated spoilage and pathogen microorganisms during more than 5 days but did not affect the growth of *S. thermophilus* [47].

In situ Experimentation

The previous section has demonstrated the antimicrobial efficiency of edible films and coatings containing essential oils *in vitro*. Even if the ADA is a reliable tool to screen for antimicrobial activity, it is not clear if this property could translate to the food model. This section will present the few studies that presented microbiological results on this subject.

Agar

When agar edible films made from algal extracts (*Gelidium corneum*) containing 0.6 % carvacrol are applied to artificially contaminated ham it allowed a reduction of 0.75 and 1.65 log for *E. coli* O157:H7 and *L. monocytogenes* respectively over 9 days of storage at 4 °C [29].

Alginate

An edible coating made from a combination of alginate and apple puree containing 0.5 to 1 % oregano or 1 to 1.5 % lemongrass essential oils significantly reduced or stopped the

growth of psychrophilic aerobes, yeast and moulds when applied to fresh-cut "Fuji" apples for 21 days at 4°C. When the same coating with essential oils was applied on artificially contaminated fresh-cut "Fuji" apples with *L. monocytogenes*, a reduced microbial count or no growth of the pathogen could be observed over the shelf-life of the product [26]. Alginate containing cinnamon, palmarosa or lemongrass essential oils with malic acid was also used to coat fresh-cut melons (*Cucumis melo* L.). This coating increased the shelf-life by 21 days. In addition, on artificially contaminated melon with *S. entereitidis*, this coating also reduced the pathogen concentration. Coatings containing 0.3 % palmarosa essential oil were the most promising and the sensorial evaluation of this product displayed a good level of acceptance by the panellists [48].

Alginate-based edible films containing 1 % Spanish oregano, Chinese cinnamon and winter savory essential oils were applied to contaminated bologna and ham slices. Films containing essential oils showed a good ability to reduce *S.* Typhimurium concentration on ham and bologna after 5 days of storage at 4 °C. However, *L. monocytogenes* was more resilient to the films and remained almost unaffected by it [49]. Alginate-based edible films containing 1 % Spanish oregano, Chinese cinnamon and winter savory essential oils were applied to contaminated whole beef muscle. Films containing essential oils showed a good ability to reduce *S.* Typhimurium and *E. coli* concentration after 5 days of storage at 4 °C [50].

Chitosan

Chitosan coatings containing rosemary or olive oleoresin at a concentration of 1 % and applied to butternut squash did not produce any significant antimicrobial effect [30]. Artificially contaminated strawberries coated with chitosan or palmitoylated chitosan containing red thyme, peppermint or limonene essential oil at 0.2 % have shown a reduced decay rate over 14 days of storage [51]. Another study used chitosan and palmitoylated chitosan based coatings containing limonene and peppermint essential oil to cover the surface of papaya (*Carica papaya* L.). Native chitosan coatings containing peppermint essential oil showed the most promising results by reducing the spoilage of the papaya [52].

Gelatin

Fresh catfish coated with catfish gelatin containing 0.5 to 2 % origanum essential oil showed a reduction of *S.* Typhimurium and *E. coli* O157:H7 during storage at 4°C and 10°C up to 12 days. Reductions of bacterial populations caused by the coating with essential oils compared to the control were more significant for *S.* Typhimurium than *E.* coli at both temperatures [53]. When fish skin gelatin containing clove essential oils was applied to raw sliced salmon stored at 2°C a reduction of 2 log in total bacteria, 4 log in *Pseudomonas* spp., 2 log in lactic acid bacteria and 8 log in *enterobacteria* was observed after 11 days of storage compared to the control fish without film [36, 37].

Mesquite Gum

Coating papayas with a mesquite gum emulsion containing thyme or Mexican lime essential oils at 0.1 and 0.5 % respectively could reduce incidence of *Colletotrichum gloeosporioides* by 100% thus reducing the severity index of fruit degradation from 3 to 0.2 [54].

Pectin

Apple-based edible films with pectin containing 0.5 to 3 % cinnamaldehyde or carvacrol showed antimicrobial activity on raw chicken breast innoculated by *S. enterica* and *E. coli* O157:H7 stored at either 4 or 23 °C for 72h. The log reduction was ranging from 0.1 to 4.6 for *S. enterica* and 0.2 to 6.8 for *E. coli* O157:H7 as compared to the control depending on the concentration of the essential oil used. The films were more antimicrobial at 23 than at 4°C and carvacrol compared to cinnamaldehyde at the same concentration seemed to be more efficient at reducing the microbial count. When apple-based edible films containing pectin and 0.5 to 3 % cinnamaldehyde or carvacrol were applied to ham inoculated with *L. monocytogenes* stored at 4 and 23 °C for 72 h a reduction up to 3 log could be observed [55].

Soy Proteins

On freshly-ground beef, soy protein films containing 5 % oregano or thyme essential oils showed a reduction in coliforms and *Pseudomonas* spp. but did not have any effect on total viable counts of lactic bacteria and *Staphylococcus* spp [42].

Whey Protein Isolate

Fresh cut beef wrapped in sorbitol-plasticized whey protein isolate film containing 0.5 to 1.5 % oregano essential oil had significantly lower specific growth rate (μ_{max}) for the total aerobe mesophile bacteria, *Pseudomonas* spp. and lactic acid bacteria over 12 days of storage at 5°C [56]. Ouattara *et al.* [13] have demonstrated that the use of an edible coating based on milk proteins containing thyme essential oil and *trans*-cinnamaldehyde and applied by immersion on shrimps was able to extend the shelf life by 12 days. It was also demonstrated that a synergistic inhibitory effect can be obtained by using a combination of coating with irradiation [57].

Future Trends for Edible Films and Coating and Recommendations.

Since in most solid foods, contamination occurs on the surface of the foods, the use of edible coatings can target the pathogens during the food post processing [12]. The control of the diffusion of the active molecules to the food is very important and depends of many factors such as electrostatic interactions, interaction between the active molecules and the polymer, ionic osmosis and structural changes induced by the presence of active compounds in the polymer formulation [12]. These parameters should be considered in future research and developments. Another aspect of the research that is often ignored by most researchers is the impact on the organoleptic properties of the food. Since edible coatings are going to be eaten by the consumers special attention to this topic should be taken. Also, essential oils haven strong impact on the taste and the odour of the product and if the concentrations used are too high, it might impair the food to a point where it won't be eaten by the customer where it nullifies all the efforts to extend the shelf life of the produce. Biodegradable and edible films are very promising packaging for the

improvement of food quality and preservation during processes and storage, and appear to be a successful key for tomorrow's food packaging [58]. Compared to plastic packaging, their cost is higher (10-50 times more) than those made from synthetic-based films. However this should not be a handicap to their development since the quantity used is low and these types of films are especially used for very specific goals in value-added products. Knowledge of edible polymers and that of synthetic materials should be used synergistically for the development of new applications, new biodegradable materials and new environmental approaches [58]. Finally, polymers produced by microorganisms or genetically modified bacteria like bacterial cellulose or polylactic acids are in progress [58].

Conclusion

This chapter showed that various edible films and coatings were evaluated to control the growth of bacteria and pathogens. The development of new technologies in order to improve the water resistance of biodegradable or edible films and the control of the release of compounds is a priority for the future in order to commercialize these types of films. Functionalization, crosslinking, immobilization and new formulation development will allow us to reach these objectives. The efficiency of the combined treatments in order to increase the bacterial sensitivity to the treatment in order to reduce the time of the treatment and enhance the protection of the nutrients will be also a priority in the future.

Acknowledgements

We wish to acknowledge the assistance of the staff librarian, M. Michel Courcelles from the INRS-Institute Armand Frappier library (Quebec, Canada) for his assistance with the literature review.

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Chapitre de livre 2: Food Packaging Containing Biological Control Agents

Chapitre publié:

M. Lacroix and D. Dussault, 2012, Food Packaging Containing Biological Control Agents, In: Current Trends in Biotechnology, Lambert Academic Publishing Santosh Kumar Tiwari et Bijender Singh eds. 301-334.

Contribution des auteurs

J'ai écris en collaboration avec ma directrice de recherche Prof. Monique Lacroix ce chapitre de livre. Une première ébauche de la publication a été écrite par la Prof. Monique Lacroix. Par la suite j'ai fait la revue de la littérature afin de la mettre à jour. J'ai aussi réalisé la mise en forme de la publication.

Résumé en français

Contaminant d'origine alimentaire : En dépit des nouvelles technologies et des concepts de sécurité modernes tel que le HACCP, le nombre de maladies d'origine alimentaire est toujours croissant. Selon Santé Canada, le coût annuel pour traiter les maladies d'origine alimentaire est estimé à 1 milliard de dollars au Canada et entre 5 et 86 milliard au États-Unis. Cette section présentera les bactéries responsables des contaminations des aliments.

Antimicrobiens naturels : Les huiles essentielles servent depuis longtemps comme additifs de saveurs dans les aliments et breuvages. Vu leur composition variée en agents antimicrobiens et antioxydant, ces extraits ont un potentiel pour devenir des agents de conservation naturels pour les aliments. Les bactéries lactiques peuvent aussi produire des métabolites antimicrobiens tels que les acides organiques et les bactériocines. Certains acides organiques sont déjà utilisés à grande échelle dans l'industrie alimentaire. Cette section présentera l'application de ces composés naturels dans les systèmes alimentaires afin de détruire les pathogènes et augmenter le temps de conservation des aliments.

Emballage actif biodégradable : Les composés actifs des huiles essentielles peuvent être ajoutés à l'emballage des aliments pour augmenter le temps de conservation, conserver la couleur et augmenter les qualités nutritionnelles. L'utilisation de films comestibles offre la possibilité d'augmenter l'efficacité des composés antimicrobiens et de prolonger la conservation en assurant un relargage contrôlé des molécules actives dans l'aliment. Cette section discutera des applications dans les systèmes alimentaires et des perspectives futures des emballages actifs antimicrobiens.

Abstract

Food contamination

In spite of modern technologies and safety concepts, such as HACCP, the reported numbers of food-borne illnesses and intoxications are still increasing. According to Health and Welfare Canada, the annual costs to treat foodborne illness are estimated at $1 billion in Canada and from $5 to $86 billion in the United States. This section will present the most important bacteria responsible for food contaminant.

Natural antimicrobials

Essential oils have long served as flavouring agents in food and beverages, and due to their versatile content of antimicrobial and antioxidant compounds; they possess potential as natural agents for food preservation. Lactic acid bacteria can also produce important antimicrobial metabolites called organic acids and bacteriocins. Other organic acids are also widely used in food systems. This section will review some applications of these natural compounds in food system in order to eliminate pathogens in food and also to increase the shelf life.

Biodegradable active packaging

Active compounds can be added to food packaging in order to extend the shelf life, preserve the color and improve the nutritional value of foods. The use of active packaging offers the possibility to improve the efficiency of the antimicrobial compounds and prolong the shelf life of foods by assuring a controlled release of the active compounds. This section will discuss the methods to produce antimicrobial polymers, the major applications in food system and the future perspectives in the field of antimicrobial packaging.

Keys words: active food packaging, natural antimicrobials, food innocuity

Introduction

In spite of modern technologies and safety concepts, such as HACCP, the reported numbers of food-borne illnesses and intoxications are still increasing (O'Sullivan et al., 2002). According to the United Nations, more than 30% of the mortality rate world-wide is caused by alimentary diseases. According to Health and Welfare Canada, the annual costs to treat foodborne illness are estimated at $1 billion in Canada and from $5 to $86 billion in the United States (Anon, 1994). *Campylobacter*, *Listeria*, *Shigella*, *Escherichia coli* and *Salmonella* are the most important bacteria responsible for foodborne illness in Canada. However, *Salmonella*, *Campylobacter jejuni*, *E. coli* 0157:H7, *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium botulinum* can contaminate meat, poultry, eggs, and seafood and dairy products. The desire of most countries to make food safer for consumption requires better food preservation and production techniques. In this regard, the use of natural antimicrobial compounds is an interesting alternative to be considered.

The use of natural products as antimicrobial compounds seems to be an interesting way to control the presence of pathogens in food. Spices are rich in phenolic compounds such as flavonoids and exhibit a large range of biological effects, including antimicrobial and antioxidant properties. Lactic acid bacteria (LAB) have been also used for centuries to preserve food using fermentation. Antimicrobial activities of LAB have been demonstrated in various species and their antagonistic actions have been demonstrated against numerous intestinal and food-borne pathogens. The preservative ability of LAB in foods is attributed to the production of anti-microbial metabolites. One of the more important antimicrobial metabolites proced by LAB are bacteriocins, which are comprised of small peptides. Bacteriocins have attracted attention as potential substitutes for antibiotics to cure and/or prevent bacterial infections and are widely employed in food preservation. The inclusion of essential oils and bacteriocins in food packaging should lead to beneficial effects such as improved safety, quality and flavour; and potential in the biopreservation of food (Lacroix, 2008).

Critical review

Essential oils

Essential oils are secondary metabolites of plants and are used in many applications of the spice and food industry. Each variety of plant has different enzymatic fonctions, genetically codified, which directs biosynthesis by a preferential formation of the components (Russo *et al.*, 1998). They are commonly concentrated in one particular region of the plant such as leaves, bark or fruit, and when they occur in various organs in the same plant, they frequently have different composition profiles. Essential oils have long served as flavouring agents in food and beverages, and due to their versatile content of antimicrobial compounds, they possess potential as natural agents for food preservation (Conner, 1993). The major active compounds present in essential oils are terpenoids and phenolic compounds (Oussalah *et al.*, 2007a).

Essential oils from spices and herbs have been shown to possess antimicrobial functions and could serve as a source of antimicrobial agents against food pathogens (Kim *et al.*, 1995; Deans and Ritchie, 1987). More particularly, essential oils and their components are known to be active against a wide variety of microorganisms, including Gram-negative bacteria (Helander *et al.*, 1998, Skaltsa *et al.*, 2003) and Gram-positive bacteria (Kim *et al.*, 1995). The use of essential oils as natural antibacterial compounds seems to be an interesting way to control the presence of pathogenic bacteria and to extend the shelf life of food (Cragg, 1997; Jantova *et al.*, 2000).

Several studies have been done on the antimicrobial activity of essential oils (Araujo *et al.*, 2003; Burt and Reinders, 2003; Delaquis *et al.*, 2002; Helander *et al.*, 1998; Panizzi *et al.*, 1993). According to these studies, the antimicrobial activity of essential oils is assigned to a number of small terpenoids and phenolic compounds, which also in pure form have been shown to exhibit higher antibacterial or antifungal activity (Suppakul *et al.*, 2003; Conner, 1993). The antibacterial properties of these compounds are normally associated with their lipophilic character, leading to accumulation in membranes and to subsequent attack of the integrity of the membrane, energy depletion (Conner, 1993;

Sivropoulou *et al.*, 1996), significant damage of external envelope (Rhayour *et al.*, 2003) or plasma membrane (Lambert *et al.*, 2001).

Chemical analysis of the most efficient oils like thyme, oregano and cinnamon had shown that the principal active constituents are principally carvacrol, thymol, citral, eugenol, 1-8 cineole, limonene, pinene, linalool and their precursors (Demetzos and Perdetzoglou. 2001; Juliano *et al.*, 2000; Sikkema *et al.*, 1995).

Clove, coriander (*Coriandrum sativum L.Vernacular*), cinnamon, cardamom (*Elettaria cardamomum L.*), thyme, tea tree (*Melaleuca alternifolia*), marjoram (*Pelargonium spp.*),, ho leaf (*Acanthopanax sieboldianus*), rosemary, peppermint (*Mentha X piperita*), palmarosa (*Cymbopogon martinii*), lemon grass (*Cymbopogon citratus*) and sage EOs in the range of 50 – 500 µg/ml possessed stronger antimicrobial activity against yeast than bacterial cultures (Hili *et al.* 1997). Vanillin (2000 ppm), a major constituent of vanilla beans, has a significant inhibitory effect against spoilage yeast like *Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii*, *Debaryomyces hansenii* in apple puree (Cerutti and Alzamora 1996). Garlic culture inhibits the growth of *Candida*, *Cryptococcus*, *Thodotorula torulopsis* and *Trichosporon* (Moore and Atkins 1977). The addition of ground oregano, thyme and their extract in growth media inhibit the production of aflatoxins (B_1 and G_1) of *A. parasiticus* (Salmeron *et al.* 1990). Thymol and carvacrol (0.0025 and 0.05%) at pH 5.5 can inhibit completely the growth of *A. flavus*, *A. niger*, *Geotricum candidum*, *Mucor* spp., *Penicillium* spp. in potato dextrose agar (Akgul and Kivanc 1988). Cinnamon at 0.02, 0.2, 2.0 and 20% inhibited the growth of *A. parasiticus* by 16, 23, 31 and 100% and aflatoxin production by 25, 68, 97, and 100%, respectively (Bullerman 1974). Mustard oil can inhibit the growth of *Saccharomyces ellipsoideus*, *S. cerevisiae* and *Mycoderma vini* in pickles and sauerkraut (Shelef *et al.*, 1984). EOs of spices damages the structural and metabolic enzymes, and inhibits the repair of heat-injured yeasts (Conner and Beuchat 1984).

Oussalah *et al.* (2006a) evaluated the mechanism of the antimicrobial action of Spanish oregano, Chinese cinnamon and savory (*Satureja montana*) EOs. The integrity of cell

membranes and walls of bacteria was studied by the measurement of the intracellular pH (pH_{in}) and ATP concentration, the release of cell constituents and the electronic microscopy observations of the cells when these EOs at their minimal inhibitory concentration (MIC) were in contact with *E. coli* O157:H7 and *L. monocytogenes*. Treatment with these EOs at their MIC (from 0.025 to 0.05%) was able to affect the membrane integrity of bacteria and to induce depletion of the intracellular ATP concentration. An increase of the extracellular ATP concentration was observed only when Spanish oregano and savory oils were in contact with *E. coli* O157:H7 and *L. monocytogenes*. Also, a significantly higher ($P \leq 0.05$) release of cell constituents was observed in the supernatant when *E. coli* O157:H7 and *L. monocytogenes* cells were treated with Chinese cinnamon and Spanish oregano oils. Chinese cinnamon oil was more effective in significantly reducing the pH_{in} of *E. coli* O157:H7, whereas Chinese cinnamon and Spanish oregano more significantly decreased the pH_{in} of *L. monocytogenes*. Electron microscopy observations revealed that except for the cells treated with cinnamon, the cell membranes of both treated bacteria was significantly damaged. These results suggested that degradation of the cytoplamic membrane involves the toxic action of EOs. The concentration of hight molecular weight membrane muropeptide was 6 times higher in the murein of cells treated with EOs. Also, the concentration of lower molecular weight muropeptide in the presence of the EOs at their MIC was 2 times weaker than in the presence of 0.5 of the MIC concentration (Caillet *et al.* 2005, Caillet and Lacroix, 2006). Transmission electron microscope observations revealed that EOs significantly affect the cell wall structure. The authors concluded that the murein was not disrupted by antimicrobial treatments, but the composition and relative percentage of muropeptides were severely modified so that they become critical for the physical integrity of the cell wall.

There are often large differences in the reported antimicrobial activity of oils from the same plant. The reasons of this variability can be due to the different geographical sources, the harvesting seasons, the genotype, the climate, the drying procedure and the distilled part of the plant. All of these variabilities influence the chemical composition and the relative concentration of each constituent in the essential oils (Juliano *et al.*,

2000). A number of essential oils constituents exhibit significant antimicrobial properties when tested separately (Lambert *et al.*, 2001; Ultee *et al.*, 2000a,b). However, there is evidence that essential oils are more strongly antimicrobial than is accounted for by the additive effect of their major antimicrobial components; minor components appear, therefore, to play a significant role (Lataoui and Tantaoui-Elaraki, 1994).

Essentials oils are considered as safe (GRAS) food additives (Lambert *et al.*, 2001), however, their use is often limited to organoleptical criteria. For this reason, it is necessary to determine the minimal concentration necessary to inhibit (MIC) the growth of pathogenic bacteria. The essential oils which exhibit a low MCI have a potential to be added to food as an antimicrobial compound without affecting the sensorial quality of the food. Oussalah *et al.* (2007a) have evaluated twenty eight essential oils for their antibacterial properties, against four pathogenic bacteria *Escherichia coli* O157:H7, *Listeria monocytogenes* 2812 1/2a, *Salmonella* Typhimurium SL 1344 and *Staphylococcus aureus*. Between them, *Corydothymus capitatus, Cinnamomum cassia, Origanum heracleoticum, Satureja hortensis*, *Satureja montana*, and Cinnamomum verum (bark) showed the lowest MIC ($\leq$ 0.05 %) for all bacteria tested. *Thymus vulgaris thymoliferum, Thymus serpyllum, Thymus satureioides*, *Cymbopogon martinii*, *Pimenta dioica*, *Cinnamomum verum* (leaf), *Eugénia caryophyllus* showed a lower antimicrobial activity showing a MIC ranging between 0.05% and 0.4% (vol/vol) against the four bacteria tested. Also thirteen others essential oils were less active showing a MIC value $\geq$ 0.8% (vol/vol) (Table 1).

Table1: Minimal inhibitory concentrations and maximum tolerated concentrations of selected essential oils against pathogenic bacteria.

Plant species	Origin	Distilled part	Main compounds (%)	MIC/MTC (%)			
				E. coli O157:H7	*S.* Typhimurium	*S. aureus*	*L. monocytogenes*
Cinnamomum cassia	China	Leaf-branch	Cinnamaldehyde (65), methoxy-cinnamaldehyde (21)	0.05/0.013	0.025/0.013	0.025/0.013	0.05/0.025
Cinnamomum verum	Sri Lanka	Bark	Cinnamaldehyde (87)	0.025/0.006	0.05/0.025	0.025/0.013	0.05/0.013
Cinnamomum verum	Madagascar	Leaf	Eugenol (63), β-caryophyllene (5)	0.1/0.013	0.1/0.013	0.05/0.013	0.2/0.006
Coriandrum sativum	Russia	Fruit	Linalool (70), α-pinene (6)	0.2/0.006	0.2/0.003	0.2/0.1	>0.8/0.2
Corydothymus capitatus	Spain	Flowering plant	Carvacrol (76)	0.025/0.003	0.025/0.006	0.013/0.006	0.025/0.013
Cymbopogon citratus	Guatemala	Herb grass	Geranial (45), neral (32), limonene (9)	>0.8/0.1	0.8/0.1	0.1/0.05	0.4/0.006
Cymbopogon flexuosus	India	Herb grass	Geranial (46), neral (31)	>0.8/0.4	0.4/0.1	0.1/0.025	0.4/0.1
Cymbopogon martinii var. motia	India	Herb grass	Geraniol (80), geranyl acetate (9)	0.2/0.1	0.2/0.025	0.1/0.025	0.2/0.013
Cymbopogon nardus	Sri Lanka	Herb grass	Geraniol (19), limonene (10), camphene (9)	>0.8/0.1	0.8/0.013	0.4/0.1	0.8/0.013
Cymbopogon winterianus	Vietnam	Herb grass	Citronellal (34), geraniol (21), Citronellol (11)	>0.8/0.05	0.4/0.1	0.05/0.025	0.4/0.2
Eugenia caryophyllus	Madagascar	Flower bud	Eugenol (78), eugenyl acetate (14)	0.1/0.013	0.1/0.025	0.05/0.025	0.2/0.006
Inula graveolens	France	Flowering plant	Bornyl acetate (51), borneol (23), camphene (7)	>0.8/0.013	>0.8/0.4	0.2/0.1	0.8/0.1
Lavandula hybrida reydovan	France	Flowering plant	Linalool (51), linalyl acetate (19), camphor (8)	>0.8/0.1	0.4/0.1	0.8/0.4	>0.8/0.2
Lavandula latifolia spica cineolifera	France	Flowering plant	Linalool (34), 1.8-cineole (22), camphor (15)	>0.8/0.025	0.8/0.2	0.2/0.1	>0.8/0.4
Melaleuca linariifolia	Australia	Leaf	Terpine-4-ol (30), γ-terpinene (19), 1.8 cineole (14)	>0.8/0.025	0.8/0.4	0.4/0.2	>0.8/0.1
Origanum compactum	Morocco	Flowering plant	Carvacrol (22), γ-terpinene (23), thymol (19)	0.025/0.006	0.05/0.006	0.013/0.006	0.1/0.013
Origanum heracleoticum	France	Flowering plant	Carvacrol (54), paracymene (14), γ-terpinene (14)	0.025/0.006	0.05/0.013	0.013/0.006	0.05/0.006
Origanum majorana	Egypt	Flowering plant	Terpinene-4-ol (26), γ-terpinene (12), thuyanol (10)	>0.8/0.013	0.4/0.025	0.2/0.05	>0.8/0.8
Pimenta dioica	Antilles	Leaf	Eugenol (48), myrcene (27), geraniol (10)	0.1/0.025	0.1/0.025	0.1/0.025	0.2/0.006
Satureja hortensis	France	Flowering plant	Carvacrol (41), γ-terpinene (33), *p*-cymene (6)	0.05/0.006	0.05/0.003	0.013/0.006	0.1/0.006
Satureja montana	Slovenia	Flowering plant	Thymol (43), *p*-cymene (12), γ-terpinene (9)	0.05/0.013	0.05/0.013	0.013/0.006	0.05/0.013
Thymus mastichina	Spain	Flowering plant	1.8 cineole (47), linalool (24), limonene (7)	>0.8/0.003	>0.8/0.4	0.8/0.4	>0.8/0.006
Thymus satureoides	Morocco	Flowering plant	Borneol (26), camphene (9), carvacrol (7)	0.2/0.05	0.2/0.025	0.05/0.025	0.4/0.1
Thymus serpyllum	Albania	Flowering plant	Cavacrol (23), *p*-cymene (20), γ-terpinene (18)	0.1/0.025	0.1/0.05	0.05/0.025	0.2/0.006
Thymus vulgaris carvacroliferum	France	Flowering plant	Cavacrol (32), *p*-cymene (24), thymol (12)	0.05/0.003	0.05/0.006	0.025/0.013	0.1/0.013
Thymus vulgaris linaloliferum	France	Flowering plant	Linalool (60), linalyl acetate (10)	>0.8/0.025	0.2/0.1	0.1/0.05	>0.8/0.4
Thymus vulgaris thuyanoliferum	France	Flowering plant	Thujanol 4 (44), myrcene 8-ol (13)	0.8/0.05	0.4/0.1	0.4/0.2	>0.8/0.4
Thymus vulgaris thuyanoliferum	France	Flowering plant	Thymol (38), *p*-cymene (19), γ-terpinene (17)	0.05/0.003	0.1/0.013	0.025/0.006	0.2/0.013

Data from Oussalah et *al.* (2007a). MTC= maximum tolerated concentration; MIC= minimum inhibitory concentration

Bacteriocins

Fermentation is a well known process that is used in order to preserve food quality. Lactic acid bacteria (LAB) have been used for centuries in the fermentation of variety of dairy products (O'Sullivan *et al.* 2002). LAB include the genera *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus*, *Carnobacterium* and *Proprionibacterium* (Savadogo *et al.* 2007). Antimicrobial activities of LAB have been demonstrated in various species and their antagonistic actions are demonstrated against numerous intestinal and food-borne pathogens such as *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium difficile* and others (Mahoney and Henriksson 2003; Millette *et al.* 2007a). The preservative ability of LAB in foods is attributed to the production of anti-microbial metabolites. These metabolites include organic acids, diacetyl, carbone dioxide, hydrogen peroxide, reuterin, derivatives of lactic acid such as hydroxyl lactic acid and also small peptides designated bacteriocins (Millette *et al.* 2007b).

Bacteriocins have been shown to have potential in the preservation of different types of foods (Chen and Hoover 2003). When screened for food applications, the producing strain should be GRAS; the bacteriocin should have a broad spectrum of inhibition; the bacteriocin should be heat-stable and have no associated health risks; the inclusion of the bacteriocin in the food products should lead to beneficial effects such improved safety, quality and flavour; it should have high specificity and potential in the biopreservation of food (Cotter *et al.* 2005).

The incorporation of bacteriocins as a biopreservative ingredient into model food systems has been studied extensively and has been shown to be effective against the growth of pathogens and spoilage microorganisms in foods. Numerous bacteriocins with potential biopreservatives are produced by LAB (Twomey *et al.* 2002; Ennahar and Deschamps, 2000; Duffes *et al.* 1999). Millette *et al.* (2007c, 2008) were the first to isolate bacteriocin-producing strains of *P. acidilactici* MM33 and *Lactococcus lactis* subsp. *lactis* MM19 from human intestine. Their study showed that the supernatant of the culture

have important antimicrobials properties. The antimicrobial metabolites were heat-stable, and were active at a large pH range (2-10).

Nisin is an antimicrobial peptide or Class Ia bacteriocin, produced by several strains of *Lactococcus lactis*. This bacteriocin is approved for use in over 40 countries and has been in use as a food preservative for more than 50 years (Cleveland *et al.* 2001). Nisin is also recognized as GRAS by the United States Food and Drug Administration as stated in the Code of Federal Regulations (CFR section 184.1538). Nisin is now known to form poration complexes in target cell membranes through a multi-step process that includes binding of the C-terminal via electrostatic interaction, then, the N-terminal part of nisin is inserted into the lipid phase of the bilayer. A depletion of the transmembrane potential ($\Delta\psi$) and /or the pH gradient, result in the leakage of cellular materials (Okereke and Montville 1992). It then results in the rapid efflux of small cytoplasmic compounds, e.g. amino acids, potassium, inorganic phosphate, and ATP, and finally to the cell death (Bauer and Dicks 2005).

The effectiveness of nisin on meat is dependent on the meat system and the type of micro-organisms (Davies *et al.* 1997). The antimicrobial efficacy of nisin is dependent upon numerous factors including salt and fat content, basic pH, presence of curing agents and food particle size (Jung *et al.* 1992). The binding of nisin to food components makes it unavailable to inhibit the groeth of microorganisms or reduces its solubility and its dispersion throughout the foodstuff resulting in a decrease in its antimicrobial capacity. Nisin is also relatively insoluble due to its hydrophobic nature and loses its efficacy at pH >5 (Pol and Smid 1999). Storage temperature, modified atmosphere packagaging (MAP), state of the meat (raw or cooked) and the presence of other preservatives can also affect nisin efficacy (Murray and Richards 1998). According to Rose *et al.* (1999), nisin could be inactivated by the presence of small amounts of glutathione, a low molecular mass thiol compound present in fresh raw ground beef. Cutter and Siragusa (1996) observed a lack of effectiveness to control the growth of *Brochothrix thermosphacta* when nisin was applied directly on beef carcass surfaces for a long period of storage. Nisin can also bind sulfhydryl groups or meat particles and interacts strongly with phospholipids which limits

its activity in meat with a high fat content (Chung *et al.*, 1989). However, nisin is stable at low temperature and could be used for meat preservation.

Organic acids

Organic acids such as lactic and acetic acids are widely used especially in processed meat and remains the most effective hurdle against *Listeria monocytogenes* (Shelef, 1994). However, research on the potential use of organic acids as post-processing antimicrobial solutions has been limited (Samelis et *al.*, 2005). According to the same authors, concentrations of 2.5-5 g/100 ml in dipping solutions, lactic acid, acetic acid, sodium acetate, sodium diacetate, potassium sorbate and potassium benzoate may provide extensive inhibition of *Listeria monocytogenes* in refrigerated bologna (Samelis *et al.*, 2001). A combination of 5000 IU/ml of nisin in combination with acetic acid or sodium diacetate (3-5 g/100 ml), or 3 g/100 ml of potassium benzoate was able to inhibit the growth of *Listeria monocytogenes* for more than 90 days.

Antimicrobial compounds in combination

Some compounds are not effective alone, however, when combined together, a synergistic effect could be found. The addition of essential oils under acidic conditions can dissolve and/or attack to the lipid phase of the bacterial membrane (Skandamis and Nychas 2000). *p*-Cymene is not an effective antimicrobial compound, however, when combined with carvacrol, synergism has been observed against *B. cereus* (Ultee *et al.* 2000a, 2000b). It seems that *p*-cymene, incorporated in the lipid bilayer of bacteria, facilitates transport of carvacrol across the cytoplasmic membrane (Ultee *et al.* 2002). Furthermore, most of the EOs are slightly more active against Gram-positive than Gram-negative bacteria (Lambert *et al.* 2001). It is proposed that the outer membrane surrounding the cell wall of Gram-negatives restricts the diffusion of hydrophobic compounds through its lipopolysaccharide covering (Ratledge and Wilkinson 1988). Another study carried out by Mangalassary *et al.* (2008) showed that the combination of nisin (2 mg) with lysozyme (10 mg/ml) applied in package just before the pasteurization of ready to eat turkey and bologna (65°C, 32 s) was able to reduce *Listeria*

monocytogenes below the level of detection. When pasteurisation was applied without antimicrobial agents, this treatment was able to reduce the levels of this microorganism by only 50%.

Pre-treatment with natural antimicrobials can also increase the susceptibility of nisin to inhibit the growth of pathogen when used in combination. The antimicrobial activity of organic acids was increased upon addition of nisin to fresh sausage formulations (Buncic and Arendt, 1997). Increased inhibition of *Listeria monocytogenes* on fresh meat treated with lactic acid or sorbate has been also reported (Ariyapitipun *et al.*, 2000; Avery and Buncic, 1997).

Lysozyme

Lysosyme is another popular natural antimicrobial. This molecule is an enzyme of 129 amino acids that is cross-linkes with four disulfide bonds. Egg white is a good source of lysosyme. This enzyme is stable at 100°C at pH 5.3 and is more active against Gram-positive bacteria (Cagri *et al.*, 2004). Rao *et al.* (2008) have evaluated the efficiency of lysozyme in presence of chitooligosaccharides produced by the irradiation of chitosan. The combination of these compounds showed synergistic effects between the compounds when evaluated in meat system. These compounds were efficient against *Escherichia coli*, *Pseudomonas fluorescens* and *Bacillus cereus* and resulted in an increase of the shelf life of meat for more than 15 days.

Lactoferrin and Lactoferrin hydrolysate

Lactoferrin, lactoferrin hydrolysate and lactoperoxidase were found to exhibit antimicrobial properties (Shah, 2000). Lactoferrin is a single-chain glycoprotein with a molecular weight of about 80 kDa (Baker *et al.*, 2002). This compound inhibits microorganisms by binding iron and making this essential component unavailable to microorganisms (Tomita *et al.*, 2002). Lactoferrin hydrolysate produced by an enzymatic reaction using pepsin contains an antimicrobial peptide named lactoferricin which has greater antimicrobial activity than lactoferrin (Tomita *et al.*, 2002). It is believed that this compound can inhibit microorganisms by damaging the outer cell wall (Murdock and

Matthews, 2002; Yamuchi *et al.*, 1993). Andersson *et al.*(2000) have also reported antifungal properties of lactoferrin hydrolysates.

Lactoperoxidase

Lactoperoxidase is another natural antimicrobial. This compound catalyzes the oxidation of thiocyanate ion (SCN^-) and iodide (I^-), generating oxidizing products such as hypothiocyanite and hypoiodite, which inhibit the growth of microorganisms. This compound is also known for its antifungal properties (Kussendrager and van Hooijdonk, 2000).

Since the active compounds are not stable over time and during processes, some studies have evaluated the possibility of protecting the compound during storage and also to assure a controlled release during storage time. The inclusion of natural antimicrobial compounds in food systems should also lead to beneficial effects such as improved safety, quality and flavour, and potential in the biopreservation of food.

Packaging formulation

The principal role of food packaging is to protect food products from outside influences and damage, to contain the food and to provide consumers with ingredient and nutritional information (Coles, 2003). Food packaging can also maintain the quality and the safety of the food and provide protection against chemical, biological and physical external influences (Marsh and Bugusu, 2007).

The use of plastics in food packaging is very popular due in part for the low cost of materials and its functional advantages (Lopez-Rubio et al., 2004). Polyethylene and polypropylene are the two most widely used plastics in food packaging. They are flexible, strong, light, stable, resistant to moisture and chemicals, permeable to gas and easy to form and process. Plastics are made by condensation polymerization or addition polymerization of monomer units (Marsh and Bugusu, 2007).

Biodegradable films are derived from replenishable agricultural feedstock, animal sources, marine food processing industry wastes, or microbial sources (Marsh and Bugusu, 2007). Edible films typically contain three major components: proteins, polysaccharides and lipids. These composite films may be of heterogenic nature and be formed via a mixture of protein/polysaccharide/plasticizer and lipid (Lacroix and Cooksey, 2005). This approach allows a better exploitation of the functional properties of each of the film's components. Edible films are freestanding structures, first formed and applied to foods. They are formed by casting and drying film-forming solutions on a levelled surface, drying a film-forming solution on a drum drier or using traditional plastic processing techniques, such as extrusion. The film requirements for food are complex. Unlike inert packaged commodities, foods are often dynamic systems with limited shelf-life and very specific packaging needs. In addition, since foods are consumed to sustain life, the need to guarantee safety is a critical dimension of their packaging requirements. While the issue of food quality and safety is first and foremost in the mind of the food scientist, a range of other issues surrounding the development of any food package must be considered before a particular packaging system becomes a reality. Secondary packaging is often used for physical protection of the product. It may be a box surrounding a food packaged in a flexible plastic bag. It could also be a corrugated box containing a number of primary packages in order to ease handling during storage and distribution, improve stackability, or protect the primary packages from mechanical damage during storage and distribution. Due to the barrier properties of edible films; they may not require high-barrier packaging materials. Therefore, the entire packaging structure can be simplified, while satisfying the barrier requirement. The barrier functions include oxygen, moisture and aroma blocks, as well as physical damage prevention. Edible film and coatings must meet the criteria that apply to conventional packaging materials associated with foods. These relate to barrier properties (water, gases, light, aroma), optical properties (e.g. transparency), strength, welding and moulding properties, marking and printing properties, migration/scalping requirements, chemical and temperature resistance properties, disposal requirements, antistatic properties as well as issues such as the user-friendly nature of the material and whether the material is price-competitive. Edible film materials must also comply with food and packaging legislation,

and interactions between the food and packaging material must not compromise food quality or safety. In addition, the intrinsic characteristics of edible film materials, for example whether or not they are biodegradable or edible can place constraints on their use for foods (Lacroix and Le Tien, 2005).

Edible films based on proteins were found to possess satisfactory mechanical properties (Peyron, 1991). However, their predominantly hydrophophilic character results in poor water barrier characteristics (Mc Hugh, 2000). The increase of cohesion between protein polypeptide chains was thought to be effective for the improvement of the barrier properties of the films. For instance, the cross-linking of proteins by means of chemical, enzymatic (transglutaminase) or physical treatments (heating, irradiation) was reported to improve the water vapor barrier as well as the mechanical properties and the resistance to proteolysis of films (Sabato *et al.*, 2001; Ouattara *et al.*, 2002a; Ressouany *et al.*, 2000; Ressouany *et al.*,1998; Brault *et al.*, 1997). When entrapment in cellulose of cross-linked whey proteins is done, this combined treatment generate insoluble films with good mechanical properties, high resistance to attack by proteolytic enzymes and a decrease in water vapor permeability (Le Tien *et al.*, 2000). The addition of lipids in the film formulations can act as a good moisture barrier (Mc Hugh, 2000). Although interesting, protein-lipid films are often difficult to obtain. For example, bilayer film formation requires the use of solvents or high temperatures, making production more costly. Furthermore, separation of the layers may occur with time. For films cast from aqueous, lipid emulsion solutions, the process is complex and the incorporation of lipids is limited. However, the addition of emulsifying agents or surfactants can improve the emulsion stability (Everett, 1989). Finally, decreased mean particle diameters of the emulsion resulted in linear decreases in water vapor permeability values (Mc Hugh *et al.*, 1994). The addition of a polysaccharide in a film formulation could improve the moisture barrier, the resistance and the mechanical properties of the films (Letendre *et al.*, 2002a; Ressouany *et al.*, 1998). It is believed that some polysaccharides such as carboxymethylcellulose, alginate and pectin form charge-charge electrostatic complexes with proteins (Letendre et al., 2002a; Sabato *et al.*, 2001; Thakur *et al.*, 1997; Shih, 1994; Imeson *et al.*, 1977). Under certain conditions, polysaccharides like pectin may form

cross-links with proteins (Thakur *et al.*, 1997). Heat treatment may enhances protein-polysaccharide interactions resulting in a three dimensional network with improved mechanical properties. Untreated polysaccharides keep their ordered structure, thus preventing any unfavourable interactions between their functional groups and those of the proteins (Letendre *et al.*, 2002a). The addition of starch in film formulation can improve the oxygen and oil barrier (Kroger and Igoe, 1971). Alginate can reduce dehydration and retard oxydative off-flavors in meat (Wanstedt *et al.*, 1981). Pectin and chitin can also reduce bacterial growth in food (Chen *et al.*, 1998).

Milk proteins, such as whey and caseinate proteins were extensively studied, owing to their excellent nutritional value and their numerous functional properties, which are important for the formation of edible films (Chen, 1995). Caseinate can easily form films from aqueous solutions due to their random-coil nature and ability to form extensive intermolecular hydrogen, electrostatic and hydrophobic bonds, resulting in an increase of the interchain cohesion (Mc Hugh and Krochta, 1994). Moreover, edible films based on milk proteins were reported to be flavourless, tasteless and flexible, and depending on the formulation, they varied from transparent to translucent (Chen, 1995). Whey and caseins are the main milk protein fractions. Caseins represent 80% of the total composition of milk proteins with a mean concentration of 3% in milk (Dalgleish, 1989).

Collagen is the most popular film used commercially. The film forming collagen has been traditionally used in the meat industry, for example, in the production of edible sausage casing. Collagen is the most commercially successful edible protein film. This protein has largely replaced natural gut casings for sausages. Collagen films are not as strong and tough as cellophane but have a good mechanical properties (Hood, 1987). Collagen film is an excellent oxygen barrier at 0% relative humidity, but oxygen permeability increases rapidly with increasing relative humidity in a manner similar to cellophane (Lieberman and Gilbert, 1973). Different crosslinking chemical agents have been used to improve the mechanical properties, reduce the solubility and improve the thermal stability of films. Carbodiimide, microbial transglutaminase or glutaraldehyde are most widely used (Taylor *et al.*, 2002; Takahashi et al., 1999). Gelatin has been known to form clear, flexible,

strong and oxygen-impermeable films when cast from aqueous solutions in presence of plasticizers (Gennadios *et al.*, 1994).

Myofibrillar proteins represent the main component of muscles (>50% of the total muscle weight) (Cuq, 2002). The use of myofibrillar from under-utilized fish for edible film development was proposed to identify new applications particularly for meat and fish but particularly for fish surimi (Cuq *et al.*, 1998a). Myofibrillar proteins can only be used for film forming applications after purification and concentration from meat or fish (Cuq, 2002). Modification of the physico-chemical properties is possible by using a combination of glycerol and PEG as plasticizers in film formulations based on myofibrillar proteins (Tanaka *et al.*, 2001). However, high molecular weight proteins are generally insoluble or only slightly soluble in water. Despite this shortcoming, these films are transparent and the oxygen permeability is excellent representing a potential for forming a good water-resistant films (Cuq, 2002; Cuq *et al.*, 1998b).

The use of egg white surplus product from egg-breaking food industry for edible films and coatings development was proposed to identify new applications in food industry. Edible packaging from egg white is clear and transparent and their properties are similar to other proteins (Gennadios *et al.*, 1996). Formation of cross-linked films was also observed when proteins were treated under UV resulting in lower total soluble matter and better mechanical properties (Rhim *et al.*, 1999).

Gelatin has been also evaluated for the film-forming capacity. The physico-chemical properties of these films depend in part of the composition of amino acid composition of the substrate used (Gómez-Guillén et al., 2009).

The use of polysaccharides presents advantages due to their availability, low cost and their biodegradability. However, due to the hydrophilic nature of polysaccharides, polysaccharide based films exhibits limited water vapour barrier ability (Gennadios and Kurth, 1997). Also, films based on polysaccharides like alginate, cellulose ethers, chitosan, carrageenan or pectin exhibit good gas barrier properties (Baldwin *et al.*, 1995).

The gas permeability properties of such films result in desirable modified atmospheres, thereby increasing product shelf life without creating anaerobic conditions (Baldwin *et al.*, 1995). Polysaccharide films are used in Japan for meat products, ham and poultry packaging before smoking and steaming processing. The film is dissolved during the process and the coated meat exhibits improved yield, structure and texture and reduced moisture loss (Stollman *et al.*, 1994).

Polysaccharides are obtained from a variety of sources such cellulose derivatives (carboxymethylcellulose, methylcellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, microcrystalline cellulose) seaweed extracts (agar, alginates, carrageenans, furcellaran), various plant and microbial gums (arabic, ghatti, karaya, tragacanth, guar, locust bean, xanthan, gellan, pullulan, levan, elsinan), to connective tissue extracts of crustaceans (chitosan) (Nisperos-Carriedo, 1994) and they are used mostly as stabilizers, emulsifiers and texturizers (Tharanathan and Kittur, 2003). Biodegradable films made from cellulose and starches are is the most common cellulose-based polymers. Starch-based polymers, which swell and deform when exposed to moisture, include amylose, hydroxypropylated starch and dextrin. Other starch-based polymers are polylactide, polyhydroxyalkanoate (PHA), polyhydroxybuterate (PHB) and copolymer of PHB and valeric acid (PHB/V) (Marsh and Bugusu, 2007). Fermentation of starch derivatives by bacterial action can produce polylactide (Auras *et al.*, 2004). PHA, PHB and PHB/V can also be produced by bacterial action on starches (Krochta and DeMulder-Johnston, 1997).

Chitin is one of the most abundant biopolymers in nature (Kenawy *et al.*, 2007). It is found in the shells of crustaceans, the cuticles of insects and the cell walls of fungi (Cho *et al.*, 1999). Chitosan is a deacetylated product of chitin and this polymer has interesting antimicrobial properties and is nontoxic. According to Dutta *et al.* (2009), the antimicrobial activity of chitosan was observed against a wide variety of microorganisms including fungi, and some bacteria. The antimicrobial efficiency of chitosan is influenced by many factors such as the type of chitosan, the degree of chitosan polymerization, the natural nutrient constituency and the environmental conditions. Chitosan was grafted on a

modified poly (ethylene terephthalate) (PET) polymer, and it was shown that this new polymer had a high antimicrobial property against *Staphylococcus aureus*. It seems that the antimicrobial properties could be attributed to the timed-release of the chitosan during long incubation periods (Huh *et al.*, 2001).

Chitin and chitosan are natural antimicrobial compounds against different group of microorganisms such as bacteria, yeast and fungi (Ouattara *et al.*, 2000). Also, the efficiency of the antimicrobial properies of chitosan depends of the degree of polymerization and the degree of acetylation (Chen *et al.*, 1998). Sulfonated chitosan has a minimal inhibitory concentration (MIC) of 100 ppm for *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* and *Vibrio parahaemolyticus*. A MIC of 200 ppm was observed for *Pseudomonas aeruginosa* and *Shigella dysenteriae* (Chen *et al.*, 1998). Chitosan can also reduce the growth of numerous fungi. Chitosan with an $-NH_2$ content of 7.5% markedly reduces the radial growth of *Botrytis cinerea* and *Rhizopus stolonifer* in strawberries (El Ghaouth *et al.*, 1991). Coatings and films based on chitosan and its *N,O*-carboxymethyl derivatives was used to reduce water loss, respiration, and fungal infection in peaches, Japanese pears, kiwi-fruits, strawberries, tomatoes, bell peppers, cucumbers, banana and mangoes (El Ghaouth *et al.*, 1991). Sebti *et al.* (2005) have shown that chitosan can inhibit *Aspergillus niger* growth at a concentration as low as 0.1%. A concentration of 3% could also inhibit *Clostridium perfringens* growth in cooked ground beef and turkey (Juneja *et al.*, 2006). Chitosan has good mechanical properties, flexibility, and is difficult to tear. Chitosan films also have a moderate water vapour permeability and are good barriers to the permeation of oxygen (Tharanathan and Kittur, 2003). Makino and Hirata (1997) have shown that a biodegradable laminate consisting of chitosan-cellulose and polycaprolactone can be used in modified atmosphere packaging of fresh produce. Chitosan was incorporated in Konjac glucomannan (a polysaccharide derived from the konjac tuber) based-films and these films showed antibacterial effects against *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus* (Li *et al.*, 2006a).

Chitosan could also be modified with biologically active moieties by grafting onto its amino groups. Kenawy *et al.* (2005) have observed that these new polymers can be effective against fungi like *Aspergillus flavus* and bacteria such as *Escherichia coli* and *Staphylococcus aureus*. Acidic solutions of chitosan were also used as a dipping solution for irradiated polypropylene films. Carboxymethyl chitosan and carboxymethyl chitin were found to adhere to the irradiated polypropylene films; increasing the amount of chitosan increased the antimicrobial property of the film. These films were used for tomato packaging and were able to keep the produce almost intact with no apparent rotting infection for more than 13 days (Elsabee *et al.*, 2008). Chitosan chemically modified to produce quaternary ammonium salts derivatives was carried out to produce (*N,N,N*-trimethylchitosan TMC). Hydroxypropylcellulose with chitosan or with TMC exhibited a total inhibition of *Listeria monocytogenes* and *Salmonella* Typhimurium (Belalia *et al.*, 2008). Octyl gallate and dodecyl gallate grafted on chitosan based films showed antimicrobial properties against *Listeria monocytogenes* and *Staphylococcus aureus* (Vartiainen *et al.*, 2008) with an observed reduction of 1.4 and 4.9 log, respectively observed.

Generally, polymers in the native state are sensitive to the humidity and soluble to water and consequently, certain modifications are necessary to improve these properties. In certain formulations of film or coating, a functionalization agent is included to increase the hydrophobicity of the polymer in order to improve its moisture barrier properties (gas and water vapour permeability). It is also possible that polymer functionalization permit an increase of the mechanical properties of the film or coating. The functionalization agent can be defined as a substance that it is covalently linked to a polysaccharide matrix, with or without the help of a coupling agent. It generally includes glyceraldehyde, acyl chlorides, fatty acids and anhydrides. Covalent modifications of the polysaccharide for the coating formulations can be obtained by esterification, etherification, carboxymethylation, etc. (Mulhacher *et al.*, 2001; Brode, 1991; Brode *et al.*, 1991; Rutenberg and Solarek, 1984).

Cross-linking between proteins and polysaccharides or treatments used to improve interactions between proteins and polysaccharides may be used to improve functional properties and the resistance of films (Letendre *et al.*, 2002a; Le Tien *et al.*, 2000; Ressouany *et al.*, 2000; Mezgheni *et al.*, 1998a,b). Pectin may form cross-links with proteins under certain conditions (Thakur *et al.*, 1997). Autoclaving enhances protein-polysaccharide, pectin-protein or agar-protein interactions, resulting in a three dimensional network with improved mechanical properties. Letendre *et al.* (2002b) have pointed out the interaction of pectin with calcium and whey proteins after heating treatments.

A major challenge for the material manufacturer is the improvement of water vapour barriers for food applications. When comparing the water vapour transmittance of various polysaccharides to materials based on mineral oil, it becomes clear that it is difficult to produce an edible film or coating with water vapour permeability rates comparable to the ones provided by some conventional plastics. However, if a high water vapor barrier material is required, very few biomaterials could be applied. Consequently, developments are currently focusing on this problem and future biomaterials must also be able to mimic the water vapour barriers of the conventional materials known today (Butler et al., 1996).

The application of nanocomposites also promises to extend the uses of edible and biodegradable films (Sinha and Bousmina 2005). Until now, only few studies have suggested the possibility of incorporating nanoparticles to improve the physical properties of food packaging. This new generation of composite films exhibits significant improvements in modulus, dimensional stability and solvent or gas resistance with respect to the pristine polymer. Nanocomposites also offer extra benefits like low density, transparency, good flow, better surface properties and recyclability (Lacroix, 2009). Various inorganic nanoparticles have been recognized as possible additives to enhance polymer performance. Some examples of these particles are synthetic polymer nanofibers, cellulose nanowhiskers and carbon nanotubes. However, until now, only the layered inorganic solids like clay have attracted some attention (Sorrentino et al. 2007). Nanocomposite films prepared from gelatin and montmorillonite exhibit significantly

improved mechanical properties (Zheng et al. 2002). The incorporation of Cu nanoparticles in chitosan matrix was able to improve the barrier properties of films, decreasing the oxygen permeability as well as water vapour permeability and increasing the protection against UV light. It was also observed that this new composite film has an improved antimicrobial activity against *Staphylococcus aureus* and *Salmonella enterica* serovar Typhimurium. A 4 and 3 log (CFU/ml) reduction was respectively observed as compared to only 1 log (CFU/ml) reduction for the chitosan –based film for the same bacteria (Cárdenas *et al.*, 2009).

Analysis

Natural antimicrobials and active packaging

Contamination by pathogens is a great concern in food sciences. Most of the class I recalls result from postprocessing contamination during subsequent handling and packaging (Cagri *et al.*, 2004). Organic acids, essential oils, salts, organic acids, lipids, spices and bacteriocins (nisin) have been largely studied for their efficiency to control microorganism growth and pathogenic bacteria in foods (Ouattara *et al.*, 2002b, 2001; Padgett *et al.*, 1998; Ming et al., 1997; Siragusa and Dickson, 1992).

Various kinds of packaging used to protect the food against bacterial contamination can be sterilized by irradiation treatment (Brault *et al.*, 1997). Active packaging containing natural antimicrobials may provide protection of perishable foods but also of the packaging itself during storage. In active packaging systems, the choice of incorporative components is often limited by the incompatibility of the component with the packaging material or by the heat liability of the component with the packaging material (Lee *et al.*, 2003). It has been demonstrated that whey protein isolates or soya protein – based films have great potential for acting as an excellent carrier for bioactive compounds in an active food packaging system (Lee *et al.*, 2008; Sabato *et al.*, 2001). Disulfide crosslinking induced by thermal denaturation is an essential step in these investigations (Sabato *et al.*, 2001; Chen 1995). The use of γ-irradiation in order to produce cross-linking via the formation of bi-tyrosine between the protein chains has permitted a significant increase of

the mechanical properties of the films (Cieśla *et al.*, 2006; Mezgheni *et al.*, 1998b; Ressouany *et al.*, 1998). Moreover, glycerol was found to play a double role in enhancing the formation of cross-links within casein chains (Brault *et al.*, 1997). Whey proteins can also be enzymatically crosslinked to form films using transglutaminase (Chen, 1995).

The development of new technologies (functionalization, cross-linking, immobilization etc.) to improve the film properties (controlled release of active molecules, bioactivities protection, resistance to water etc.) of active packaging and coatings represent a need for the future. One strategy to reduce the rate of diffusion is to entrap the antimicrobial in the polymeric matrix (Cagri *et al.*, 2004).

The coverage of Chitosan/layered films with organosillicic was demonstrated to increase their stability during storage (Darder *et al.*, 2003). These nano-particles also can be used to stabilize food additives and efficiently control their diffusion into the food. The controlled release of these compounds is important for long term storage of foods or for imparting specific desirable characteristics, such as flavour, to a food system (Sorrentino *et al.*, 2007). The development of this technology will likely further facilitate development of stable functional and intelligent packaging systems. Also, the use of nanomaterials will most likely enhance the ability to produce more efficient active packaging systems. For example, Rhim *et al.* (2006) found that chitosan-based nanocomposite films blended with some organically modified montmorillonite exhibited antimicrobial activity against Gram-positive bacteria. Whey protein isolate-based nanocomposite films showed also beneficial bacteriostatic effect against Gram-positive bacteria like *Listeria monocytogenes* (Sothornvit *et al.*, 2009).

Plant extracts and active packaging

Essential oils from spices or herbs or liquid smoke can be added to films to modify the flavour but also to introduce antimicrobial properties. The use of these compounds are however limited to their strong flavour. The selection of extracts active at low concentrations represents an interest in food system.

Ouattara *et al.* (2001), have demonstrated that the use of an edible coating based on milk proteins containing natural antimicrobial compounds (e.g. thyme oil and *trans*-cinnamaldehyde) and applied by immersion on shrimps was able to extend the shelf life by 12 days. It was also demonstrated that a synergistic inhibitory effect can be obtained by using a combination of coating with irradiation (Lacroix and Ouattara, 2000). The incorporation of antimicrobial compounds extracted from spices in edible films reduced lipid oxidation and –SH radical production during post-irradiation storage of ground beef (Ouattara *et al.*, 2002b). Salmieri and Lacroix (2006) have incorporated Spanish Oregano, Chinese cinnamon or winter savory as natural antioxidant and antimicrobial agents in alginate/polycaprolactone-based films. The antioxidant properties have shown that the oregano-based films had the highest antiradical properties. Oregano-based films showed also a better level of active compounds in films during storage of whole beef, ham and bologna as compared to cinnamon or savory based-films (Oussalah *et al.* 2006b, 2007b). These results mean a better controlled release of the active compounds during storage. Zein based-films were used to immobilize thymol and these films were evaluated against *Bacillus cereus*, *Pseudomonas* sp., *Candida lusitaniae* and *Streptococcus thermophilus*. Results showed that the films were effective in inhibiting the growth of the investigated spoilage and pathogen microorganisms during more than 5 days but did not affect the growth of *S. thermophilus* (Del Nobile *et al.*, 2008). Carvacrol was immobilized in apple-based films and the efficiency of the films was evaluated against *Escherichia coli* 0157:H7 as well as the stability of the films (Du *et al.*, 2008). A concentration of 1% of carvacrol was able to inhibit the growth of this pathogen over a period of storage up to 49 days. The results also showed that the carvacrol had a dual benefit. It could be used to both impart antimicrobial activities and enhance barrier properties of edible films (Du *et al.*, 2008). *Gelidium corneum*-gelatin blend films were used to immobilize grapefruit seed extract (GFSE) or tea extract (GTE) and these films were evaluated on pork loin inoculated with *E. coli* and *Listeria monocytogenes*. Films containing 0.08% (GFSE) or GTE (2.8%) showed a decrease of the microbial population from 0.69 to 1.11 log CFU/g for *E.coli* and from 1.05 to 1.14 log CFU/g for *L. monocytogenes* after 4 days of storage and improved the quality of the pork (Hong *et al.*, 2009). Chlorophyllins are semi-synthetic porphyrins obtained from chlorophyll and used as food colorants and dietary

supplements. These compounds were incorporated inside gelatin as a polymer matrix for film development (López *et al.*, 2008). This study demonstrated that the film containing sodium magnesium chlorophyllin and water soluble copper chlorophyllin reduced the growth of *Staphylococcus aureus* and *L. monocytogenes* by 5 log and 4 log respectively. Films containing sodium magnesium chlorophyllin were assessed on cooked frankfurters showed that it was possible to reduce by 1 log, the level of *S. aureus* and *L. monocytogenes* after 15 min of contact with the product (López *et al.*, 2008).

Synthetic based-films were also used to develop active packaging. For example, low-density polyethylene-based films were used to immobilize linalool or methylchavicol as antimicrobial compounds. Cheddar cheese, wrapped with films containing methylchavicol or linalool, showed a dramatic reduction in the bacterial population. During the first two days of storage, a 1.90 and 2.14 log units reduction of total mesophilic aerobic bacteria was observed. Also, on inoculated cheese, a decrease by a factor of 47 and 28 was respectively observed for the content of *E. coli* after 15 days of storage at 4°C. For the presence of *L. innocua* a decrease by a factor of 3.4 and 2.7 was observed during storage for the same respective active films (Suppakul *et al.*, 2008). Low density polyethylene / polyamide films were used to immobilize sorbic acid, carvacrol, *trans*-cinnamaldehyde, thymol and rosemary oleoresin. All films showed inhibition zones in an agar diffusion test against *L. innocua* and *E. coli*. Moreover, irradiation treatment of the film (1-3 kGy) was able to improve the moisture barrier functionality of the films (Han *et al.*, 2007). Polyamide-coated low density polyethylene films were also used to immobilize *trans*-cinnamaldehyde. This film was then irradiated to induce the crosslinking in the polymer. This new crosslinked polymer was then able to assure a controlled release of the compound during time. This study demonstrated that irradiation could be used as a controlling factor for the release of active compounds and potential applications in the development of antimicrobial packaging system (Han *et al.*, 2008). Lemon extract was also tested on mozzarella cheese in an immobilized alginate based-film. A 1 and 2 log reduction was respectively observed for *Pseudomonas* and coliforms during storage (10 days at 15 °C). Moreover, a prolongation of the lag time was recorded on coliforms (Conte *et al.*, 2007a).

Farmhouses without refrigerated storehouses have been suffering losses of agricultural products due to the putrefaction related to respiration, transpiration and microbial attack (Kim *et al.*, 2005). The same authors have coated packaging papers with zeolite and a botanical antimicrobial agent containing grapefruit seed extracts. It was found that the zeolite was able to adsorb the gases and that the botanical extract was able to significantly reduce significantly the microbial growth during storage at 15 °C. Cinnamaldehyde and carvacrol were also immobilized in soy protein isolates (SPI) and octenyl-succinate modified starch (OSA) based coating to develop antimicrobial paper. SPI based coatings showed a better ability to retain the active compound on the surface of the paper. Also, a quantity of 5 mg of carvacrol or cinnamaldehyde resulted in *E. coli* growth inhibition from 4-5 log and a growth delay of *Botrytis cinerea* for up to 21 days (Arfa *et al.*, 2007).

Bacteriocins and active packaging

Nisin is the first bacteriocin used in the food industry. This bacteriocin is recognized as a safe biological food preservative. Nisin is a protein of 34 amino acids produced by *Lactococcus lactis* subsp. *lactis* and is inhibitory to a wide range of gram-positive bacteria including *Listeria monocytogenes* and could be efficient against some gram-negative bacteria when a chelating agent is added (Cagri *et al.*, 2004). Nisin is also heat stable, targets Gram-positive bacteria, disrupting their membranes and also interferes with peptydoglycan synthesis (Holzman, 2008). Nisin is one of the most heavily investigated bacteriocins in antimicrobial edible film studies (Jin *et al.*, 2009; Nguyen *et al.*, 2008; Sebti *et al.*, 2007; Li *et al.*, 2006b; Cooksey, 2005).

In order to protect nisin against deleterious agents in fresh beef and to improve the efficiency of this bacteriocin, nisin (500 or 1000 IU/mL) was immobilized into palmitoylated alginate-based films or in activated beads in order to control a pathogen in meat products. Results demonstrated a 2 log reduction of *S. aureus* after one week of storage of sliced meat covered by the film containing 1000 IU/mL of nisin (Millette *et al.* 2007b). The palmitoylation of alginate can be used to produce hydrophobic and water-vapor resistant beads and films. These matrices act also as a control delivery vehicle or to

protect the bioactivity of the immobilized compound (Le Tien *et al.*, 2004). Nisin was also immobilized in plastic film for food application. Nisin was also incorporated into polylactic acid (PLA) polymer in order to control food pathogens i.e. *L. monocytogenes*, *E. coli* 0157:H7 and *Salmonella* Enteritidis. The incorporation of nisin in PLA films reduced pathogens from 2 to 4 log for up to 72 hours (Holzman, 2008, Jin and Zhang, 2008). According to Jin *et al.* (2009), pectin allowing higher amounts of nisin adherence and enhancing the bacteriocin activity against *L. monocytogenes*. Pectin and polylactic based films were able to reduce by 2, 4 and 3.7 log the level of *L. monocytogenes* in pectin plus broth, liquid egg white and orange juice (Jin *et al.*, 2009). Also, a reduction of more than 7.5 log of *E. coli* was observed in orange juice and a 2 log reduction of *Staphylococcus aureus* was observed in liquid egg white when packed in polylactic acid package containing nisin (Jin and Zhang, 2008). Nisin (2500 and 7500 IU/ml) was also found to be an effective antimicrobial against *L. monocytogenes* on processed meats when immobilized in cellulose based-films and have shown, for example a 2 log/g reduction after 14 days of storage as compared to the control (Cooksey, 2005; Nguyen *et al.*, 2008). Guerra *et al.* (2005) have observed a 1.5 log units reduction of the total aerobic count on chopped meat, resulting of a shelf life extension of more than 12 days at 4°C. Scannell *et al.* (2000) investigated the immobilisation of nisin in polyethylene-polyamide based films to form a stable bond with the bacteriocin and the activity was maintained for three months. This active packaging was able to extend the shelf life of ham and reduce the level of *L. innocua* and *S.aureus*. Hydroxypropylmethylcellulose containing nisin (250 µg/mL)-based film was efficient in inhibiting *Aspergillus niger* and *Kocuria rhizophila* food deterioration microorganisms (Sebti *et al.*, 2007). Chitosan was incorporated in films based on glucomannan and chitosan. A concentration of 42 000 IU/g nisin in film was found to have antimicrobial activity against *S. aureus*, *L. monocytogenes* and *Bacillus aureus*. However, in this study, the addition of nisin did not have a synergistic effect with chitosan (Li *et al.*, 2006b). Nisin (2000 IU/cm^2) was also used to coat plastic films in order to control *L. monocytogenes* on vacuum-packaged cold-smoked salmon. This film was able to reduce by 3.9 log CFU/ cm^2 the level of *Listeria* when compared to the control (Neetoo *et al.*, 2008).

Pediocins are another commonly studied group of bacteriocins for edible film due in one part for their wide spectrum of antimicrobial activity and their effectiveness over a wide range of pH values and temperature (Cagri *et al.*, 2004). Films based on cellulose acetate containing 50% of pediocins were able to reduce by 2 log cycles the level of *L. monocytogenes* and a slight reduction of *Salmonella* after 15 days of storage at 12°C was observed (Santiago-Silva *et al.*, 2009).

Enterocins A and B and sakacin K are bacteriocins whose antilisterial activity has been shown in a meat homogenate and have been applied experimentally as ingredients in several meat products (Aymerich *et al.*, 2005, 2000). The antimicrobial packaging containing one of these bacteriocins, nisin, potassium lactate with nisin or lactate alone was applied on ham and then the product was treated with 400 MPa high hydrostatic pressure treatments. The product was analyzed to verify the efficiency of the combined treatment to inhibit *Salmonella*. It was found that nisin was the only treatment that completely inhibited *Salmonella* 24 h after pressurisation and was the most effective treatment to achieve growth inhibition absence when high level inocula is considered (Jofré *et al.*, 2008). Enterocins were immobilized in alginate –based films. These films were used to package cooked ham artificially inoculated with *L. monocytogenes* and the product was treated with high-pressure processing. Packaging with films containing 2000AU/cm^2 of enterocins significantly delayed ($P \leq 0.05$) the growth of *L. monocytogenes* at 6 °C, extending the lag phase for more than 8 days and the level of the pathogen was reduced by around 1 log (from 4 to 3 log CFU/g) during this time period. The combined treatment with high pressure was however able to reduce the level of microorganisms to 0.6 logs CFU/g in *L. monocytogenes* and allowed an extension of the lag phase of *L. monocytogenes* until day 22 (Marcos *et al.*, 2008).

Organic acid and active packaging

Organic acids like acetic acid, lactic acid or sodium diacetate can be incorporated in films preparation. Lactic acid was evaluated on meat carcasses and has shown that this compound is more effective on gram-negative psychrotrophs. This organic acid can be used also as an acidulant in chitosan and collagen based films or to modify the tensile

strength and antimicrobial properties (Cagri *et al.*, 2004; Dickson and Anderson, 1992). Chitosan based films containing lactic acid or citric acids are soft and elastic, however, the addition of formic or acetic acid make them hard and brittle (Cagri *et al.*, 2004). Ye *et al.* (2008a) have incorporated sodium lactate, sodium diacetate, potassium sorbate, and sodium benzoate in chitosan based films. According to his study, the film incorporating sodium lactate was the most effective and was able to inhibiting the growth of *L. monocytogenes* during ten days of storage on cold-smoked salmon. When applied on ham, a growth inhibition was observed for more than 12 weeks of storage (Ye *et al.*, 2008b). Sodium benzoate, nisin and lysozyme were immobilized in swellable polymers based films for their potential to inhibit the growth of *Micrococcus lysodeikticus* and *Saccharomyces cerevisiae*. A growth inhibition of *M. lysodeikticus* was observed at around 10 h in presence of 500 mg of lysozyme and the growth inhibition for *S. cerevisiae* was observed after 200 hours in presence of sodium benzoate (100 mg) in the films. Also results indicated that the release kinetics of the compound could be modulated through the degree of cross-linking of the polymer matrix and a multilayer structure need to be used to control the release of sodium benzoate (Buonocore *et al.*, 2004).

Enzyme and active packaging

Lysozyme is one of the most frequently used antimicrobial enzymes. This enzyme is active mainly against gram-positive bacteria by splitting the bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptydoglycan in their cell walls (Güçbilmez *et al.*, 2007). Zein based films containing partially purified lysozyme in combination with chickpea albumin extract have shown to be effective against the growth of *E. coli* and *B. subtilis* (Güçbilmez *et al.*, 2007). Cellulose acetate was also used to immobilize lysozyme. A concentration of 1.5% of lysozyme in presence of EDTA was able to inhibit the growth of *E. coli* and assure a controlled release of the active compound during more than 25 hours (Gemili *et al.*, 2009). Polyvinylalcohol- based film containing immobilized lysozyme was tested against *Alicylobacillus acidoterrestris,* which is recognized as a cause of spoilage of acidic beverages. Results showed that this film was able to slowing the growth of this bacteria and spores (Conte *et al.*, 2006) and the antimicrobial activity increased as the amount of enzyme incorporated increased

(Conte *et al.*, 2007b). Chitosan- lysozyme composite film was applied on inoculated mozzarella cheese with *E. coli*, *Pseudomonas fluorescens*, mold and yeast. This film was able to completely inhibit the growth of these microorganisms (Duan *et al.*, 2007).

Lactoferrin and active packaging

Lactoferrin is an iron-binding glycoprotein present in bovine milk. This molecule contains 25 amino acid residues and can inhibit bacteria at concentrations of 0.3 - 150 µg/ml (Cagri *et al.*, 2004). Edible whey protein isolate based-films were used to immobilize lactoferrin (LF), lactoferrin hydrolysate (LFH) and lactoperoxidase (LPOS). A concentration of $\geq$ 10 mg/ml of LF or LFH were able to inhibit the growth of *Penicillium commune* in potato broth and films containing LF and LFH were not effective against this microorganism. However, films containing LPOS containing 59 mg/g film inhibited the growth of *P. commune* (Min and Krochta, 2005).

Future perspectives and conclusions

This review showed that various active packaging were evaluated to control the growth of bacteria and pathogens. Since in most solid foods, contamination occurs on the surface of the foods, the use of active packaging can target the pathogens during the food post processing (Cagri *et al.*, 2004). Controlling the diffusion of the active molecules to the food is very important and depends of many factors such as electrostatic interactions, interaction between the active molecules and the polymer, ionic osmosis and structural changes induced by the presence of active compounds in the polymer formulation (Cagri *et al.*, 2004).

Biodegradable and edible films are very promising packaging for the improvement of food quality and preservation during processes and storage, and appear to be a successful key for tomorrow's food packaging (Daraba, 2008). Compared to plastic packaging, their cost is higher (10-50 times more) than those made from synthetic-based films. However, according to the same author, cost is not a handicap to their development because the quantity used is low and these types of films are especially useful for very specific goals in value-added products. Knowledge of edible polymers and that of synthetic materials

should be used synergistically for the development of new applications, new biodegradable materials and new environmental approaches (Dabara, 2008). However, the development of new technologies in order to improve the water resistance of biodegradable or edible films and the control of the release of compounds is a priority for the future in order to commercialize these types of films. Functionalization, crosslinking, immobilization and new formulation development is undergoing. The efficiency of the combined treatments in order to increase the bacterial sensitivity to the treatment in order to reduce the time of the treatment and enhance the protection of the nutrients will be also a priority in the future. Finally, polymers produced by microorganisms or genetically modified bacteria like bacterial cellulose or polylactic acids are in progress (Dabara, 2008).

Acknowledgements

We wish to acknowledge the assistance of the staff librarian, M. Michel Courcelles from the INRS-Institute Armand Frappier library (Quebec, Canada) for his assistance with the literature review.

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Mise en contexte du projet de doctorat

Le présent projet de recherche a été réalisé en collaboration avec la compagnie Les Ingrédients alimentaire BSA. J'ai été récipiendaire de la bourse BMP innovation pour les trois premières années du doctorat ce qui impliquait une collaboration en industrie pour la majeure partie de mon doctorat. Il est à noter que les décisions au niveau du projet de recherche ont été prises en étroite collaboration entre ma directrice de recherche, Prof. Monique Lacroix, les membres de la compagnie BSA responsables du projet, Nathalie Rivard, Claude Benoît et Jocelyn Piette, et ainsi que moi-même. La recherche réalisée devait répondre aux critères d'innovation, de faisabilité industrielle, de coûts et des intérêts commerciaux de l'industriel.

La problématique

La sécurité alimentaire est un enjeu prenant de plus en plus d'importance dans notre société. Ainsi, la problématique principale est de développer et caractériser une solution antimicrobienne d'origine naturelle empêchant la croissance des bactéries pathogènes dans les aliments.

Hypothèse

L'hypothèse de travail stipule que l'incorporation de certains composés d'origine naturelle à l'intérieur d'aliments à base de viande peut limiter la croissance de *Listeria monocytogenes* et autres pathogènes présents dans les alimetns au cours de l'entreposage de l'aliment tout en n'affectant pas les propriétés organoleptiques et en respectant la législation canadienne.

Objectifs

a) Sélectionner *In vitro* des molécules pour leurs propriétés antimicrobiennes contre les bactéries *Escherichia* coli O157:H7, *Salmonella* Typhimurium, *Bacillus cereus*, *Pseudomonoas aeruginosas*, *Staphyloccocus aureus* et *Listeria monocytogenes.*

b) Évaluer l'effet antimicrobien des molécules sélectionnées précedemment contre les bactéries de la flore totale de la viande dans un modèle *in situ*.

c) Évaluer l'effet antimicrobien d'extraits de plantes et de métabolites bactériens dans un modèle *in situ* de viande prêt-à-manger contre la bactérie *L. monocytogenes*.

d) Mettre au point un modèle mathématique démontrant l'impact de certains paramètres physico-chimiques clés d'un aliment sur l'efficacité antimicrobienne de molécules sélectionnés sur la croissance de *L. monocytogenes* dans un modèle de viande prêt-à-manger.

Moyens pour atteindre les objectifs

a) Les propriétées antimicrobiennes de 67 huiles essentielles et extraits de plantes ont été évaluées selon la méthode de dilution en bouillion tel que défini par Gutierrez, Barry-Ryan et Bourke (2008).

b) L'inhibition de la croissance de la flore microbienne de la viande a été évaluée dans un modèle alimentaire à base de viande crue pour démontrer l'efficacité d'un fermentat de bactéries en combinaison avec un traitement physique.

c) Les propriétées antimicrobiennes de différentes molécules ont été évaluées dans un modèle d'aliment prêt-à-manger à base de viande afin de déterminer l'impact sur la croissance de *L. monocytogenes*.

d) Un modèle mathématique a été mis au point en utilisant la méthode des surfaces de réponses de deuxième ordre à composé central afin d'optimiser les différentes formulations antimicrobiennes selon la méthode de Seman, Borger, Meyer, Hall et Milkowski (2002) .

Partie 1: Criblage *In vitro*

Mise en contexte de la Partie 1

Cette section a pour but de présenter les différentes études de criblage effectuées afin de sélectionner des composés potentiels pour le contrôle des pathogènes occasionnellement présents dans les aliments. Le but premier de la recherche était de sélectionner des composés d'origine naturelle pouvant être distribués par le partenaire industriel. La compagnie BSA est spécialisée dans l'importation d'épices des quatre coins de la planète. Vu l'expérience précédente du laboratoire sur la recherche des propriétés antimicrobiennes des huiles essentielles et la collection importante d'huiles essentielles de la compagnie BSA il était de mise d'explorer cette avenue. Étant donné le commencement du projet plusieurs pathogènes différents ont été étudiés.

Au cours du projet de doctorat une opportunité c'est présentée afin d'évaluer le potentiel antimicrobien et anti-cancer d'extrait d'algues et de cyanobactéries de la région de Hawaii. Pour cette raison, le deuxième article présente le criblage de ces différents composés contre plusieurs pathogènes alimentaires.

Il est important de noter que les acides organiques ont été évalués *in vitro* de la même façon que les autres composés. Puisque les résultats n'étaient pas concluants (absence d'efficacité) aucun article n'a été présenté dans cette section. Tel que décrit dans la revue de la littérature, la méthodologie est à l'origine de ces résultats et les acides organiques ont été criblés *in situ* pour ne pas perdre de temps inutilement sur le développement d'une méthode. À l'instar des huiles essentielles, les acides organiques sont moins nombreux et l'évaluation *in situ* directement est donc possible.

Article 1: *In vitro* evaluation of antimicrobial activities of various commercial essential oils, oleoresin and pure compounds against food pathogens and application in ham.

Publié dans Meat Science, 2014, 96 (1), pp. 514-520.

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Running title: *Essential oil and oleoresin antibacterial activity*

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Contribution des auteurs

J'ai imaginé, mis au point et réalisé toutes les expériences de cette étude. Par la suite, j'ai rédigé le manuscrit scientifique. Khanh Dang Vu m'a assisté lors de la révision du manuscrit suite à la rédaction. La Prof. Monique Lacroix était la responsable scientifique et la coordinatrice du projet de recherche. De plus, la Prof. Lacroix a supervisé l'élaboration des protocoles, les discussions scientifiques entourant ce projet et elle a révisé le manuscrit.

Résumé en français

La contamination des aliments par des bactéries pathogènes et de dégradation est un problème sérieux pour la santé humaine. Le contrôle de ces bactéries pourrait toutefois être fait en utilisant des produits antimicrobiens d'origine naturelle. Lors de cette étude, 67 huiles essentielles et oléorésines commerciales ont été évaluées *in vitro* pour leur activité antimicrobienne contre six bactéries pathogènes et de dégradation. Ces huiles essentielles ont été préalablement criblées avec l'utilisation de l'épreuve de diffusion sur disque. Ce premier test a permis de sélectionner 41 produits démontrant une activité antimicrobienne. Ces produits ont été par la suite caractérisés pour leur concentration minimale inhibitrice et leur concentration maximale tolérée contre les six bactéries à l'étude. Des différents produits analysés, cinq se sont démarqués (allylisothiocyanate, cannelle chinoise cassi, oléorésine de cannelle, origan et thym rouge) pour leur spectre large d'inhibition contre toutes les bactéries utilisées incluant la bactérie *Pseudomonas aeruginosa*. Six autres huiles essentielles (citral, coriandre, ail, citronnelle et romarin) ont démontré une activité contre les bactéries Gram positives seulement alors que sept autres produits (cannelle ceylan, clou, laurier, pimento et sariette) possédaient des propriétés antimicrobiennes contre les Gram positifs et négatifs mais étaient sans effet sur la bactérie *P. aeruginosa*. Des analyses sur modèle alimentaire *in situ* ont permis de démontrer que la croissance de *L. monocytogenes* était réduite au cours du temps de 19 et 10% pour l'huile essentielle d'origan et de cannelle respectivement. Dans cette optique, les

meilleures huiles pourraient être combinées à d'autres traitements antimicrobiens pour assurer l'innocuité des aliments.

Abstract

The purpose of this research was to evaluate the application of commercially available essential oils (EOs) and oleoresins to control bacterial pathogens for ready to eat food. In this study, sixty seven commercial EOs, oleoresins (ORs) and pure compounds were used to evaluate in vitro their antimicrobial activity against six food pathogens. These products were first screened for their antimicrobial activity using disk diffusion assay. Forty one products were then chosen for further analysis to determine their minimum inhibitory concentration against 6 different bacteria. There were 5 different products (allylisothiocyanate, cinnamon Chinese cassia, cinnamon OR, oregano and red thyme) that showed high antimicrobial activity against all tested bacteria. Further analysis examined the effect of four selected EOs on controlling the growth rate of mixed cultures of *L. monocytogenes* in ham. A reduction of the growth rate by 19 and 10% was observed when oregano and cinnamon cassia Eos were respectively added in ham at a concentration of 500 ppm.

Keywords: Essential oils, oleoresin, foodborne pathogen, *Listeria monocytogenes,* ham

Introduction

Foods are important vectors of many harmful agents (prions, virus, bacteria, parasites, toxins, heavy metal, etc.) that can cause foodborne diseases. The food contaminants are responsible for various symptoms related to gastroenteritis and can lead to serious consequences such as stomach pain, meningitis, organs failures and even death. In the United States, major known pathogens were responsible for 9.4 million illnesses, 56 000 hospitalizations and 1350 death (Scallan, et al., 2011). In the United States, the economic cost of the foodborne illness is estimated to 77.6 billion $ a year (Scharff, 2012). *Salmonella* sp. *Staphyloccocus* sp. *Escherichia coli*, *Bacillus cereus* account for 28%, 7%, 5% and 2% of foodborne bacterial infection. *Listeria monocytogenes* is one of the bacterial species with the highest mortality rate (30%) (Scallan, et al., 2011).

Essential oils (EOs) are volatile liquids that are extracted from plants. They are mainly used for their organoleptic properties in many industrial domains. Oleoresins (ORs) are naturally occurring mixture of oils and resins extracted from various plants and contain different concentration of EO. In *in vitro* analysis, many EOs have shown antiviral, antimycotic, antitoxigenic, antiparasitic and insecticidal properties (Burt, 2004). The antimicrobial properties of EOs have been recognized since long time (Atkinson & Brice, 1955). But over the last decades, consumers have changed their interests towards using food products containing natural flavoring agents, especially, EOs. EOs are complex mixtures of chemical molecules. Some of these molecules are known to have poor stability at room temperature for an extended time. Even if the sensorial characteristic of the EOs seems to be unaffected during storage; the potent antimicrobial molecules of EOs could be destroyed or modified during that time. It is recommended that EOs would be preserved at room temperature (from 5 to 40 °C) depending on the time of the year and the condition of the warehouse, in a dry place and away from sunlight. Also, the shelf life of EOs is expected to be around 1 to 2 years depending on the manufacturers. These storage conditions could alter the EOs properties and reduce their antimicrobial potentials in comparison to freshly extracted products which are usually used for most antimicrobial studies.

It is known that bacteria express different degree of resistance to the presence of EOs. Due to their hydrophobic nature, EOs tends to affect by a greater number the Gram positive bacteria (i.e. *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes*) followed by Gram negative bacteria (i.e. *E. coli*, *S.* Typhimurium and *Pseudomonas aerugionosa)* (Burt, 2004). Lypopolysaccharide (LPS) component of outer membrane surface of the Gram negative bacteria repels EOs from them. *P. aeruginosas* is resistant to many antiseptics and disinfectants since it poses a higher level of Mg2+ in its outer membrane. Higher levels of magnesium in the membrane will increase the crosslinking between the LPS therefore reduce the size of the porines and ultimately limit the migration of antimicrobial molecules through the bacterial membrane (McDonnell & Russell, 1999). Also, bacteria that have the ability to produce biofilms (such as *P. aerugionosas)* have an additional protection from their hydrophilic expolysaccharides against a wide range of antimicrobials (Kavanaugh & Ribbeck, 2012).

Many EOs extracted from spices or seasoning plants are considered as GRAS by the FDA (Burt, 2004), however, their uses in food as preservatives are normally limited due to the flavor aspect, especially, at higher concentration (Lambert, Skandamis, Coote, & Nychas, 2001). Moreover, very few studies have evaluated the antimicrobial efficiency of EOs in cooked meat products against *L. monocytogenes* using low concentration of EOs. Therefore, the first objective of this study was to screen the most potent products of a commercial collection of EOs, ORs and pure compounds (PC) for their antimicrobial activity. Secondly, based on previous work (Oussalah, Caillet, Saucier, & Lacroix, 2007), selected compounds were quantitatively evaluated for their antimicrobial efficiency, by determining minimal inhibitory concentration (MIC) and maximal tolerated concentration (MTC) against six foodborne pathogens. Two concentrations of bacteria (10^3 and 10^6 CFU/ml) were evaluated to determine if bacterial population density had an effect on the antibacterial efficiency. Finally, the most potential EOs with high antimicrobial activities (low MIC values) against food pathogens were selected for evaluation of their capacity in controlling the growth rate of *L. monocytogenes* in a meat model.

Materials and Methods

Essential oils, oleoresins and pure compounds

Sixty seven EOs, ORs and PC were selected from a list of most used products from a food ingredient supplier (BSA Food Ingredients s.e.c/l.p, Montreal, Qc, Canada). The selected EOs, ORs and PC with their respected common name, major components, plant variety, part of the plant material used for the extraction and volatile oil content are presented in Table 1. All selected EOs were produced by steam distillation. All EOs, ORs and PC were kept at room temperature in an amber glass bottle away from sunlight.

In vitro experiments

Microorganisms and growth condition

Three Gram positive bacteria (*S. aureus, L. monocytogenes*, and *B. cereus*) and three Gram negative bacteria (*E. coli, S.* Typhimurium, and *P. aeruginosa*) were used as target bacteria. Except for *P. aeruginosa*, other bacteria were all selected due to their significant roles causing foodborne illness. *P. aeruginosa* was used as more resistant bacterium to disinfectants. *E. coli* O157:H7 EDL 933, *S. aureus* ATCC 29213, *B. cereus* LSPQ 2872, *S. enterica* serovar Typhimurium SL 1344, *L. monocytogenes* HPB 2812 *and P. aeruginosa* ATCC 15442 were subcultured in tryptic soy broth (1%, v/v) (TSB, Difco Laboratories, Detroit, USA) at 37 °C for 24 h from the stock cultures which were maintained at −80 °C in TSB containing glycerol (20%, w/v). Prior to the experiment, 1 mL of culture was incubated for 24 h at 37 °C in TSB to obtain a working culture containing approximately 10^9 CFU/ml. The bacterial culture was centrifuged at 2,000 × g for 15 min at 4 °C and washed with NaCl (0.85%, w/v) and then resuspended in TSB and incubated for 24 h preceding the experiment. On the day of the broth dilution assay, bacterial cultures were incubated in Mueller-Hinton broth (M-H; Difco Laboratories) for 2h at 37 °C to obtain bacterial cells in the logarithm growth phase.

Disk diffusion assay

The disk diffusion assay is a modified version of the Kirby-Bauer antibiogram assay (Bauer, Kirby, Sherris, & Turck, 1966). Mueller-Hinton agar (M-H; Difco Laboratories) plates where inoculated with one of the selected bacterial specie using a sterile swab dipped into a bacterial suspension containing 10^7 CFU/ml. Then a sterile paper disk (5 mm ID, Whatmann #1) was placed onto the center of the agar plate and 5 µl of EO was placed onto the center of the disk. Plates were then incubated at 37 °C for 24h. Following incubation, growth inhibition zones were observed. Each experiment was done in triplicate.

Antimicrobial broth dilution assay

The influence of varying concentration of EOs, ORs and PC antimicrobial efficacy was assessed against two concentrations (10^3 and 10^6 CFU/ml) of microorganisms using 96-wells (flat bottom wells) micro titer plates (8 × 12; Sarstedt Inc. Montréal, Canada) broth dilution assay according to a modified protocol of (Gutierrez, Barry-Ryan, & Bourke, 2008). On the day of the experiment, the compounds were emulsified in water at a concentration of 2.5% (w/w) using 5% of Tween 80. First column of the plate was filled with 200 µl of M-H broth and columns 2 to 12 was filled with 150 µl M-H broth. Following, 58 µl of stock solution (2.5%) was added to the first column. Two fold serial dilutions we done from column 1 to 11. Row 12 served as a positive control without antimicrobial. Finally, 15 µl of a bacterial suspension (10^4 and 10^7 CFU/ml) was added to each well prior to 24 h incubation at 37 °C. Bacterial concentrations were standardized using optical density ratios that were calculated for each specific bacterial strain. Each row from 1 to 3 and 5 to 7 contained a different bacterial strain and row 4 and 8 served as negative turbidity control without bacteria. On the following day, 15 µl of iodonitrotetrazolium chloride (INT, 0.5 mg/ml in 70% ethanol) were added to each well and the microplates were again incubated at 37°C for 60 min, and in those wells, where bacterial growth occurred, INT changed from clear to purple. Bacterial growth was detected by optical density at 540 nm using a micro titer plate reader (ELISA reader, CLX800-BioTek Instruments) and compared to the negative turbidity control (Valgas, de Souza, Smania, & Smania, 2007). The lowest concentration

that inhibited the growth of the bacteria (the minimum concentration of ~~HE~~EO where the OD of the sample was equal to the OD of the turbidity control) was determined as the minimal inhibitory concentration (MIC). The MIC was determined using a bacterial concentration of 10^6 CFU/ml. Moreover, in this study the maximal tolerated concentration (MTC) was also determined. The MTC is described as the maximal concentration of antimicrobial agents that permit the bacteria to grow without being affected (the maximum concentration of HE where the OD of the sample was equal to the OD of the positive control) (Oussalah, et al., 2007). The MTC was determined using a bacterial concentration of 10^3 CFU/ml.MTC investigated was undertaken in order to characterize the level of HE that did not affect the growth of the target bacteria. Since the ultimate goal of this experiment is to combine multiple antimicrobial compounds in a lower concentration the MTC investigation was important to our research. The MIC and MTC values were determined based on the average MIC or MTC of 3 separate growth kinetic and were expressed in part per million (ppm or mg/l). As a control, a solution of Tween (10%; w/w) was assayed and it did not show any growth inhibition against target bacteria. In this study, the results obtained for the PC were used to compare our results with other experiments published in the literature. Each experiment was done in triplicate.

Inhibitory effect of the selected products against *L. monocytogenes*

Meat Preparation

The *in situ* experiment is a modified version of the ham contamination experiment described by (Seman, Borger, Meyer, Hall, & Milkowski, 2002). The products required for the experiment were formulated using extra lean pork ground meat purchased from a local butcher shop (Metro, Laval, Canada) on the day of preparation of each sample. Products also contained sodium chloride (1.5%), sodium erythorbate (750 ppm), sodium nitrite (192 ppm), tripolyphosphate (0.43%; BSA Food Ingredients, St-Leonard, Canada) and water (amounts varied depending on the desired finished-product formulation). The EOs from garlic, oregano, thyme (red) and cinnamon cassia were used at a concentration of 500 ppm. The EOs concentration was determined as it was the maximum concentration that could be added to ham based on sensorial analysis (results not shown). All

ingredients except meat were mixed together for at least 30 min using a magnetic stirrer in order to prepare the brine solution. Tween 80 at a concentration of 0.1% was added to the brine solution in order to emulsify the EO before mixing with the meat. Ground meat (62.5%) was then mixed with the brine solution (37.5%) for 2 min in a tabletop mixer (A907D, Keenwood, Mississauga, Canada) in order to simulate a high yield boneless ham with 60% injection rate (i.e. 60 kg of brine for 100 kg of meat). The ~~injected~~ meat preparation (≈ 1 kg) was then poured into a rectangular shape disposable aluminium mold (20.3 × 9.8 × 6.3 cm) and cooked in an oven (Mesallion 850, GE, Missisauga, Canada) at 163°C for around 1.25 hours until internal temperature reached 74°C. Products were chilled for 24 h in a refrigerator maintained at 4°C. After chilling, product was divided into 20 g portions and placed into sterile 50 ml Falcon tubes under atmospheric air condition.

Microorganisms and growth condition

For the *in situ* test on meat, *L. monocytogenes* was used as a target food bacterium. Five *Listeria monocytogenes* strains (Health Canada, Health Products and Food Branch (HPB), Ottawa, Canada) were used in this study. All bacterial strains were isolated from contaminated food samples: HPB 2558 1/2b from beef hot dogs, HPB 2818 1/2a from homemade salami, HPB 1043 1/2a from raw turkey, 2569 1/2a from cooked cured sliced turkey and HPB 2371 1/b from raw turkey. Prior to the innoculation procedure, the bacteria were subcultured in tryptic soy broth (TSB, Difco Laboratories, Detroit, USA) at 37 °C for 24 h from the stock culture maintained at −80 °C in TSB containing glycerol (20%, w/v).

Meat inoculation procedure

Ham was used as a meat model. After an initial period of 24h hours of incubation in TSB, the bacteria were resuspended in TSB and incubated for 24 h preceding the experiment to obtain the working cultures of approximately 10^9 CFU/ml. On the day of inoculation of *L. monocytogenes* into ham, 1 ml of each culture of five strains of *L. monocytogenes* were combined. The mixed culture was then diluted 10^4 folds in peptone water (0.1%, Difco) in order to prepare an inoculation solution (between 10^4 and 10^5 CFU/ml). A volume of 500 µl of the contamination solution was then poured on top of

each ham cube and spread by rotating the Falcon tubes for 15 sec in order to achieve a final concentration of 10^3 CFU/g of ham. These tubes were then stored at 4°C in the horizontal position for the duration of the experiment (35 days).

Microbiological analysis

The microbiological analysis of meat samples was conducted every week during five weeks. Three random samples of each recipe were analysed on each day of analysis. Samples were diluted 5 folds using peptone water (0.1%, w/v) in a sterile stomacher filter bag (710 ml, Whirlpak, Nasco, Fort Atkinson, USA) and mechanically blended for 1 min at 260 rpm (400circulator, Seward Laboratory Systems Inc., Port Saint Lucie, USA). Then samples were appropriately diluted 10 folds in peptone water (0.1%, w/v) and 100 µl of each dilution was plated on PALCAM agar (Alpha Bioscience, Baltimore, USA) containing acrylflavine (5 mg/l) polymixin B (10 mg/l) and ceftazidime (8 mg/l) and the plates were incubated at 37 °C for 48 h. After incubation, colonies producing a black precipitate on the agar plate were considered as *L. monocytogenes*. According to the current protocol the detection limit was 50 CFU/g.

Data analysis

The effect of selected EOs on controlling *L. monocytogenes* in meat samples was evaluated based on growth rate (µ) of *L. monocytogenes* in meat samples. The *L. monocytogenes* growth rate was calculated over a period of 5 weeks. The equation (1) was used to describe the *L. monocytogenes* population over time.

$$Y = xe^{\mu t} \quad (1)$$

Where x is the initial population, µ the growth rate of *L. monocytogenes* in Ln CFU /day/g, and t the number of days since the inoculation. The day of inoculation is considered as day 0. At first, CFU counts were transformed using natural logarithm. Then the slope function (Excel 2007, Microsoft, Mississauga, Canada) using equation (2) was used to do a linear regression of the linear part of the growth curve to estimate µ. The RSQ function (Excel 2007) using equation (3) correlation factor R^2 between the estimated growth rate and the experimental data.

$$\mu = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2} \quad (2)$$

$$R^2 = \left(r = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{\sum(x-\bar{x})^2 \sum(y-\bar{y})^2}}\right)^2 \quad (3)$$

Results and discussion

In vitro antimicrobial activities of EOs, ORs and PC

The screening process of antimicrobial activities of sixty seven EOs, ORs and PC against six target bacteria was assessed using the disk diffusion assay. Products that showed inhibition zones against any target bacterial strains in the disk diffusion assay were further selected for evaluation of their MIC and these products are listed in Table 2. From the 67 products that were initially tested, 26 of them (allspice, basilic OR, capsicum OR (1 and 2), celery OR, coriander OR (1, 2), curcuma OR, fennel OR, ginger OR, jalapeno OR, lemon, mace OR (1, 2), majoram OR, mustard (artificial), citrus (1 and 2), oregano OR, paprika OR, pepper (black), pepper OR (black), pepper OR (white), rosemary OR (2), star anise, tarragon OR) did not show any zone of inhibition that were greater than the size of the paper disk (5.5 mm). Therefore, these products were not selected for evaluation of their MIC values against the 6 target bacteria.

Due to the nature of the EOs that have astringent taste at high concentrations, a maximum concentration of 5000 ppm was used as the maximum threshold since the main goal of this study was to screen the most potent products for the development of an antimicrobial formulation that could be added to food.

Minimum inhibitory concentration (MIC) of EOs, ORs and PC

The compounds with their respective MIC values against six bacterial strains (*E. coli* O157:H7, *S. aureus, B. cereus, S. enterica* serovar Typhimurium, *L. monocytogenes* and *P. aeruginosa*) are presented in Table 2. Since the standard broth dilution method usually use a bacterial concentration of 10^6 CFU/g, the comparison of antimicrobial efficiency was done at that concentration using their respective MIC. From the broth

dilution assay, 22 of 41 products showed a MIC values that was greater than 5000 ppm for most of all target bacteria evaluated.

For *L. monocytogenes*, the EOs, ORs and PC showing the highest antimicrobial activities (MIC values ≤ 1000 ppm) were allylisothiocyanate (AITC) (pure) > oregano > cinnamon Chinese cassia = cinnamon OR = winter savory > thyme (red > citral (pure). The EOs and ORs showing average antimicrobial activities (1000 ppm < MIC values ≤ 2500) were lemongrass = rosemary OR (3) > clove (leaf) > clove (buds) > cinnamon ceylan = pimento (berry) = pimento (leaf) = laurel.

For *S. aureus*, the EOs showing the highest activities (MIC values ≤ 1000 ppm) were thyme (red) = citral (pure) > oregano > AITC (pure) = cinnamon Chinese cassia = winter savory = lemongrass > garlic. The EOs and ORs with average antimicrobial activities (1000 ppm < MIC values ≤ 2500) were cinnamon OR = clove (buds) > rosemary OR (3) = cinnamon ceylan = pimento (berry) = pimento (leaf) = laurel = rosemary OR (1) > clove (leaf) > coriander.

For *B. cereus*, the EOs and PC showing the highest antimicrobial activity (MIC values ≤ 1000 ppm) were lemongrass > cinnamon Chinese cassia > oregano = cinnamon OR > AITC (pure) = winter savory = citral (pure) > garlic > thyme (red). The EOs and ORs with average antimicrobial activities (1000 ppm < MIC values ≤ 2500) were clove (buds) = pimento (berry) = laurel > rosemary OR (3) = clove (leaf) = cinnamon ceylan = pimento (leaf) = rosemary OR (1) > coriander. The EOs and ORs with lowest antimicrobial activity (2500 ppm < MIC values ≤ 5000) was caraway. *B. cereus* seemed to get the lowest MIC values for most of the EOs and ORs as compared to *L. monocytogenes* and *S. aureus*.

For *S.* Typhimurium, the most effectives EOs and PC were AITC (pure) > Cinnamon Chinese cassia > oregano > cinnamon OR > winter savory = clove (leaf) = clove (buds) = cinnamon ceylan = pimento (berry) = pimento (leaf) > thyme (red) = laurel. In case of *E. coli*, the antimicrobial profile of the EOs and PC was as follow: AITC (pure) > cinnamon Chinese cassia > oregano > cinnamon OR > winter savory = thyme

(red) = clove (leaf) = clove (buds) = cinnamon ceylan = pimento (berry) = laurel > pimento (leaf).

There were only five products demonstrating MIC values smaller than 5000 ppm against *P. aeruginosa*: AITC (pure) > cinnamon Chinese cassia = cinnamon OR > oregano > thyme (red). Also, these products were found to have the smallest MIC against all tested bacteria (Gram positive, Gram negative and *P. aeruginosa*). These results confirm the fact that *P. aeruginosas* is a more resistant specie to EOs than other Gram negative bacteria. These products can also be grouped in three distinctive categories: the first one contains AITC extracted from mustard; the second one is composed of cinnamon based products and the third one regroup oregano and thyme EOs that both compositions contain thymol and carvacrol in varying concentrations. AITC is the main constituent of the EO extracted from mustard seed. In our study, the MIC values of AITC against all tested microorganisms were the lowest ones observed. Lin, Preston, & Wei (2000) have reported similar antibacterial properties of AITC. In this same study, it was also found that the antimicrobial activity of AITC was similar against Gram negative bacteria and Gram positive bacteria. Our results can confirm the same observation since the MIC of all tested organisms except *P. aeruginosas* is 313 ppm.

Trans-cinnamaldehyde is the main antimicrobial component usually found in Chinese cinnamon cassia EO. The similar result between the EO and the OR can be explained by the fact that the OR is composed of 60% EO. The composition of Chinese cinnamon (*trans*-cinnamaldehyde (30%), 3-methoxy-1,2-propanediol (29%), o-methoxy-cinnamaldehyde (25%) and coumarin (6%)) has been reported in the literature (Wang, Wang, & Yang, 2009). Ooi, et al. (2006) showed that Chinese cinnamon had antibacterial properties with the MIC values ranging from 75 to 600 ppm depending on the tested bacterial strains. These authors also demonstrated that cinnamon cassia had the highest antimicrobial potential as compared to other types of cinnamon (Ooi, et al., 2006). We have also observed in this study that cinnamon cassia had a smaller MIC value as compared to cinnamon ceylan.

Our results showed broad spectrum antibacterial activity against all groups of bacteria for oregano and thyme EOs. Oregano EO contains carvacrol (66%) and p-

cymene (17%) as their mains constituents (Bounatirou, et al., 2007). Oregano has been reported in the literature to have antibacterial properties against a wide range of microorganisms. Gutierrez, et al. (2008) showed that *B. cereus*, *E. coli*, *L. monocytogenes* and *P. aeruginosa* were sensitive to the oregano EO. There seemed to be no difference in antimicrobial effects between Gram positive and Gram negative bacteria. Although, our results show MIC values for *P. aeruginosa* were up to 10 folds higher than other Gram negatives bacteria. In previous experiments, red thyme has been found to be principally made of thymol (36 %), *p*-cymene (22%) and γ-terpinene (17%) (Hudaib, Speroni, Di Pietra, & Cavrini, 2002). Red thymes also showed antimicrobial activity against *S. aureus*, *B. cereus*, *S.* Typhimurium and *P. aeruginosas* and in general the Gram positive were more sensitive than the Gram negative (Al-Bayati, 2008).

It can be observed that there were seven EOs (cinnamon ceylan, clove (leaf and buds), laurel, pimento (berry and leaf) and winter savory) that were active against both Gram positive and negative bacteria. They showed a wide antimicrobial spectrum but were ineffective against *P. aeruginosa*. The EO from cinnamon ceylan usually contains from 70 to 90% eugenol as its main component. It is reported that cinnamon ceylan EO has antibacterial properties against most types of bacteria. Usually, *trans*-cinamaldehyde is responsible for the high antimicrobial activity of cinnamon EO. A higher concentration of *trans*-cinnamaldehyde is normally found in the EO distilled from the bark than the leave. Eugenol has less antimicrobial activity than *trans*-cinnamaldehyde and could be explained by the lower MIC values found for Chinese cinnamon than Ceylan (Jayaprakasha & Rao, 2011). Clove EO is mainly composed of eugenol (77%) and β-caryophyllene (17%) (Jirovetz, et al., 2006). (Magina, et al., 2009) have shown that clove EO possess antimicrobial activity with MIC values ranging from 156 to 1100 ppm for *S. aureus*, *E. coli* and *P. aeruginosa* and they also found that *S. aureus* was the most sensitive bacterium and *P. aeruginosa* was the most resistant bacterium (Magina, et al., 2009). Laurel EO is mainly composed of eugenol (55%) and myrcene (27%) (Ayedoun, et al., 1996). It was reported that EO from laurel showed antimicrobial properties against Gram positive bacteria (*Bacillus* spp. and *Staphylococcus aureus)* with MIC values of 150 to 200 ppm. The MIC values against Gram negative bacteria (*S.* Typhimurium, *E. coli* and *P. aeruginosa*) were from 200 to 500 ppm (Saenz, Tornos, Alvarez, Fernandez,

& Garcia, 2004). The major molecule found in pimento EO is eugenol (80%) (Minott & Brown, 2007). Winter savory composition was reported as follow: carvacrol (62 %), thymol (10%), p-cymene (7%), c-terpinene (6%) (Grosso, et al., 2009). Winter savory EO showed antimicrobial activity in the disc diffusion assay against *E. coli*, *S.* Typhimurium, *P. aeruginosa* and *L. monocytogenes* (Serrano, et al., 2011). Recently, Klein et al. (2013) have evaluated the antimicrobial activity of six EO components such as thymol, carvacrol, linalool, 1,8-cineol, α-pinene or α-terpineol against 4 bacteria (*E. coli*, *Aeromonas hydrophila*, *Pseudomonas fragi*, *Brochothrix thermosphacta*. The authors found that the most susceptible bacterium was *A. hydrophila* (Gram negative), then, *B. thermosphacta* (Gram positive) and *E. coli* (Gram negative). Thus, it seems that the EO components express the specific antimicrobial activity against different target bacteria.

It can be observed that six products (citral, coriander 2, garlic, lemongrass, rosemary OR (1, 3)) were only active against Gram positive bacteria. Previous studies have shown that coriander EO possess antimicrobial properties. Coriander EO is mainly composed of linalool (73 %) and a minimal amount (less than 5%) of α-pinene and p-cymene (Zoubiri & Baaliouamer, 2010). Coriander EO showed an MIC ranging from a 1000 to 16000 ppm against a wide range of microorganisms where *Pseudomonas aeruginosa* was the most resistant, *Salmonella* Typhimurium showed intermediate resistance (4000 ppm) and *Bacillus cereus* was the most sensitive (Silva, Ferreira, Queiroz, & Domingues, 2011). Garlic EO is composed principally of di-2-propenyltrisulfide (60 %, di-2-propenyl disulfide (13%), methyl 2-propenyl trisulfide (11%) and di-2-propenyltetrasulfide (5%) (Sowbhagya, Purnima, Florence, Rao, & Srinivas, 2009). In fact, garlic EO showed antimicrobial properties in the disc diffusion assay against *S. aureus* and *S.* Enteritidis (Benkeblia, 2004). Lemongrass is mainly composed of α-citral (geranial) (43%) and β-citral (neral) (37%) (Leimann, Goncalves, Machado, & Bolzan, 2009). (Kotzekidou, Giannakidis, & Boulamatsis, 2008) showed that lemongrass EO have antimicrobial activity against Gram positive *S. aureus* and *L. monocytogenes* but did not show any effect on the Gram negative *S.* Typhimurium and *E. coli*. It can be observed that the main antimicrobial activity of lemongrass EO comes from its main component citral since most of the CMI values were almost identical for the two (Table 2). Rosemary EO is mainly composed of 1,8 cineole (44%), Camphor (12%), α-pinene (10%), β-pinene (5%), β-

caryophyllene (5%). It was also reported that rosemary EO showed antimicrobial activity against Gram positive bacteria but lack the same properties against Gram negative microorganisms (Ait-Ouazzou et al., 2011).

Minimum Tolerated Concentration of EOs, ORs and PC

The MTC is described as the maximal concentration of antimicrobial agents that permit the target bacteria to grow (Oussalah et al., 2007). The tested compounds with their respective MTC values against a low concentration (10^3 CFU/ml) of six bacterial strains (*E. coli* O157:H7, *S. aureus, B. cereus, S. enterica* serovar Typhimurium, *L. monocytogenes* and *P. aeruginosa*) are presented in Table 2. Most of the compounds showed antimicrobial activity, and the MTC values observed are 1 to 2 times smaller than the MIC values. However, AITC and garlic showed a MTC value of more than 4 times smaller than their respective MIC values.

Therefore, our obtained results are in line with previous studies in which EOs, ORs and PC have shown various degrees of antimicrobial activity against foodborne pathogens. The main mechanism of action that has been proposed for the antibacterial effects is the disruption of bacterial membrane by the hydrophobic compounds that they are principally composed of EOs and ORs (Burt, 2004). In this experiment, it could be found that there was no antibacterial activity that was specific against only one bacterial species. The only kind of specificity that could be observed was the absence of inhibition for Gram negative in comparison to Gram positive. These results would confirm the hypothesis of membrane disruption since the membrane composition is the properties differentiate the two groups (Gram positive and negative).

In this study, most of the products that showed antimicrobial activity were EOs. ORs were in all cases less effective than the corresponding EOs. One exception could be observed when comparing the cinnamon OR to the cinnamon Chinese cassia EO since both products had the same MIC values. This could be explained by the fact that the OR had a high level of EO (62%) (Table 1). In order to test the feasibility of the addition of EOs in meat products, four of the best candidates were selected to test *in situ* in ham inoculated with *L. monocytogenes*.

Effects of selected EOs on controlling the growth rate of *L. monocytogenes* in a meat model

Four EOs were selected for their antimicrobial properties and the feasibility of their addition into meat products. AITC was not selected for further studies because of its high volatility and hazardous properties when handled at high concentration. Garlic, oregano, thym (red) and cinnamon cassia were selected to be used at 500 ppm (0.05 % v/w) since this concentration is organoleptically acceptable based on the sensory evaluation (data not shown). The concentration of a mix of 5 strains of *L. monocytogenes* in ham containing EOs is presented in Figure 1. Respective growth rate of *L. monocytogenes* in control ham and hams containing EOs of garlic, oregano, thym (red) and cinnamon cassia are 0.404, 0.396, 0.328, 0.396, and 0.362 ln CFU/g/day (Table 3). Growth rate inhibition presented in Table 3 is the difference of the test group compared to the control group. Growth rate for hams containing EOs of garlic, and thyme (red) were not significantly different ($P > 0.05$) from the control ham. However, hams containing EOs of oregano and cinnamon cassia showed a reduced growth rate significantly ($P \leq 0.05$) by 0.076 and 0.042 ln CFU/g/day, which were equivalent to 19 and 10 % of growth inhibition, respectively (Table 3). It is also interesting to note that the ham containing oregano EO showed a lag phase of 7 days before showing a positive growth rate for *L. monocytogenes*. Other studies have reported the antimicrobial efficiency of EOs in meat products. However, many of these researches were done using uncooked meat products. In presence of oregano EO at a concentration of 8000 ppm, it was reported that *L. monocytogenes* population in raw beef filet was reduced by 1 log CFU at day 1. In that same experiment, in presence of the raw meat autochthonous bacterial flora, modified atmosphere and oregano EO, *L. monocytogenes* concentration diminished below the detection limit over the shelf-life (Tsigarida, Skandamis, & Nychas, 2000). Our research has demonstrated that antimicrobial effect of EO can still be detected when using lower concentration (500 ppm).

Conclusion

In conclusion, commercially available EOs possesses potent antimicrobial activity. In comparison with their related ORs, EOs are much more effective against tested bacteria in

term of MIC values. Although, even if numerous EOs and ORs possess antibacterial properties their strong flavoring properties will ultimately limit their usage as food antimicrobial in food. Following our research, only a few potent EOs could be used in combination with other antimicrobial treatments to inhibit the growth of food pathogens. Current sensorial limit is too low to obtain satisfactory microbial inhibition when EOs are used alone. Further analysis, in artificially contaminated ham with *L. monocytogenes* showed a reduction of the growth rate when using oregano and cinnamon cassia EOs. These EOs could be used for food preservation against food pathogens in combinations with other treatments to increase the antimicrobial effects as well as to reduce the concentration of EOs required to be used. Other antimicrobial compounds such as organic acids or bacterial metabolites or other physical methods such as ionizing irradiation or high pressure processing can be used in combination with the most potent EOs to achieve the required bactericidal effect without impairing the taste of the food products.

Acknowledgements

This research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and BSA Food Ingredients s.e.c/l.p (Montreal, Qc, Canada) under a research contract agreement with the INRS. The NSERC, le Fond de Recherche sur la Nature et les Technologies (FQRNT) and BSA Food Ingredients s.e.c/l.p supported Dominic Dussault through the Industrial Innovation Scholarships BMP Innovation. The Armand-Frappier Fondation also supported Dominic Dussault through the doctoral scholarship.

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Table 1: Description of essential oils and oleoresin selected for the study

#	common name	Plant variety	part of plant	vol oil (%)*
1	Allspice	*Pimenta officinalis*	berry	20
2	Allylisothiocyanate (pure)	Allyl isothiocyanate	seed	100
3	Basilic	*Ocimum basilicum* L.	leaf	100
4	Basilic OR	*Ocimum basilicum* L.	leaf	20
5	Capsicum OR 1	*Capsicum annuum*	fruit	n.a.
6	Capsicum OR 2	*Capsicum annuum*	fruit	n.a.
7	Caraway	*Carum carvi* L.	seed	100
8	Celery	*Apium graveolens* L.	seed	100
9	Celery OR	*Apium graveolens* L.	seed	10
10	Cinnamon ceylan	*Cinnamomum zeylanicum*	leaf	100
11	Cinnamon Chinese cassia	*Cinnamomum cassia*	bark	100
12	Cinnamon OR	*Cinnamomum cassia*	bark	62
13	Citral (pure)	Citral (2,6-octadienal, 3, 7-dimethyl)	lemongrass oil or lemon oil	100
14	Clove (leaf)	*Eugenia* spp.	leaf	100
15	Clove (buds)	*Eugenia* spp.	bud	100
16	Coriander OR 1	*Coriandrum sativum* L	leaf	15
17	Coriander OR 2	*Coriandrum sativum* L.	seed	6
18	Coriander	*Coriandrum sativum* L.	seed	100
19	Cumin	*Cuminum cyminum* L.	seed	100
20	Cumin OR	Cuminum cyminum L.	seed	60
21	Curcuma OR	*Curcuma Longa* L.	root	n.a.

22	Dill	*Anethum graveolers* L.	weed	100
23	Fennel (sweet)	*Foeniculum vulgare* Mill.car.dulce	seed	100
24	Fennel OR	*Foeniculum vulgare*	seed	10
25	Garlic	*Allium sativum* L.	crushed bulb and clove	100
26	Ginger	*Zingiber officinale roscoe*	root	100
27	Ginger OR	*Zingiber officinale roscoe*	root	28
28	Jalapeno OR	*Capsicum annum*	fruit	n.a.
29	Lemon	*Citrus limon* L.	peel	100
30	Lemongrass	*Cymbopogon citratus* DC and *Andropogon nardus* var. ceriferus	grass	100
31	Laurel	*Pimenta racemosa*	leaf	100
32	Lime	*Citrus latifolia*	peel	100
33	Mace OR (1)	*Myristica fragrans* Houtt	ground ripe seed	25
34	Mace OR (2)	*Myristica Fragrans* Houtt	ground ripe seed	100
35	Marjoram	*Thymus mastichina*	leaf	100
36	Marjoram OR	*Origanum majorana* L.	leaf	40
37	Mustard (artificial, 12 % AIT)	*Brassica nigra* L. Koch.	seed	n.a.
38	Mustard (spray dried)	*Brassica juncea* L.	seed	20
39	Nutmeg	*Myristica fragrans* Houtt	seed	100
40	Onion	*Allium cepa* L.	bulb	n.a.
41	Onion (roasted)	*Allium cepa* L.	bulb	n.a.
42	Citrus 1	mixed	peel	100
43	Citrus 2	mixed	leaf, peal and twig	100
44	Oregano OR	*Origanum Vulgare L.*	leaf	5
45	Oregano	*Thymus capitatus* Hoff.	leaf	100
46	Paprika OR 1	*Capsicum annuum* L.	fruit	n.a.

47	Paprika OR 2	*Capsicum annuum* L.	fruit	n.a.
48	Parsley	*Petroselinum sativum* Foffm.	leaf	100
49	Pepper (black)	*Piper nigrum* L.	berry	100
50	Pepper (black) OR	*Piper nigrum* L.	berry	18
51	Pepper (white)	*Piper nigrum* L.	berry	100
52	Pepper (white) OR	*Piper nigrum* L.	berry	10
53	Pimento (berry)	*Pimenta officinalis* Lindley	berry	100
54	Pimento (leaf)	*Pimenta officinalis* Lindley	leaf	100
55	Peppermint	*Mentha Piperita* L.	leaf	100
56	Rosemary (1)	*Rosmarinus officinalis*	leaf	10
57	Rosemary (2)	*Rosemarinus officinalis*	leaf	100
58	Rosemary OR (1)	*Rosmarinus officinalis*	leaf	n.a.
59	Rosemary OR (2)	*Rosmarinus officinalis*	leaf	n.a.
60	Rosemary OR (3)	*Rosmarinus officinalis*	leaf	n.a.
61	Sage (dalmatian)	*Salvia officinalis* L.	leaf	100
62	Sage OR	*Salvia officinalis* L.	leaf	30
63	Star anise	*Illicium Verum Hooker Filius*	dried ripe fruit	100
64	Tarragon OR	*Artemisia dracunculus* L.	leaf	12
65	Thyme (red)	*Thymus vulgaris* and *Thymus zygid* L. var gacilis Boissier	leaf	100
66	Thyme OR	*Thymus vulgaris* L.	leaf	10
67	Winter savory	*Satureia montana* L.	weed	100

n.a.= non-available; *volatile EO refers to the % of volatile compound present in the product that was used for the experiment; concentrations were taken from the manufacturer's specification sheet

Table 2: Minimal inhibitory concentration and maximal tolerated concentration of selected essential oils, oleoresins ans pure compounds against foodborne bacteria

		MIC (ppm)						MTC (ppm)					
#	common name	*L. mono*	*S. aureus*	*B. cereus*	*Salmo*	*E. coli*	*Pseudo*	*L. mono*	*S. aureus*	*B. cereus*	*Salmo*	*E. coli*	*Pseudo*
2	Allylisothiocyanate (pure)	313	313	313	313	313	78	39	78	39	39	156	39
3	Basilic	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	1875	>5000	>5000	>5000
7	Caraway	>5000	>5000	4167	>5000	5000	>5000	>5000	1875	625	2083	1667	>5000
8	Celery	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
10	Cinnamon ceylan	2500	1250	1250	1250	1250	>5000	1250	1250	1042	1250	1250	3333
11	Cinnamon Chinese cassia	625	625	208	417	417	1250	156	313	104	313	156	625
12	Cinnamon OR	625	1042	261	625	625	1250	261	625	208	417	261	833
13	Citral (pure)	938	313	313	5000	5000	>5000	365	469	469	1042	833	1250
14	Clove (leaf)	1667	1667	1250	1250	1250	5000	1250	1667	1042	1250	1042	2083
15	Clove (buds)	1875	1042	1042	1250	1250	>5000	1250	1250	1250	1250	1042	5000
18	Coriander	>5000	2500	2500	>5000	5000	>5000	2500	1875	1250	1875	1250	>5000
19	Cumin	>5000	>5000	5000	>5000	>5000	>5000	>5000	>5000	1250	>5000	3750	>5000
20	Cumin OR	>5000	>5000	5000	>5000	>5000	>5000	>5000	>5000	1563	>5000	5000	>5000
22	Dill	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
23	Fennel (sweet)	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
25	Garlic	>5000	729	365	>5000	>5000	>5000	26	52	52	>5000	>5000	>5000
26	Ginger	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
30	Lemongrass	1250	625	156	>5000	5000	>5000	313	313	208	2917	1667	>5000
31	Laurel	2500	1250	1042	2083	1250	>5000	1667	1250	729	1250	1250	>5000

32	Mace OR	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
35	Marjoram	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	2500	5000	5000	5000
38	Mustard (spray dried)	>5000	>5000	>5000	>5000	>5000	>5000	>5000	2500	2500	>5000	>5000	>5000
39	Nutmeg	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
40	Onion	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
41	Onion (roasted)	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
45	Oregano	521	417	261	625	625	2083	313	104	313	313	417	1042
47	Paprika OR 2	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
48	Parsley	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
51	Pepper (white)	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
53	Pimento (berry)	2500	1250	1042	1250	1250	>5000	1250	1250	833	1250	1250	3333
54	Pimento (leaf)	2500	1250	1250	1250	1667	>5000	1250	1250	1042	1250	1667	2500
55	Peppermint	>5000	>5000	>5000	>5000	>5000	>5000	2083	625	833	1042	833	1458
56	Rosemary (1)	>5000	>5000	5000	>5000	>5000	>5000	>5000	2500	2500	>5000	>5000	>5000
57	Rosemary (2)	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	625	5000	2500	5000
58	Rosemary OR (1)	5000	1250	1250	>5000	>5000	>5000	625	469	625	>5000	>5000	2500
60	Rosemary OR (3)	1250	1250	1250	>5000	>5000	>5000	833	625	91	4167	>5000	3333
61	Sage (dalmatian)	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
62	Sage OR	>5000	>5000	>5000	>5000	>5000	>5000	5000	>5000	>5000	>5000	>5000	>5000
65	Thyme (red)	833	313	417	2083	1250	3333	313	130	261	417	521	521
66	Thyme OR	>5000	5000	5000	>5000	>5000	>5000	3750	3750	1250	3750	>5000	>5000
67	Winter savory	625	625	313	1250	1250	>5000	313	78	78	625	313	5000

3 L. mono = *L. monocytogenes*; Salmo = *Salmonella* Typhimurium; Pseudo = *P. aeruginosa*

Table 3: Growth parameters of *L. monocytogenes* mixed cultures in ham

	μ*	R^2	Growth inhibition*	Growth inhibition (%)
Control	0.404	0.99	0.00	-
Garlic	0.396	0.98	-0.01	2
Oregano	0.328	0.95	-0.08	19
Thym (red)	0.396	0.97	-0.01	2
Cinnamon cassia	0.362	0.99	-0.04	10

μ*= (ln CFU/g/day), R^2= Correlation coefficient of the linear regression used to estimate μ

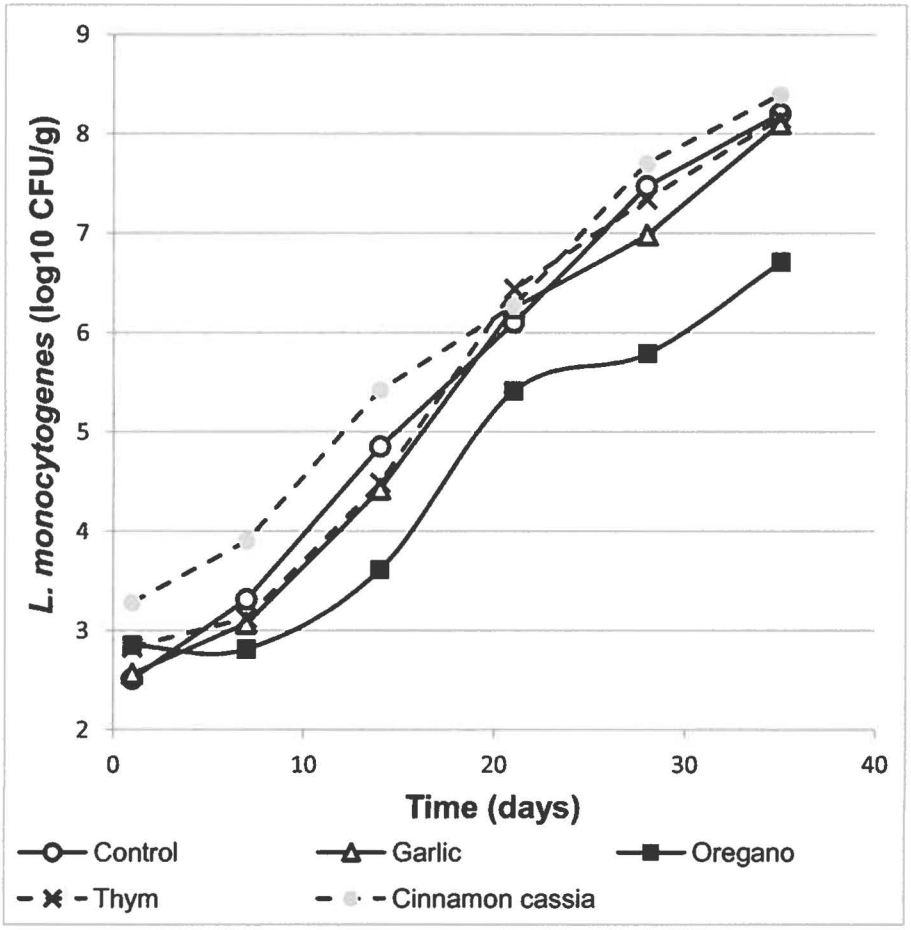

Figure 1: Growth inhibition of *L. monocytogenes* by garlic, oregano, thyme and cinnamon cassia EOs at 500 ppm in ham stored at 4°C during 35 days under aerobic conditions.

Article 2: Antimicrobial and Potential Cancer Preventive Substances from Marine Algae and Cyanobacteria Collected in Hawaii and the Caribbean

Préparé pour la soumission à Food Chemistry

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Contribution des auteurs

J'ai imaginé, mis au point et réalisé les expériences de l'activité antimicrobienne et de l'activité anticancer de cette étude en collaboration avec Khanh Dang Vu. Le matériel végétal a été produit par les laboratoires du Dr. F. David Horgen du Hawaii Pacific University. Tifanie Vansach a participé à la préparation des échantillons au Hawaii Pacific University. Par la suite, Khanh Dang Vu et moi avons rédigé le manuscrit scientifique. Les Dr. F. David Horgen et Dr. Clarissa Gerhauser ont participé à la révision du manuscrit. La Prof. Monique Lacroix était la responsable scientifique et la coordinatrice du projet de recherche. De plus, la Prof. Lacroix a supervisé l'élaboration des protocoles, les discussions scientifiques entourant ce projet et elle a révisé le manuscrit.

Résumé en français

L'environnement marin est une source connue de molécules structurellement complexes et de composés bioactifs. Nous avons étudié l'effet d'une collection d'extraits et d'isolats d'origine marine sur des analyses biologiques qui touchent deux aspects importants de la santé humaine tels la réduction des maladies d'origine alimentaire et la prévention du cancer. Premièrement, dix isolats cyanobactériens et neuf extraits organiques provenant d'organismes recueillis dans les Caraïbes et les eaux hawaiiennes ont été testés contre des bactéries pathogènes dans un assai de dilution en bouillon. Les résultats ont démontrés que certains extraits (*Padina* sp. et *Ulva* sp.) et composés (antillatoxin B, laxaphycins A, B and B3 et malyngamides A, C, I et J) possèdent des activités antimicrobiennes contre les pathogènes Gram positifs (*Listeria monocytogenes*, *Bacillus cereus* et *Staphylococcus aureus*) à faible concentrations (≤500 ppm). Deuxièmement, nous avons analysé l'effet des extraits et isolats d'origine marin sur l'induction de la NAD(P)H quinone réductase (NQO1; EC 1.6.5.5) sur des cellules d'un hépatome murin Hepa 1c1c7. La NQO1 est une enzyme de métabolisation des drogues de phase II qui supprime l'activation métabolique des composés aromatiques procarcinogènes en réduisant les intermédiaires quinones. Les résultats ont démontré que les extraits *de Gracilaria salicornia*, *Ligora* sp. et *Ulva* sp. possèdent un potentiel élevé pour l'induction de NQO1. Cette recherche démontre le

potentiel de certains composés marins comme sources de substances biologiquement actives pour contrôler les pathogènes alimentaires et la prévention de certains cancers.

Abstract

The marine environment is a proven source of structurally complex and biologically active compounds. We investigated the effects of a small collection of marine-derived extracts and isolates on biological assays available in our lab that relate to two very different but important health concerns, the reduction of foodborne illness and prevention of cancer. First, we determined 10 cyanobacterial isolates and 9 organic algal extracts from organisms collected in Caribbean and Hawaiian waters against foodborne pathogens using a broth dilution assay. Results showed that several extracts (*Padina* sp. and *Ulva* sp.) and compounds (antillatoxin B, laxaphycins A, B and B3 and malyngamides A, C, I and J) showed antimicrobial activity against Gram positive foodborne pathogens (*Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*) at low concentrations (≤500 ppm). Secondly, we analyzed the effects of the marine-derived samples on the induction of NAD(P)H:quinone reductase (NQO1; EC 1.6.5.5) in Hepa 1c1c7 murine hepatoma cells. NQO1 is a phase II drug-metabolizing enzyme, which suppresses the metabolic activation of aromatic procarcinogens by reducing quinone intermediates. Results showed that extracts of *Gracilaria salicornia*, *Liagora* sp. and *Ulva* sp. demonstrated high potential to suppress carcinogen metabolic activation through induction of NQO1. This research demonstrated the potential of several cyanobacterial isolates as sources of biologically active substances for the control of foodborne pathogens and for potent inducers of an important enzyme in reducing carcinogen activation.

Keywords: Antimicrobial, Cancer preventive, Marine algae, Cyanobacteria

Introduction

Foods are important sources of many different diseases (prions, virus, bacteria, parasites, toxins, heavy metal, etc.). Food contaminants are responsible for various symptoms related to gastroenteritis and can lead to serious consequences such as stomach pain, meningitis, organ failures and even death. In the United-States, it was estimated that between 76 million people are victims of food diseases and five thousand of them die from it every year. Known pathogenic organisms cause 9.4 million infections per year and the three most important groups are virus, bacteria and parasites responsible for 59 %, 39 % and 3 % respectively (Scallan et al, 2011). With increasing importation of food from all around the world, food safety has become a major concern for the world population.

Cyanobacteria and algae are both photosynthetic aquatic organisms that have a history of human consumption and toxicity. Several species of microalgae and cyanobacteria are used as food supplements around the world (Gantar & Svircev, 2008), which reflects the importance of many species as sources of nutrients and anti-oxidants. At the same time, aquatic microorganisms and higher algae represent a pool of under-investigated secondary metabolites. Collectively, these compounds possess "drug-like" physicochemical properties by virture of their ability to interact with biological targets and thereby mediate ecological functions, such as toxic effects on consumers, feeding deterrence, competition for space, and intra- and inter-species communication. There are numerous examples of toxins from photosynthetic aquatic species, especially form cyanobacteria that pose significant risks to human health. At the same time, many potential beneficial uses have been associated with extracts and compounds from cyanobacteria and algae. These include antioxidant, immunomodulatory, antimicrobial, and antitumor activities (Plaza, Herrero, Cifuentes, & Ibanez, 2009). In fact, a number of algal and cyanobacterial derived compounds have progressed to preclinical and human clinical trials for cancer (Newman & Cragg, 2004). In recent years, there has been increasing consumer interes for functional foods because they can promote health benefits (Mollet & Roland, 2002), and extracts and isolates from cyanobacteria and algae hold the potential as beneficial food additives in controlling food pathogens and providing protection against chronic disease.

Cancer is a leading cause of death in most industrialized countries. Cancer can be either treated after its manifestation in the human body or can be prevented before its apparition. It has been demonstrated that many food products such as fruits and vegetables reduce the risk of developing cancer (Paredes-Lopez, Cervantes-Ceja, Vigna-Perez & Hernandez-Perez, 2010). One mechanism for the prevention of cancer initiation is the upregulation of NAD(P)H:quinone reductase (NQO1; EC 1.6.5.5), a phase II drug-metabolizing enzyme that protects against toxic and reactive chemical species by suppressing carcinogen metabolic activation and blocking the formation of ultimate carcinogens. Induction of NQO1 can be detected by using murine hepatoma Hepa 1c1c7 cells *in vitro*. Numerous natural product extracts are known to induce NQO1 activity and therefore the NQO1 assay can be used to screen novel anticancer compounds (Kang & Pezzuto, 2004). For example, some food supplements, such as curcumin, increase the expression of NQO1 (Scapagnini et al., 2006).

The aim of this study is to evaluate the presence of novel antibacterial and anticancer compounds in the crude methanol extract and purified products of different marine algae and cyanobacteria collected in Hawaii and the Caribbean.

Materials and Methods

Collection and extraction of algae biomass.

From our collection of marine-derived algal and invertebrate extracts, 9 samples collected in waters off Oahu, Hawaii were investigated for antibacterial activity and quinone reductase induction. Algal biomass was collected by SCUBA or free diving. Algae were collected by hand leaving holdfasts in place. Dried algal voucher specimens are held in collections at Hawaii Pacific University (Department of Natural Sciences). The freshly collected material was frozen and freeze dried. Dried biomass was extracted exhaustively with methanol, and solvent was removed under vacuum. Extracts were generally eluted from a short silica vacuum liquid chromatography (VLC) column with dichloromethane/methanol mixtures to select for moderately polar metabolites, and the fractions were dried under vacuum and stored at -20 °C until use. The collection site and

date and the VLC elution solvent for each sample are as follows: *Dictyosphaeria* sp. (Chlorophyta), Pearl Harbor, adjacent to Hickam Airforce Base, August 12, 1997, dichloromethane/methanol (50:50); *Dictyota* sp. (Phaeophyta), Kailua Beach Park, November 3, 2001, dichloromethane/methanol (50:50); *Eucheuma striatum* (Rhodophyta), Kaneohe Bay, April 4, 2000, not subjected to VLC; *Galaxaura rugosa* (Rhodophyta), Kahala Beach Park, March 23, 2002, dichloromethane/methanol (50:50); *Gracilaria salicornia* (Rhodophyta), Kaneohe Bay, April 4, 2000, dichloromethane/methanol (70:30); *Laurencia nidifica* (Rhodophyta), Kahala Beach Park, March 23, 2002, dichloromethane/methanol (50:50); *Liagora* sp. (Rhodophyta), Maunalua Bay, May 9, 2001, dichloromethane/methanol (50:50); *Padina* sp. (Phaeophyta), Ulehawa Beach Park, February 3, 2001, dichloromethane/methanol (50:50); *Ulva* sp. (Chlorophyta), Ulehawa Beach Park, February 3, 2001, dichloromethane/methanol (90:10). Species were identified by the late Professor I.A. Abbott (University of Hawaii).

Isolation of cyanobacterial bioactive compounds.

Different compounds were isolated from field-collected cyanobacteria. The cyanobacteria used for isolation of bioactive compounds consisting of (1) an unidentified cyanobacterium (HPU code 071007-KAN-07) that was collected in Kaneohe Bay, Kaneohe, HI; (2) a sample of *Lyngbya majuscula* from True Blue Bay, Grenada, West Indies, on May 12, 2001; (3) a *Lyngbya* sp. collected from waters of Kaneohe Bay, Kaneohe, HI. Generally, organic extracts were subjected to reversed phase or both normal and reversed phase chromatography, and compounds were identified by comparison of their MS and ^{1}H NMR data with literature values.

Isolation of bioactive compounds from the unidentified cyanobacterium (HPU code 071007-KAN-07)

The freeze-dried sample (9.0 g dry weight) was extracted with dichloromethane then dichloromethane/methanol (50:50), and the combined extracts were dried under vacuum. The crude extract (830 mg) was subjected in several portions to reversed-phase HPLC [Luna C18(2), 10 µm, Phenomenex, 21.2 x 250 mm, acetonitrile/water gradient: 40:60 to

100:0 (0-25 min), 100:0 (25-30 min); 20 mL/min], to yield different fractions. The chemical compounds of these fractions were identified by comparing their MS and ^{1}H NMR data with literature data (Bonnard et al., 2007).

Isolation of bioactive compounds from Lyngbya majuscula

The dried sample (250 g dry wt) of extracted with dichloromethane/isopropanol (50:50), followed by methanol. The resulting organic extracts were combined, dried, and fractionated over a C_{18} medium pressure liquid chromatography (MPLC) with methanol/water mixtures (40:60, 60:40, 80:20) and finally 100% methanol. The methanol/water (70:30) fraction (600 mg) was further fractionated in several portions by reverse phase HPLC [Luna C18(2), Phenomenex, 21.2 x 250 mm, acetonitrile/water gradient: 60:40 to 100:0 (0-17 min); 20 mL/min]. Repeated HPLC of resulting fractions [Luna C18(2), Phenomenex, 10.0 x 250 mm, MeCN/H_2O gradient: 65:35 to 100:0 (0-20 min); 4.7 mL/min] yielded different components which were identified by comparison of MS and ^{1}H NMR data with reported values (Ainslie, Barchi, Moore & Myndersel, 1985; Wu, Milligan & Gerwick, 1997; Nogle, Okino & Gerwick, 2001).

Isolation of bioactive compounds from Lyngbya sp.

The freeze dried sample (7.0 g dry wt) was extracted with dichloromethane then dichloromethane/methanol (50:50). The extracts were combined extracts and dried under vacuum. The crude extract (567 mg) was subjected in multiple portions to HPLC chromatography [Luna C18(2), Phenomenex, 21.2 x 250 mm, methanol/water (80:20); 20.0 mL/min]. Similar fractions were combined and rechromatographed [Luna C18(2), Phenomenex, 10.0 x 250 mm, acetonitrile/water gradient: 50:50 to 100:0 (0-10 min), 100:0 (10-20 min); 4.6 mL/min] to yield different fractions and the chemical components of these fractions were identified by comparison of HRMS and previously reported ^{1}H NMR data (Marner, Moore, Hirotsu & Clardy, 1977; Todd & Gerwick, 1995; Kan, Sakamoto, Fujita & Nagai, 2000).

Microorganisms and growth condition.

Escherichia coli O157:H7 EDL 933, *Staphylococcus aureus* ATCC 29213, *Bacillus cereus* LSPQ 2872, *Salmonella enterica* serovar Typhimurium SL 1344, *Listeria monocytogenes* HPB 2812 and *Pseudomonas aeruginosa* 15442 were subcultured in tryptic soy broth (1%, v/v) (TSB, Difco Laboratories, Detroit, USA) at 37 °C for 24 h from the stock culture maintained at −80 °C in TSB containing glycerol (20%, w/v). Prior to the experiment, 1 mL of culture was incubated through one cycle of 24 h at 37 °C in TSB to obtain a working culture containing approximately 10^9 CFU/mL. The bacterial culture was centrifuged at 2,000 × g for 15 min at 4 °C and washed with NaCl (0.85 %, w/v) and then resuspended in TSB and incubated for 24 h preceding the experiment.

Minimal inhibitory concentration (MIC) assay in micro titer plates.

The influence of varying concentrations of methanol extract (ME) efficacy was assessed against pathogenic microorganisms (10^6 cells per mL) using 96-well micro titer plates (Sarstedt Inc., Montreal, Canada) according to a modified protocol of Gutierrez, Barry-Ryan, & Bourke, (2008). On the day of the experiment, the lyophilized ME or the isolates were solubilized in methanol at a concentration of 1 mg/mL. The first column of the microplate was filled with 200 µL of Mueller-Hinton (M-H) broth and columns 2 to 12 was filled with 150 µL M-H broth. Afterward, 58 µL of previously prepared ME or isolates was added to the first column. Two-fold serial dilutions we done from column 1 to 11 by transferring 125 µL of the mixture from the previous column. Finally, 15 µL of a bacterial suspension (10^7 cells per mL) was added to each well prior to a 24 h incubation at 37 ^{0}C. Row 12 served as a negative control without antimicrobial (broth + 5% methanol + bacteria). Each row from 1 to 5 contained a different bacterial strain and row 6 served as blank without bacteria (broth + methanol + ME or isolates). For the positive control, allylisothiocyanate was used with the same procedure as mentioned before. On the following day, bacterial growth was detected by optical density at 540 nm (ELISA reader, CLX800-BioTek Instruments) after the addition of 20 µL of an iodonitrotetrazolium chloride (INT; 0.5 mg/mL) alcoholic solution (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). The microplates were again incubated at 37 °C for

60 min, and in those wells, where bacterial growth occurred, INT changed from clear to red.

Cancer prevention assay using the quinone reductase (NQO1) assay.

The induction of NQO1 was measured in the Hepa 1c1c7 hepatoma cells (CRL-2026; American Type Culture Collection, Manassas, VA, USA) as previously described (Fahey, Dinkova-Kostova, Stephenson & Talalay, 2004). The cells were maintained in α-MEM Glutamax (Invitrogen Canada Inc.) (without ribonucleotides or deoxyribonucleosides) supplemented with 10% fetal calf serum (Thermo Scientific, Fisher Scientific Company, Ottawa, ON, Canada), 1 mM sodium pyruvate (Thermo Scientific) and 1X non-essential amino acids (Thermo Scientific) at 37 ^{0}C in a humidified atmosphere at 5% CO_2 in air. Approximately, 10 000 cells/well were placed in flat-botomed, 96-well Cellbind treated plates (Corning Incorporated, NY, USA) and incubated at 37 ^{0}C in a humidified atmosphere with 5% CO_2 for 24h. Cell culture medium was removed from each well and fresh medium containing ME extract or isolate was added for 48h of incubation under same atmospheric conditions. The final concentration of methanol never exceeded 5% and had no significant impact on the NQO1 activity compared to the control. In this assay, the blank consisted of medium plus methanol, the negative control was the medium plus murine Hepa cells plus methanol and the positive control was the medium plus murine Hepa cells, methanol and Beta-naphthoflavone (BNF, 1 μM). After 48 h of incubation, the medium was removed and followed by two washing using HBSS (Thermo Scientific). Cells were lysed in 50 μL of 0.8% digitonin (Sigma-Aldrich) and placed in a rotating agitator (100 rpm) at 37 ^{0}C for 20 min. A fraction of the cell lysate was used for protein quantification (20 μL) using BCA protein assay kit (Thermo Scientific). The remaining cell lysate was used for NQO1 activity assay (30 μL).

For the NQO1 activity measurement of NQ01 activity, 220 μL of reaction mixture (For 30 mL = 28 mL distilled water, 1.5 mL Tris-HCl buffer 0.5 M pH 7.4, 20 mg BSA, 200 μL Tween-20 1.5%, 20 μL FAD+ 7.5 mM, 200 μL glucose 6-phosphate 150 mM, 18 μL NADP+ 50 mM, 60 U glucose 6-phosphate dehydrogenase from yeast, 9 mg MTT, 30 μL menadione 50 mM) was added to the 30 μL of lysed cells. After 5 min, 50 μL of stopping

solution (0.3 mM dicoumarol in phosphate buffer 5 mM pH 8.5) was added to each well. After this step, the optical density of each well was read at 595 nm (ELISA reader, CLX800-BioTek Instruments). Specific activity was measured using modified equations (Kang & Pezzuto, 2004). Induction activity was calculated by the equation 1.

$$\text{Induction activity} = \frac{\text{Specific activity compound}}{\text{Specific activity control}} \quad (1)$$

Statistical analysis

Analysis of variance, Student t test was employed to statistically analyze all results of the NQO1 assay in order to determine the significant effects on the induction level. Differences between means were considered significant when $P \leq 0.05$. PASW Statistics 18 software (SPSS Inc., Chicago, IL, USA) was used for the analysis. Each measurement, was done in triplicate.

Results and discussion

Isolation of different compounds from cyanobacteria

The three compounds consisting of laxaphycin A (10.4 mg), laxaphycin B (4.8 mg), and laxaphycin B3 (4.5 mg) were isolated from the unidentified cyanobacterium (HPU code 071007-KAN-07) that was collected in Kaneohe Bay, Kaneohe, Hawaii and were identified by comparing their MS and ^{1}H NMR data with literature data (Bonnard et al., 2007). In case of *Lyngbya majuscula* collected from True Blue Bay, Grenada, West Indies, on May 12, 2001, the four compounds consisting of malyngamide C (12.0 mg), malyngamide C acetate (11.9 mg), malyngamide J (14.6 mg), and antillatoxin B (11.5 mg) were isolated and identified by comparison of MS and ^{1}H NMR data with reported values (Ainslie, Barchi, Moore & Myndersel, 1985; Wu, Milligan & Gerwick, 1997; Nogle, Okino & Gerwick, 2001). Finally, three other compounds such as isomalyngamide A (4.7 mg), majusculamide A (11.1 mg), and malyngamide I (6.2 mg) were isolated from *Lyngbya* sp. collected from waters of Kaneohe Bay, Kaneohe, HI, USA and were identified by comparison of HRMS and previously reported ^{1}H NMR data (Marner, Moore, Hirotsu & Clardy, 1977; Todd & Gerwick, 1995; Kan, Sakamoto, Fujita & Nagai, 2000). The chemical structures of the cyanobacterial isolates are presented in Figure 1.

Antimicrobial activity

Extracts of nine algae (2 Chlorophyta, 5 Rhodophyta, and 2 Pheaophyta) and 10 cyanobacterial isolates (Figure 1) were evaluated for their antimicrobial effects. The spectrum of bacterial test cultures includes 5 common foodborne pathogens. Three strains are Gram positives (*Bacillus cereus, Staphylococcus aureus* and *Listeria monocytogenes*) and 2 are Gram negative (*Escherichia coli* and *Salmonella enterica* serovar Typhimurium). The positive control allylisothiocyanate showed MIC values ranging from 39 to 156 ppm. These values are comparable to the other publications (Burt, 2004). None of the extracts or isolates showed antimicrobial activity against the Gram negative bacteria. This could be explained by the hydrophobicity of the compounds extracted. The Gram negative bacterial membrane contains lipopolysaccharides that create a hydrophilic barrier that prevent the apolar compounds that were extracted with dichloromethane/methanol (50:50) from entering the cell. Usually, the non-specific antimicrobial activity of hydrophobic compounds will act by disrupting the cell membrane and creating leakage of the cytoplasm (Burt, 2004).

On the other hand, Gram positive bacteria showed varying degree of sensitivity to the extracts and isolates. *B. cereus* and *S. aureus* showed a MIC of 63 and 130 μg/mL, respectively, for the *Padina sp.* extract, while *Ulva* sp. extract showed also some antimicrobial activity with a MIC of 130, 130 and 250 μg/mL against *B. cereus, S. aureus* and *L. monocytogenes*, respectively. A recent study has shown that an ethanolic extract of a Hawaiian *Ulva fasciata* demonstrated antimicrobial activity (Vijayavel & Martinez, 2010).

Similar to the extract activity, several cyanobacterial isolates showed inhibitory effects on Gram positive bacteria but no isolate inhibited the Gram negative strains. The most sensitive bacterium for all active isolates was *B. cereus*. Majusculamide A and malyngamide C acetate were inactive against all strains. The laxaphycins and antillatoxin B showed weak to moderate inhibition of the Gram positive bacteria, while several of the malyngamides inhibited *B. cereus* more potently, while being essentially inactive against *S. aureus* and *L. monocytogenes*. The most notable is isomalyngamide A, which showed a specific and low MIC of 7.8 μg/mL. To our knowledge, there is only one previous report

(Gerwick et al., 1987) of antibacterial activity for the extensive series of malyngamide-class compounds.

There are several previous studies on the antimicrobial activity of different extracts from marine algae. For example, Ely et al., (2004) have screened the antimicrobial activity of two seaweeds, collected from south east coast of India, against selected clinical isolates of bacteria and fungi. It was found that methanolic extracts of all the marine organisms demonstrated activity against one or more of microbes tested. *Sigmadocia carnosa* was the most active exhibiting a broad spectrum antimicrobial activity against each of the microbe tested with the exception of *Fusarium* species. Considerable antibacterial activity was exhibited by *Haliclona cribricutis* and *Chrotella australiensis* against *Klebsiella* species and *Vibrio cholerae*, respectively. *Petrocia testudinaria* showed equally good activity against the bacterium *V. chlorae* and the fungus *Cryptococcus neoformans*. The sponges *Callyspongia fibrosa*, *Ircinia* species and the seaweed *Stoecheospermum margilatum* are totally inactive against fungi.

Tuney et al., (2006) have tested the *in vitro* antimicrobial activity of methanol, acetone, diethyl ether, and ethanol extracts of 11 seaweed species collected from the coast of Urla, Turkey against *Candida* sp., *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus epidermidis*, *Pseudomonas aeruginosa* and *Escherichia coli* using the disc diffusion method. The authors found that diethyl ether was the best solvent for extracting the effective antimicrobial materials from the algae species, with the exception of *D. linearis*, for which ethanol was the most effective extraction solvent. Diethyl ether extracts of fresh *Cystoseira mediterranea*, *Enteromorpha linza*, *Ulva rigida*, *Gracilaria gracilis* and *Ectocarpus siliculosus* showed effective results against all tested organisms. However, diethyl ether extracts of some species, such as *Padina pavonica*, *Colpomenia sniosa*, *Dictyota linearis*, *Dictyopteris membranacea*, *Ceramium rubrum* and *Acanthophora nojadiformis*, gave different results against tested organisms. A difference in antimicrobial activity between the acetone and methanol extracts of each alga was not significant. In addition, the author also found that all tested organisms were more sensitive to fresh extracts of the algae as compared to dried extracts.

Thus, in accordance with other studies, the observed antimicrobial activity of our collected marine algal extracts and cyanobacterial isolates against food pathogens showed promise for further research on this domain, especially, isomalyngamide A isolated from *Lyngbya* sp.

Cancer chemopreventive activity

The cancer chemopreventive activity of cyanobacterial and algal ME was investigated *in vitro* using the NQO1 assay. NQO1 inducing activity of the extracts and isolates was compared to cells treated with the solvent (methanol). The induction level of the solvent (control) was 1 ± 0.12. *Beta*-Naphthoflavone at a concentration of 1 μM was used as a positive control and had an induction level of QR of 4.74 ± 0.53. The induction levels of NQO1 and the effect on the growth of Hepa1c1c7 cells by the marine algal extracts and cyanobacterial isolates are presented in Figure 2 and 3, respectively.

With the exception of extracts from *Padina* sp. and *Ulva* sp., all of the tested extracts significantly ($P \leq 0.05$) induced NQO1 activity at the highest concentration of 250 μg/mL (Figure 2A). In contrast, the extracts from *Padina* sp. and *Ulva* sp. significantly ($P \leq 0.05$) inhibited Hepa1c1c7 cell growth (Figure 2B), which was associated with a significant ($P \leq 0.05$) reduction in NQO1 activity. The extract derived from *Gracilaria salicornia* was identified as the most active extract, with significant ($P \leq 0.05$) induction of NQO1 activity by more than 1.5-fold at concentrations from 31.3 to 250 μg/ml, as compared to the solvent control without treatment. We identified that extracts from *Liagora* sp. and *Ulva* sp. also contain potent inducers of NQO1 activity. They led to a significant ($P \leq 0.05$) 1.5 fold increase in NQO1 activity at the lowest concentration of 31.3 μg/mL as compared to the control. Thus, the three extracts from *Gracilaria salicornia*, *Liagora* sp. and *Ulva* sp. are the most potent extracts and might contain interesting cancer preventive components. Based on this initial screen, their active principles for the induction of NQO1 and potentially additional enzymes regulated *via* the Nrf-2 pathway should be identified.

Most of the isolated compounds from cyanobacteria showed very low levels of NQO1 induction at the lowest concentration of 31.3 μg/mL (Figure 3). At higher concentrations (from 62.6 to 250 μg/mL), these compounds did not induce, but even significantly ($P \leq 0.05$) reduce NQO1 activity. In fact, these isolated compounds at higher concentrations were cytotoxic to the Hepa1c1c7 cells (Figure 3B). Thus, further research on induction of NQO1 using lower concentrations of these isolated compounds should be done. Also, it might be interesting to test the anti-proliferative potential of these compounds in additional cancer cell lines from colon, lung, prostate etc.

It should emphasized that nowadays, there is a growing interest on the discovery of natural antioxidants because there is epidemiological and clinical evidence suggesting that consumption of vegetables and fruits reduces the risk of developing chronic disease, for example, different types of cancer. It has been confirmed that the main sources of antioxidant are herbs, spices, and medicinal plants. Marine algae are also rich sources of antioxidant compounds (Vijayavel & Martinez, 2010). Marine algae and cyanobacteria provide for an excellent source of bioactive compounds such as carotenoids, dietary fibre, protein, essential fatty acids, vitamins, and minerals. Marine algae are being used as food supplement, source of vitamins, and as food additives (Patra *et al.*, 2008). For example, the seaweed *Asparagopsis taxiformis* contains 36 % ash, 6.1 % total protein, 13.2 % soluble carbohydrate and 4 % crude lipids and it was the most popular food additive for ancient Hawaiians (Mcdermid and Stuercke 2003).

The other example about the diversity of biological properties of algae is the unicellular algae *Chlorella vulgaris*. This alga contains many bioactive substances with medical properties. *Chlorella* contains many dietary antioxidants such as lutein, α-carotene, β-carotene, ascorbic acid and α–tocopherol (Shibata et al., 2001; Shibata et al., 2003). These bioactive compounds have the capacity to scavenge free radicals. Chemical analysis revealed that *Chlorella* extract contained 44.3 g protein, 39.5 g carbohydrates and 15.4 g nucleic acids in 100 g (dry weight) whole material (Hasegawa et al., 1995). Moreover, several studies have demonstrated the medicinal properties of *Chlorella*, such as antibacterial effects (Tanaka et al., 1986), antioxidant properties (Singh, Singh &

Bamezai, 1998) and antitumor effect (Konishi, Tanaka, Himeno, Taniguchi, Nomoto, 1985; Morimoto et al., 1995).

Thus, based on the literature as well as the results of this study, several algae or cyanobacteria could become promising candidates of natural sources for antimicrobial agents or cancer chemopreventive agents in the future. Further research on isolation of other bioactive compounds from other algae or cyanobacteria for use as natural cancer chemopreventive agents should be pursued.

Conclusions

The effects of nine algal extracts and ten cyanobacterial isolates on the reduction of foodborne pathogens and on the induction of NQO1 activity were investigated. Several algal extracts (*Padina* sp. and *Ulva* sp.) and cyanobacterial compounds (antillatoxin B, laxaphycin A, B and B3 and malyngamide A, C, I and J) were found to have the antimicrobial activity against Gram positive foodborne pathogens (*Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*) at low concentrations (≤500 ppm). These results confirmed the potential of several cyanobacterial isolates to serve as sources of bioactive substances for the control of foodborne pathogens. Moreover, some algal extracts demonstrated high potential in inducing NQO1. All the concentrations (from 31.3 to 250 μg/mL) of the extract from *Gracilaria salicornia* significantly induced NQO1 by more than 1.5 fold as compared to the control. Finally, the three extracts from *Gracilaria salicornia*, *Liagora* sp. and *Ulva* sp. are found to be the most potent extracts in terms of induction of NQO1 activity. Further studies to explore their potential in prevention of cancer should be conducted.

Acknowledgements

This research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and BSA Food Ingredients s.e.c/l.p. (Montreal, Qc, Canada) under a research contract agreement with INRS. This research was also funded by the U.S. National Institutes of Health (NIH P20 RR-016467) to FDH. NSERC, le Fond de Recherche sur la Nature et les Technologies (FQRNT) and BSA Food Ingredients

s.e.c/l.p. supported Dominic Dussault through the Industrial Innovation Scholarships BMP Innovation.

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Table 1. Minimal inhibitory activity (μg/mL) of marine algae extracts and cyanobacterial isolates

	Extracts or isolates	*B. cereus*	*S. aureus*	*L. monocytogenes*	*E. coli*	*S.* Typhimurium
organic algal extracts	*Dictyosphaeria* sp.	>500	>500	>500	>500	>500
	Dictyota sp	>500	>500	>500	>500	>500
	Eucheuma striatum	>500	>500	>500	>500	>500
	Galaxaura rugosa	>500	>500	>500	>500	>500
	Gracilaria salicornia	>500	>500	>500	>500	>500
	Laurencia nidifica	500	>500	>500	>500	>500
	Liagora sp.	>500	>500	>500	>500	>500
	Padina sp.	63	130	>500	>500	>500
	Ulva sp.	130	130	250	>500	>500
cyanobacterial isolates	Antillatoxin B	130	250	250	>500	>500
	Isomalyngamide A	7.8	500	>500	>500	>500
	Laxaphycin A	250	125	250	>500	>500
	Laxaphycin B	250	250	250	>500	>500
	Laxaphycin B3	250	500	>500	>500	>500
	Majusculamide A	>500	>500	>500	>500	>500
	Malyngamide C	130	>500	>500	>500	>500
	Malyngamide C Ac	>500	>500	>500	>500	>500
	Malyngamide I	250	>500	>500	>500	>500
	Malyngamide J	63	>500	>500	>500	>500
Posotive control	Allylisothiocyanate	39	78	39	156	39

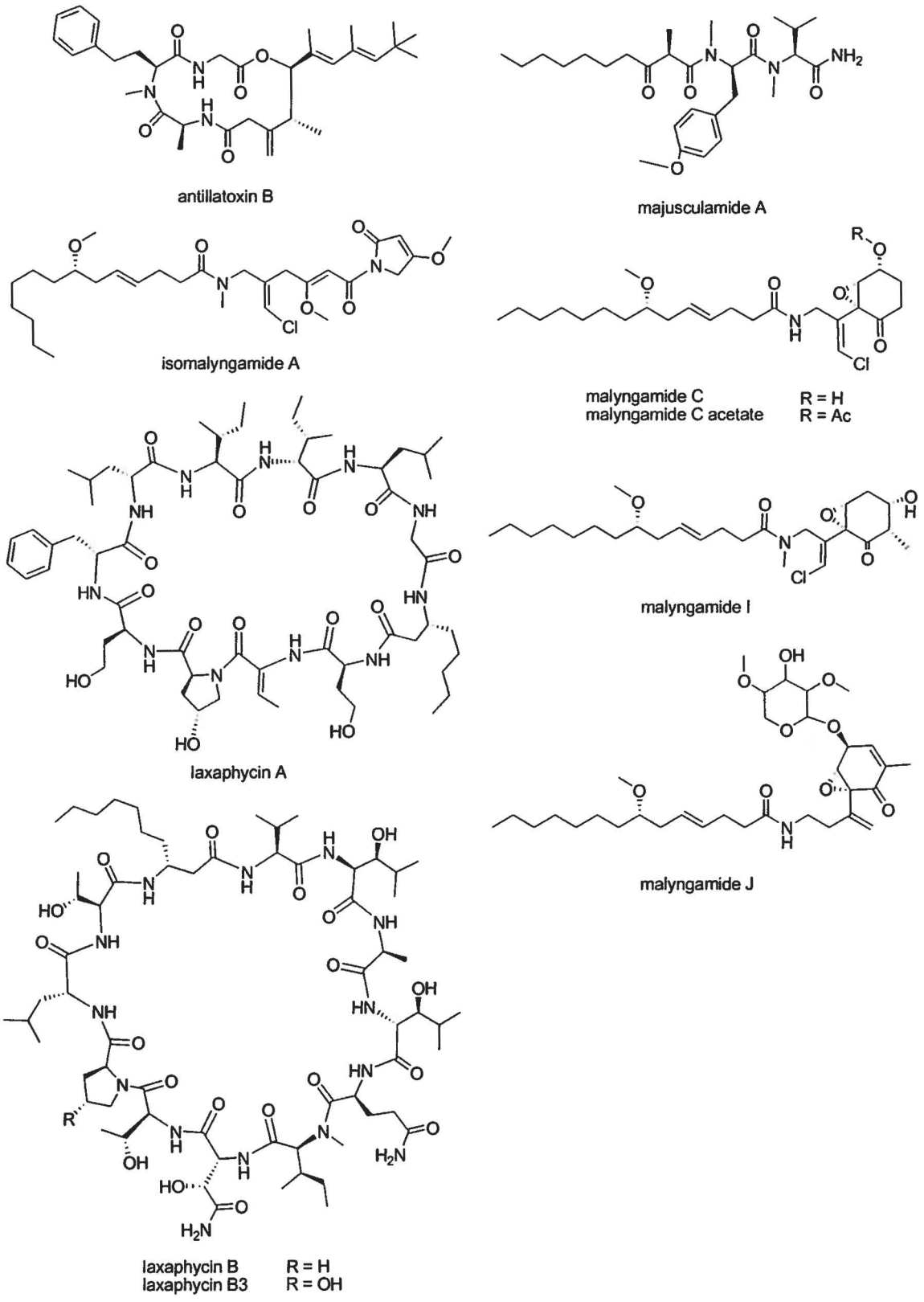

Figure 1. Chemical structure of the cyanobacterial isolates

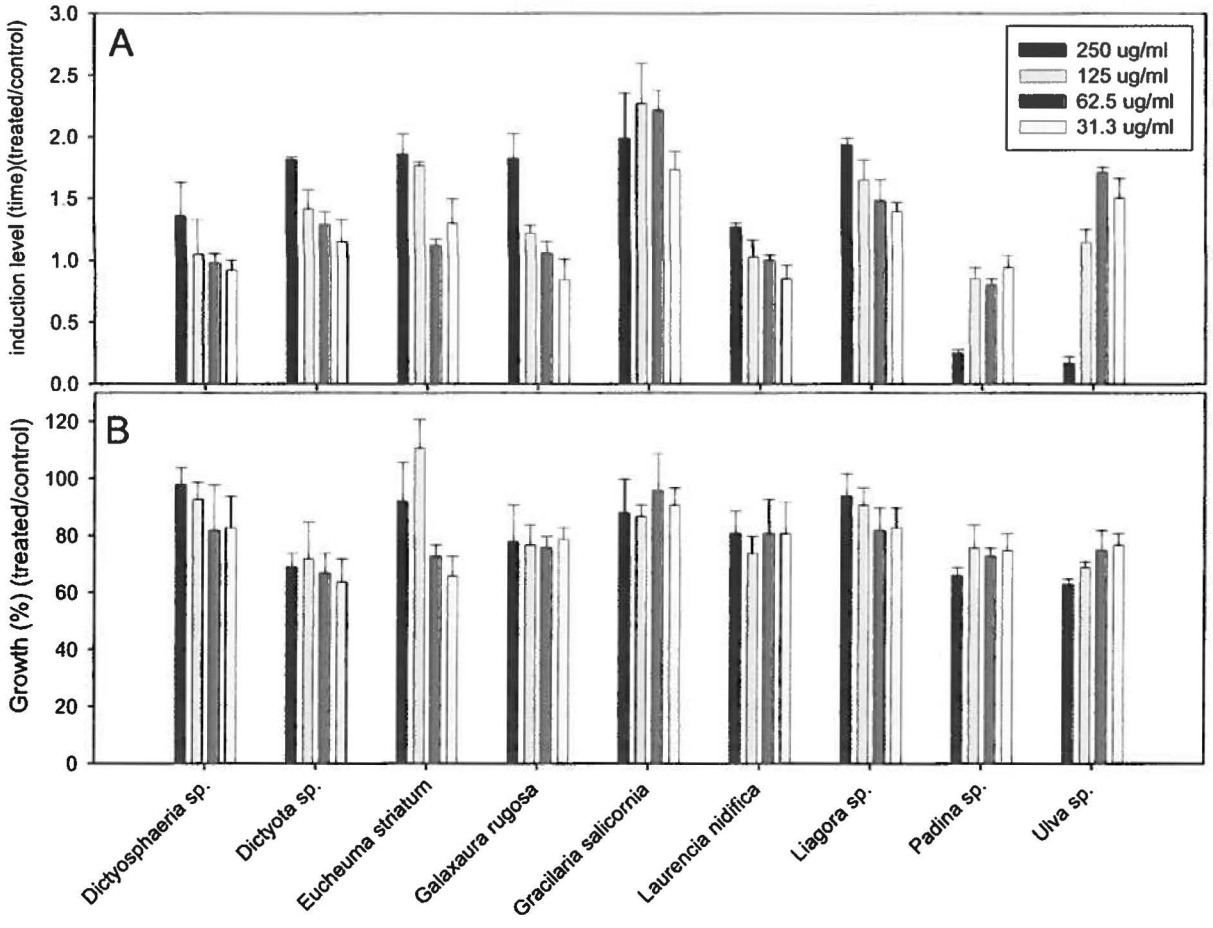

Figure 2. Induction of quinone reductase (A) and effect on growth (B) by different marine algae extracts on Hepa1c1c7 cells. Error bars are showing the standard deviation of triplicates.

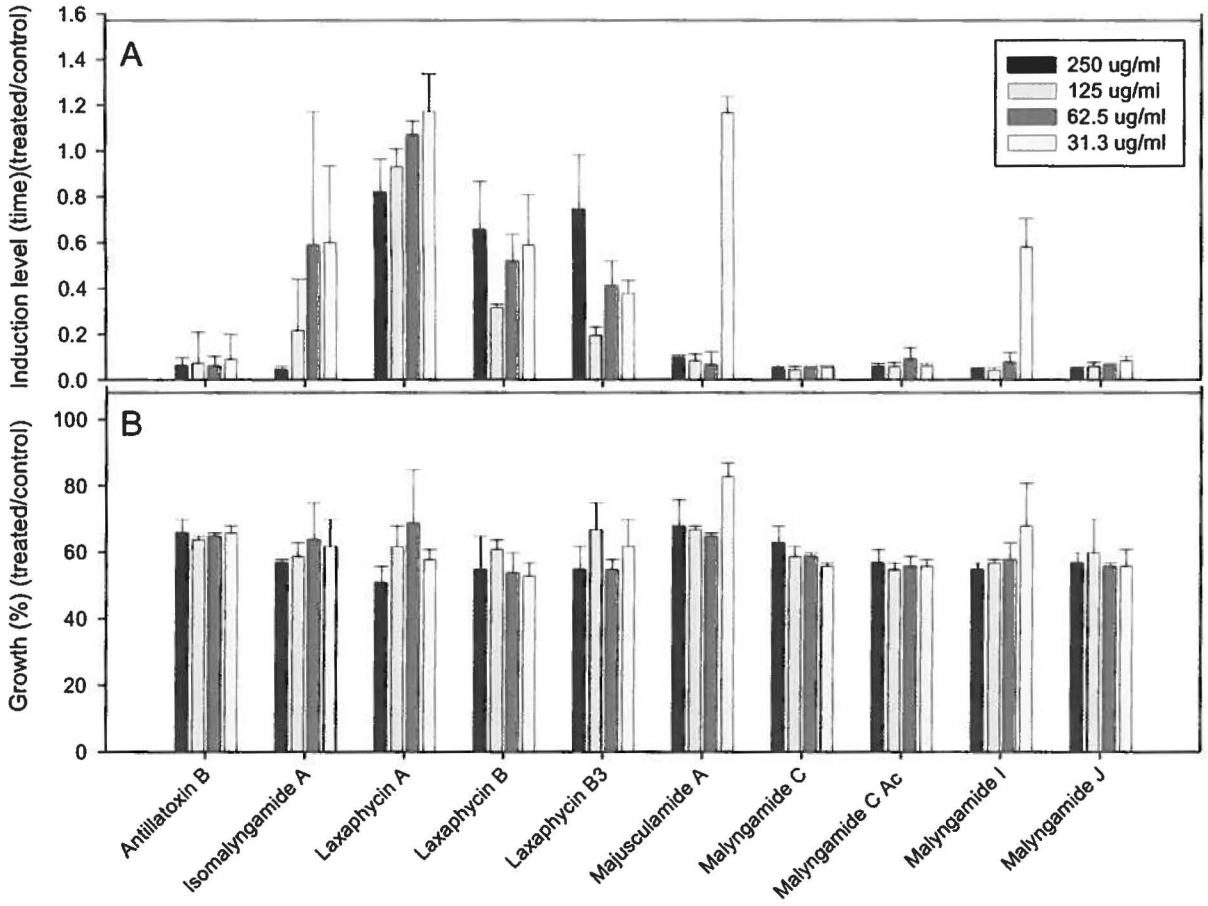

Figure 3. Induction of quinone reductase (A) and effect on growth (B) by different cyanobacterial isolates on Hepa1c1c7 cells. Error bars are showing the standard deviation of triplicates

Partie 2: Criblage *in situ*

Mise en contexte de la Partie 2

Cette section vise à présenter l'application des découvertes réalisées dans la section 1 à l'intérieur d'un modèle alimentaire. Premièrement, l'utilisation d'un fermentat de bactéries contenant des acides organiques a été évaluée pour son effet bactériostatique sur la flore microbienne d'un aliment à base de viande fraîche. Cette première étape visait à déterminer de façon simple l'effet des sels d'acides organiques sur les bactéries.

Deuxièmement, les charcuteries contaminées par *Listeria monocytogenes* étairnt la catégorie d'aliments visée principalement par la présente recherche. La crise de la listériose au Canada en 2008 a dirigé la recherche vers ce type d'aliment. Vu la complexité et le temps nécessaire à la confection d'une charcuterie il était impossible d'utiliser directement la méthode de fabrication industrielle de l'aliment. Pour cette raison, le développement d'un modèle de charcuterie contaminée a dû être développé. Le développement de ce modèle a été complexe afin de respecter les propriétés du produit. Ainsi, le modèle choisi, un jambon avec un taux d'injection de saumure de 60% (ex : 100 kg de viande pour 60 kg de saumure) avec un faible taux de sel 1.5 %, un taux d'humidité élevé 76%, contenant des nitrites de sodium, avec un pH élevé (pH 6.3) et emballé sous air a été mis au point. Ce type d'aliment respectait les spécifications des charcuteries tout en ayant modulé les paramètres pour les rendre le moins restrictif possible.

Il est à noter que la compagnie nous a transmis des concentrations maximales pour chacune des huiles essentielles sélectionnées suite à l'étude de la partie 1. Ces concentrations visent seulement le côté organoleptique de l'aliment : citronnelle : 60 ppm, ail 100 ppm, cannelle 60 ppm, origan 15 ppm et thym rouge 15 ppm. Au-delà de ces concentrations, les huiles essentielles apportent une saveur trop importante pour l'utilisation dans un produit commercial.

Article 3: Combined Effect of γ-Irradiation and Bacterial-Fermented Dextrose on Microbiological Quality of Refrigerated Pork Sausages

Publié dans Radiation Physics and Chemistry, 2012, 81, pp. 1098–1102.

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Running title: Fermented dextrose and γ-irradiation for sausages

Published in Radiation Physics and Chemistry vol. 81 (2012) p. 1098–1102

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Contributions des auteurs

J'ai imaginé, mis au point et réalisé toutes les expériences de cette étude. Par la suite, j'ai rédigé le manuscrit scientifique. Claude Benoît de la compagnie BSA m'a assisté lors de la confection des saucisses en usine pilote et de la réalisation des analyses microbiologiques. M. Benoit a aussi révisé le manuscrit suite à la rédaction. La Prof. Monique Lacroix était la responsable scientifique et la coordinatrice du projet de recherche. De plus, la Prof. Lacroix a supervisé l'élaboration des protocoles, les discussions scientifiques entourant ce projet et elle a révisé le manuscrit.

Résumé en français

L'objectif de cette étude était d'évaluer l'effet d'un glucose fermenté possédant des propriétés antimicrobiennes en combinaison avec une faible dose d'irradiation γ (1.5 kGy) sur la qualité microbiologique de saucisses de porc fraîches. Des saucisses de porc fraîches contenant le glucose fermenté (0.25, 0.5 et 0.75 %) ont été préparées dans une usine pilote et ont été irradiées avec un irradiateur UC-15A possédant une source de ^{60}Cobalt. Le traitement d'irradiation seul a été capable de réduire la population initiale de bactéries psychrophiles et mésophiles par plus de 2 log UFC/g et a maintenu le niveau de bactéries lactiques sous le seuil de détection tout au long de la durée de conservation (<100 UFC /g). Les résultats ont aussi démontré que le glucose fermenté seul a augmenté la durée de conservation de 5 à 13 jours. Au jour 13, le glucose fermenté ou l'irradiation γ ont démontrés 2 log UFC/g de moins que le contrôle pour les bactéries mésophiles. Suite à la combinaison du glucose fermenté et du traitement d'irradiation, il a été possible d'observer une diminution supplémentaire de 1 log UFC/g. En combinant les deux traitements, les résultats ont démontré un taux de croissance réduit pour les bactéries psychrophiles et mésophiles en comparaison avec les deux traitements seuls. Cette étude démontre que la combinaison d'une faible dose d'irradiation peut agir en synergie avec un glucose fermenté pour réduire la multiplication des bactéries indigènes de la viande afin d'augmenter la durée de conservation de l'aliment.

Abstract

The objective of this study was to evaluate the effect of a concentrated fermented dextrose (FD) a natural antimicrobial product, combined with low dose γ-irradiation (1.5 kGy) on the microbiological quality of fresh pork sausages. Fresh pork sausages containing the FD (0.25%, 0.5% and 0.75%) were prepared in a meat pilot plant and were irradiated using a UC-15A irradiator equipped with a ^{60}Cobalt source. The γ-irradiation treatment alone was able to reduce the initial psycrophilic and mesophilic bacteria by more than 2 log CFU/g and kept the *lactobacillus* population under the detection limit (100 CFU/g). Results also showed that the FD alone was able to extend the shelf life of the sausages from 5 days up to 13 days. At day 13, the FD or irradiation alone showed 2 log CFU/g less mesophilic bacteria than the control. After combining FD and irradiation another reduction of the microbial count of 1 log CFU/g was observed. When combining the irradiation treatment with the FD results showed a reduced growth rate of the psycrophilic and mesophilic bacteria comparing to both treatments alone. This study demonstrated that FD with low dose gamma irradiation act in synergy to reduce the multiplication of the total bacterial flora in fresh sausages.

Keywords: γ-irradiation, pork sausages, fermented dextrose, propionic acid

Introduction

Foods are important sources of diseases. The presence of prions, virus, bacteria, parasites, toxins, heavy metal, etc. can be a source of food disease. Food contaminants are responsible for various symptoms related to gastroenteritis and can lead to serious consequences such as stomach pain, meningitis, organs failures and even death. Known foodborne pathogens such as virus, bacteria and parasites are causing 38 million infections per year. Bacterial infections by pathogenic microorganisms such as *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Campylobacter* sp. are responsible for 5.2 million cases (Mead et al., 1999). It is also estimated, that 25% of the world food production is lost due to premature spoilage (Ghaly et al., 2010). To asses this problematic, food ingredient companies are developing new natural products that will inhibit growth of pathogen and spoilage microorganisms. Food fermentation is a preservation technique that has been used for centuries to extend shelf life of perishable food. Metabolites produced by lactic acid bacteria (LAB) during fermentation of milk or other substrates are known to inhibit the growth of spoilage and pathogen microorganisms (Settanni and Corsetti, 2008). Many organic acids (ex: acetic, lactic, citric and ascorbic) are considered as GRAS by the FDA and have already shown promising abilities to inhibit bacterial growth in food (Bari et al., 2005; Over et al., 2009; Samelis et al., 2005). Organic acids have an optimal antimicrobial activity under its conjugated form (RCOOH). Since the molecule is exempt of charge under this form, it can penetrate the bacterial membrane and acidify the bacterial cytoplasm (Van Immerseel et al., 2006).

Food additives rarely diminish initial food bacterial contamination and food processor must rely on physical treatment to achieve a significantly microbial reduction in food. Heat treatment has been used since the antiquity to decontaminate raw meat. Although, cooking of meat products is affecting greatly the properties of food products. New technologies such as high pressure processing, pulse electric field and ionizing irradiation are being developed in order to reach the same decontamination effectiveness without impairing the food. In order to minimize the impact on the food low dose (< 2 kGy) irradiation treatment are being used. Since irradiation of food offer only a reduction of the initial microorganisms' concentration the combination with food additive that offer protection over time could increase the preservation technique efficiency.

Acidic compounds cannot be added to meat preparation since water retention abilities are directly related to the pH of the preparation. Under a pH of 5.4, the isoelectric point of myosin, the major protein in meat, meat proteins lose their conformation and as well as their water holding capacity (Huff-Lonergan and Lonergan, 2005). In that optic, organic acids can be added as a neutralised salt (ex: RCOONa) in order to retain the initial pH of the preparation. Antimicrobial activity of organic acids salts will still be dependent on the acids conjugated form concentration and the conjugated form concentration will be dependent on the pH of the preparation and the dissociation constant (pKa) of the acids used (Carpenter and Broadbent, 2009). Therefore, a high pKa and a high concentration acid will be required to achieve similar results as more acidic product. However, using higher concentration of acid could lead to another problem: alteration of organoleptic properties of the food.

The objective of this study was to evaluate the antimicrobial efficiency of low dose γ-irradiation treatment with different concentrations of a fermented dextrose when added in fresh pork sausages during storage at 4°C.

Materials and Methods

Sausage preparation

Six formulations of sausages were prepared (1-without antimicrobial, 2-fermented dextrose (FD) 0.25%, 3-FD 0.5%, 4-FD 0.75% 5-1.5 kGy 6-1.5 kGy and FD 0.5% (w/w)) using pork meat. The fermented dextrose (PRO-LONG; BSA Food Ingredients s.e.c/l.p, Montreal, Qc, Canada) is a concentrated dextrose broth that has been fermented by a bacterium that belongs to the *Propionibacterium* genus. This bacterium is mainly used in the dairy industry but is also used for commercial production of propionic acid. Following dextrose fermentation, the product is neutralized, dried and standardized to obtain 35% of calcium propionate. Sausages were prepared in a pilot meat plant (BSA Food Ingredients, Montreal, Canada) kept at 4°C following standard small sausage maker procedures. Fresh pork (Boneless Picnic pork shoulder containing 18% fat) was ground through a 0.35-cm grinder plate (Pm 98, Mainca Equipamientos Carnicos S.L., Barcelona, Spain). Breakfast sausages seasoning and binding agents (7%) containing toasted wheat crumbs, salt, sugar, spices, silicon dioxide, polysorbate 80 and canola oil (BSA Food Ingredients) and iced

water (17%) were added to the ground meat and was hand mixed for 2 min. Then, the preparation was extruded into lamb sausage casing (22-24 mm diameter, International Casing Group Canada Inc., Montreal, Qc, Canada) using a manual sausage filler Trespade (FACEM Spa, Torino, Italy). Each type of sausage was kept in a sterile bag at 4 °C until the day of the experiment.

Irradiation

The sausages were irradiated with a mean dose of 1.5 kGy. A UC-15A irradiator (Nordion international Inc., Kanata, Ontario, Canada) equipped with a ^{60}Cobalt source was used to deliver radiation at a mean rate of 16.2 kGy h^{-1}. This irradiator was certified by the National Institute of Standards and Technology (Gaithersburg, Md.), and the dose rate was established using a correction for decays of source. Amber Oersoex 3042D (Atomic Energy Research Establishment, Harwell, Oxfordshire, UK) was used to validate the doses distributions. The radiation treatment was carried out at the Canadian Irradiation Centre (Laval, Quebec, Canada) at room temperature (20 °C).

Microbial and chemical analysis

Sausages were analysed during storage for microbial growth after 24 h, 5, 8 and 13 days. On each day of analysis, 3 sausages from each treatment were cut, mixed and diluted 10 folds with peptone water (0.1%; BD) using a mechanical homogenizer for 1 min at 200 rpm in a sterile filter sample bag (Whirl-Pak; Nasco, Fort Atkinson, WI, USA). Dilutions were then plated on tryptic soy agar (TSA; BD, Sparks, MD, USA) for the enumeration of psycrophilic and mesophilic bacteria and were incubated at 15 °C (Greer, 1981) and 37 °C respectively for 48 h. For the enumeration of *Lactobacillus* sp. present in the sausage, dilutions were plated on acidified Rogosa SL agar (BD) and were incubated under anaerobic conditions at 37 °C for 48 h. Following incubation, the colonies forming unit were count using a magnifier. Detection limit for all microbial analysis was 100 colonies forming unit (CFU)/g. Shelf life limit was considered overdue when mesophilic microbial count reached the current authorities regulation level of 5 x 10^6 CFU/g (MAPAQ, 2009). Growth rate of the selected bacterial population were

determined by calculating the linear regression (y = mx+b) of the slope of each CFU/g curve.

Remaining sausage samples from the microbial examination were kept for pH analysis. Sample (10 g) was mixed and diluted 10 folds with distilled water in a filter sample bag using a mechanical homogenizer. The filtered homogenate obtained was used for pH reading using an electronic pH meter (Accumet basic AB15, Fisher Scientific, ON, Canada).

Statistical analysis

All bacterial counts were $\log_{10}$ transformed prior to statistical analysis. Three samples per days of analysis were used for each formulation. To determine the difference between each group on the same day for the microbial counts, an analysis of variance (ANOVA) and Duncan's multiple-range tests were employed. To determine the difference between each group for the sensorial analysis, an ANOVA and Duncan's multiple-range tests were also employed. Differences between means were considered significant at $P \leq 0.05$. Stat-Packets Statistical analysis software (SPSS Base 18.0, SPSS, Inc., Chicago, Ill.) was used for the analysis.

Results and Discussion

Total psychrophilic bacteria

The objective of this study was to evaluate the antimicrobial efficiency of different concentrations of FD in combination with γ-irradiation treatment on fresh pork sausages during storage at 4°C. Microbiological analysis of psychrophilic bacteria in fresh pork sausages are shown in Fig. 1. The FD did not reduce the initial population compared to the control. On the other hand, the 1.5 kGy irradiation treatment allowed a reduction of 2.3 log CFU/g. At day 5, 8 and 13, all samples containing the FD formulations and irradiated samples showed significantly ($P \leq 0.05$) lower microbial counts as compared to the control. At day 5, there was no significant difference ($P > 0.05$) between microbial counts of all sausages containing the FD formulation nevertheless the concentration used. At day 8, formulation containing 0.5 and 0.75% of FD showed

significantly ($P \leq 0.05$) lower microbial counts than 0.25%. Irradiated treatments, showed lower microbial count for day 1, 5 and 8 independently of PL addition. At day 13 only the sausages that contained PL combined with irradiation showed less psychrophilic bacteria. Also at day 13, there was no significant difference ($P > 0.05$) between microbial counts of 0.25% and 0.5% but 0.75% FD showed significantly less psychrophilic microorganisms ($P \leq 0.05$). These results show that the addition of the FD is dose dependant but the correlation can only be observed over a 13 days period. At a smaller interval, lower concentration of the FD will have the same effect as the higher one. This phenomenon could also be observed in presence of the irradiation treatment. For days 1 and 5, addition of the FD did not show any significant difference ($P > 0.05$) versus the irradiation treatment alone but over 8 and 13 days a significant difference ($P \leq 0.05$) could be observed.

Total mesophilic bacteria

Results of microbiological analysis of mesophilic bacteria in fresh pork sausages during storage are shown in Fig. 2. Results demonstrated that with addition of the FD there was no difference on initial population of mesophilic bacteria, compared to the control ($P > 0.05$). On the other hand, the 1.5 kGy irradiation treatment allowed a reduction of 2 log CFU/g. At day 5, 8 and 13, all samples containing FD and irradiated treatments showed significant lower microbial counts ($P \leq 0.05$) as compared to the control. At day 5, there was no significant difference ($P > 0.05$) between microbial counts of all samples containing the FD. At day 8, formulation containing 0.5% and 0.75% FD showed significantly ($P \leq 0.05$) lower microbial counts than 0.25% FD.

Growth rate and microbial contamination differences of psychrophilic and mesophilic bacteria after 13 days of storage are presented in Table 1. It is observed that the growth rate of psychrophilic microorganisms was increased (+0.018 log CFU/g/day) for the irradiated sausages without FD as compared to the control. We can hypothesize that the increased growth rate is related to the fact that the remaining bacterial population is less heterogeneous, since the radiation treatment only left behind the more radioresistant species. Therefore, the remaining microorganisms have less competition for

the nutrient and can grow faster. It is well documented in the literature that bacteria competing for an energy source will modify its environment in order to limit the competitors' growth (Hibbing, 2010). For psychrophilic, the highest decrease was observed for the product containing 0.75% PL (-0.129 CFU/g/day). It is interesting to note that the combination of low dose irradiation treatment with 0.5% FD showed the same growth rate. Hence, the addition of the FD nullified the increase in the growth rate that could be observed for irradiation alone. The antimicrobial activity of the FD is related to its organic acid content. Once organic acids enter the cytoplasm of the bacteria they will acidify the pH resulting in a modification of the homeostasis and consequently reducing the rate of division of the cell (Van Immerseel et al., 2006).

The FD addition to the meat preparation seemed to have the same dose dependent effect on the psychrophilic and mesophilic population. Very few studies have demonstrated the effect of propionic acid on the total microbial flora. According to (Golden et al., 1995) concentration of 0.9% propionic acid was able to reduce by 4 log the CFU of *Listeria monocytogenes* in brain heart infusion broth at pH 3.5 at 28 °C in 65 days as compared to the acidified control that took 172 days. In fresh cut apples, dipping in solution containing calcium propionate inhibited or reduced the growth of yeast, molds and *E. coli* during storage (Guan and Fan, 2010). In cooked turkey and ham propionate was able to inhibit the growth of *L. monocytogenes* up to 12 weeks when stored at 4 °C (Glass et al., 2007).

Lactobacillus and pH

Presence of *Lactobacillus* in meat is often associated with unfresh food. LAB grow on meat producing organic acids from glucose by fermentation. This gives rise to aciduric off-odors which may be accompanied by gas and slime formation and greening of meat leading to spoilage of the food (Doyle, 2007). Important level of LAB will be associated with production of acidity therefore lowering the pH and increase the water loss from the sausage (Holmer et al., 2009). Results of microbiological analysis of LAB in fresh pork sausages during storage are shown in Fig. 3. Initial meat contamination (day 1) showed lower microbial level than the detection limit for all samples. At day 8 since

there was a huge variation among sample of the same treatments, no significant difference ($P > 0.05$) could be observed between all treatments. Since the LAB content in the sausages with 0.25% and 0.75% FD are not significantly different ($P > 0.05$) than the control but 0.5% FD is significantly different ($P \leq 0.05$) it is not possible to correlate the reduction of LAB to the addition of the FD in the sausages. Although, all samples that received the irradiation treatment showed counts under the detection limit. Lack of uniformity in the LAB counts could be explained by the fact that the detection limit of LAB counts were 100 CFU/g in the present study, the initial LAB count in the sausage might show high variation within the range of the detection limit. The efficiency of irradiation for preventing growth of LAB could be explained by the fact that the treatment was able to remove completely the presence of LAB in the product because of the initial low level of contamination. A 1.5 kGy irradiation dose could potentially reduce by several log CFU/g the LAB population. Since the sausages were around 100 g each, in order to eliminate the LAB from the sausages a theorical 4 log CFU/g decrease would be required to have less than 1 CFU per 100 g. If 1.5 kGy is divided by 4, a maximum D_{10} of 0.375 kGy for the LAB in the meat is required for their complete elimination. This observation is probable since vegetative cells with average resistance to irradiation have a D_{10} around 0.42 kGy (van Gerwen et al., 1999).

As mentioned before, pH reduction control is critical for meat preparation since a lower pH means more water loss from the food preparation (Huff-Lonergan and Lonergan, 2005). Results for pH analysis of fresh pork sausages during storage are shown in Fig. 4. At day 1, formulation containing 0.5 and 0.75% FD showed a respective pH of 6.33 and 6.36. These values are greater than the pH observed for the control and 0.25% FD (6.12 and 6.15 respectively). The irradiation treatment had no effect on the pH of the sausages. Increase in pH can be explained by the presence of calcium propionate which is a neutralized form of the propionic acid. At the end of the shelf life, the FD addition to the sausage limited the pH reduction to 0.1 units as compared to the control where a drop of 0.5 units was observed. Even if the FD was not able to suppress the growth of the *Lactobacillus* spp., the FD addition was able to stabilize the pH and limit the acid production usually associated with the growth of LAB. Irradiation treatment had a similar effect as the FD on the pH. Irradiated samples with or without FD showed a decrease of

only 0.1 pH units. Inhibition of LAB growth by irradiation can therefore be correlated with the stability of the pH during storage of the sausages. Further studies on the utilization of bacterial fermented dextrose to control foodborne pathogens would greatly benefit the usage of naturally produced antimicrobial for food preparation since standard practices to control pathogens such as *L. monocytogenes* require adding synthetic chemicals.

Conclusion

In conclusion, this study demonstrated the feasibility of adding a fermented dextrose in sausages as a natural antimicrobial product during food preparation. FD addition can efficiently reduce the growth rate of the total bacterial flora and limit the pH reduction. Irradiation alone offers a good mean decontamination of raw meat. During storage this pasteurization treatment leaves the food without protection for radiation surviving microorganisms. Combining low dose irradiation with the FD is a good mean to achieve long term preservation. Food manufacturers are looking for new innovative solution to extend shelf life of their products without adding new chemicals to their labels. Natural products require a new natural approach in order to extend their shelf life and offer comparable product quality as the similar equivalent with chemical additives.

Acknowledgements

This research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and BSA Food Ingredients s.e.c/l.p (Montreal, Qc, Canada) under a research contract agreement with the INRS. The NSERC, le Fond de Recherche sur la Nature et les Technologies (FQRNT) and BSA Food Ingredients s.e.c/l.p supported Dominic Dussault trough the Industrial Innovation Scholarships BMP Innovation. The authors thank Nordion International, Inc. for the irradiation operations.

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Table 1: Growth rate and bacterial count reduction of psychrophilic and mesophilic bacteria in refrigerated pork sausages containing fermented dextrose (FD) and submitted to γ-irradiation treatment.

	Psychrophilic			Mesophilic		
	Growth rate[1]	Δ^2 Growth rate[1]	Δ^3 log CFU	Growth rate[1]	Δ^2 Growth rate[1]	Δ^3 log CFU
Control	0.333	-	-	0.279	-	-
1.5 kGy	0.351	0.018	1.98	0.248	-0.031	2.08
0.25 % FD	0.269	-0.064	1.15	0.148	-0.131	1.63
0.5 % FD	0.258	-0.075	1.23	0.140	-0.139	2.28
0.5 % FD+ 1.5 kGy	0.259	-0.074	3.24	0.149	-0.129	3.17
0.75 % FD	0.204	-0.129	1.81	0.122	-0.157	2.19

[1]Reported as average increase of log CFU/g/day; [2]variation compared to the control in log CFU/g/day; [3]variation compared to the control in log CFU/g at day 13

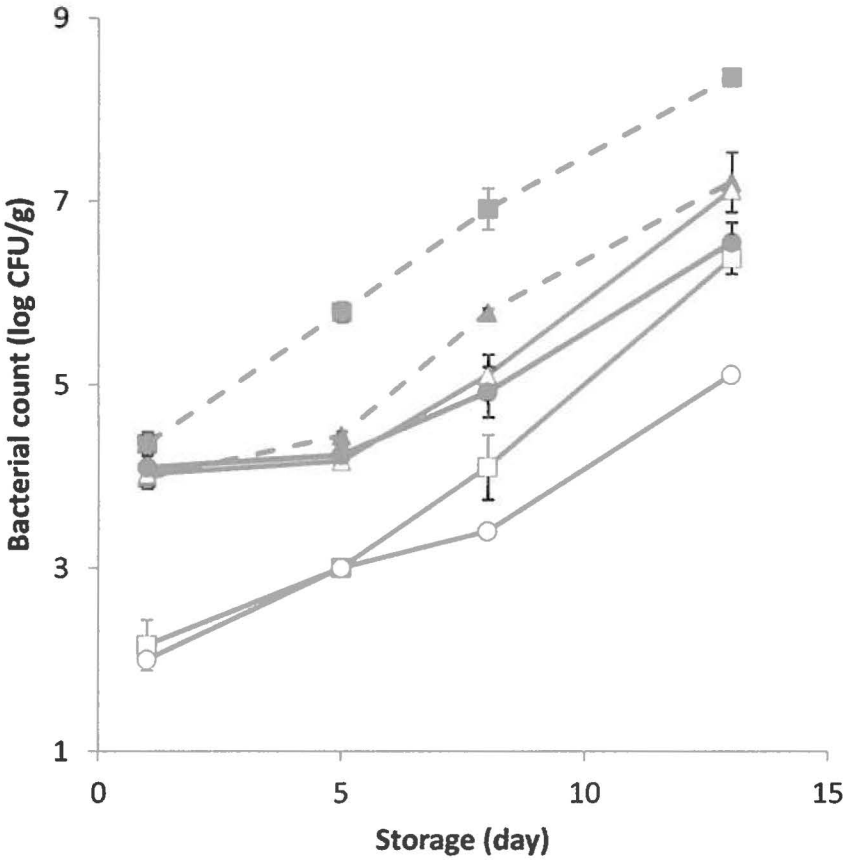


Figure 1: Population of psychrophilic bacteria in fresh pork sausages during storage at 4°C. Control (■), 1.5 kGy (□), Fermented dextrose 0.25% (▲), 0.5% (△), 0.5% + 1.5 kGy (○), and 0.75% (●).

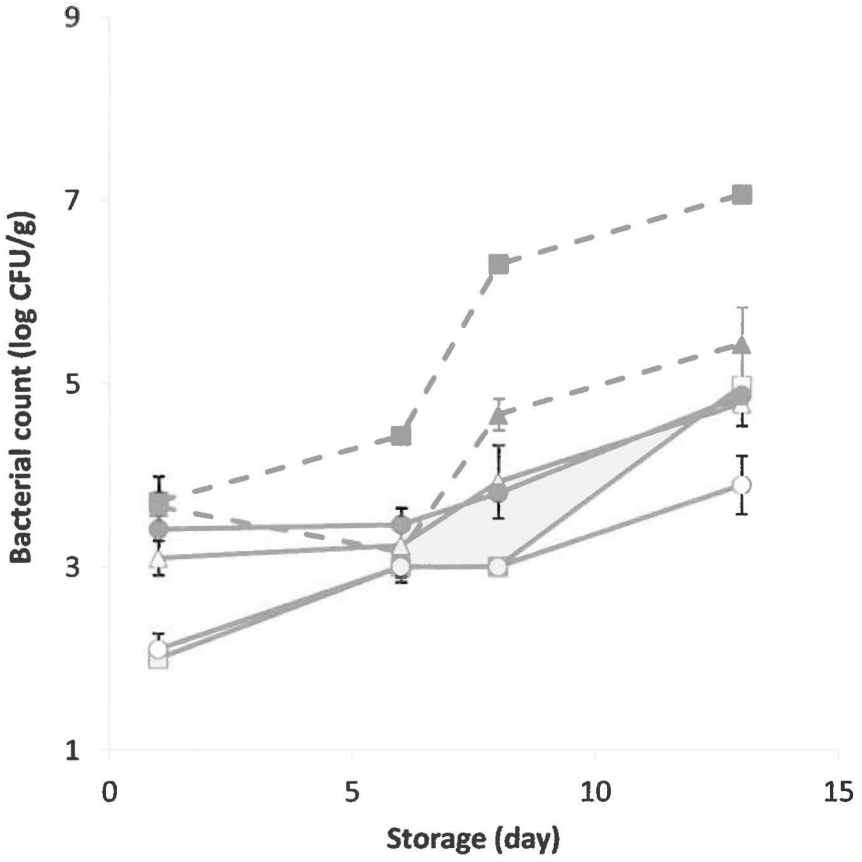


Figure 2: Population of mesophilic bacteria in fresh pork sausages during storage at 4 °C. Control (■), 1.5 kGy (□), Fermented dextrose 0.25% (▲), 0.5% (△), 0.5% + 1.5 kGy (○), and 0.75% (●).

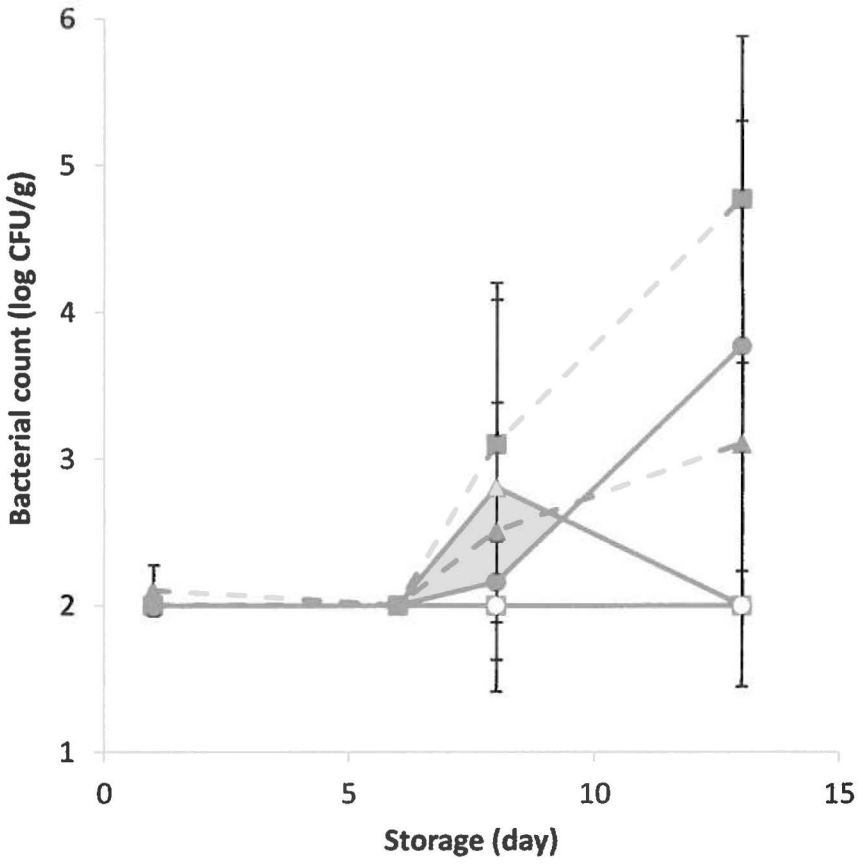


Figure 3: Population of *Lactobacillus* spp. in fresh pork sausages during storage at 4°C.

Control (■), 1.5 kGy (□), Fermented dextrose 0.25% (▲), 0.5% (△), 0.5% + 1.5 kGy (○), and 0.75% (●).

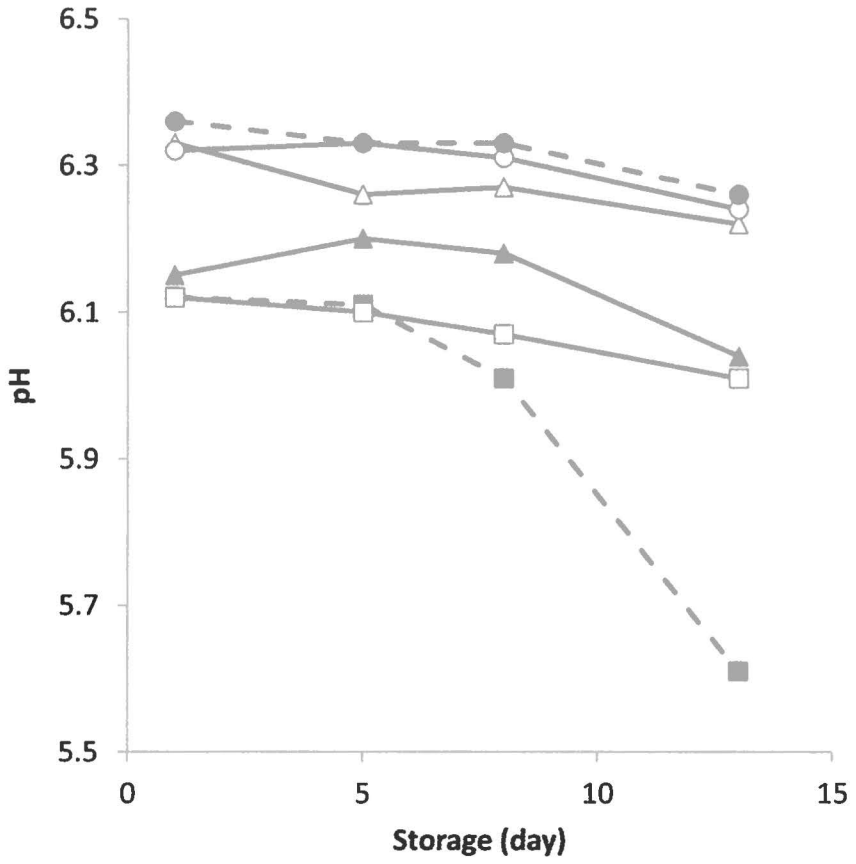


Figure 4: Effect of FD formulation on pH of fresh pork sausages during storage at 4°C. Control (■), 1.5 kGy (□), Fermented dextrose 0.25% (▲), 0.5% (△), 0.5% + 1.5 kGy (○), and 0.75% (●).

Matériel supplémentaire: Développement du modèle *in situ* et essais préliminaires.

Mise en contexte de la Partie 3

Cette partie vise à modéliser l'impact de l'addition des antimicrobiens sur la croissance de *L. monocytogenes* dans les charcuteries. D'après les résultats de la partie 2, certains additifs peuvent inhiber de façon efficace la croissance de *L. monocytogenes* dans les charcuteries. Toutefois, des paramètres intrinsèques des charcuteries peuvent moduler l'efficacité des antimicrobiens. Les antimicrobiens sélectionnés sont l'acétate, le lactate et le propionate pour les acides organiques, la nisine comme bactériocine et l'acide α du houblon pour l'extrait de plante. Puisque les acides organiques sont grandement influencés par le pH, celui a été pris en compte dans le modèle. Les nitrites et le chlorure de sodium sont aussi des antimicrobiens ajoutés dans la recette du jambon traditionnel et leurs concentrations peuvent varier donc ils ont aussi été pris en compte dans le modèle. La création du modèle mathématique permettra de mieux prévoir l'impact des différentes recettes de charcuteries sur l'efficacité des antimicrobiens. Il est à noter que la création d'un modèle mathématique ne remplace pas les études de validation *in situ*. Ce modèle permettra de limiter les recettes et les études de validation pour les industries.

Notes

Cette section a pour but de présenter les résultats préliminaires sur le modèle *in situ* du jambon. Les résultats présentés dans cette section ne seront pas publiés afin de respecter la confidentialité avec la compagnie BSA. La présentation de cette section dans la thèse est essentielle puisque qu'elle démontre les expérimentations réalisées afin de sélectionner les paramètres de l'article 4 sur la modélisation mathématique.

Introduction

Le criblage de composés démontrant une activité antimicrobienne peut être fait dans un modèle de croissance bactérien *in vitro*. Il est à noter que les analyses *in vitro* comportent des avantages et inconvénients. Les analyses *in vitro* permettent de tester une grande quantité de composés, en peu de temps et contre une vaste gamme de microorganismes. Toutefois, les analyses *in vitro* ne prennent pas en compte les interactions possibles entre la matrice alimentaire, les composés antimicrobiens et les microorganismes à l'étude. Pour cette raison, il est nécessaire d'évaluer *in situ* les meilleures molécules ayant démontré un potentiel antimicrobien *in vitro*. Il est important de mentionner que certaines molécules efficaces *in vitro* vont nécessiter des concentrations jusqu'à 100 fois plus grande dans un aliment afin de démontrer la même efficacité. L'impact de la matrice alimentaire peut expliquer cette différence.

Il est à noter que l'acide propionique en tant qu'antimicrobien dans les produits de viande standardisés n'est pas accepté. Lors de changements à la règlementation canadienne, survenus suite à la crise de la listériose en 2008, le diacétate a été ajouté à la liste des antimicrobiens pouvant être utilisés avec une concentration maximale de 0.25%. Lors de ces mêmes changements l'acétate a aussi été ajouté sans limite d'utilisation.

L'objectif de cette section était en premier lieu de développer un modèle alimentaire, facile à préparer en laboratoire et représentatif de conditions retrouvées dans un produit de charcuterie PAM. Suite à la mise au point du modèle alimentaire, celui-ci sera utilisé pour observer la croissance d'un mélange de plusieurs souches de la bactérie pathogène *Listeria monocytogenes*. Ainsi, ce modèle permettra d'évaluer *in situ* les composés

antimicrobiens sélectionnés lors des analyses *in vitro*. Dans un premier lieu, des acides organiques seuls et en combinaison ont été évalués dans le modèle de contamination. Par la suite, afin de préciser l'expérimentation lors du modèle mathématique présenté à l'article 4, l'impact de l'atmosphère d'emballage et des nitrites a été observé.

Matériel et méthodes

Préparation de la charcuterie

Cette méthodologie est aussi utilisée et décrite dans l'article 1. Le type d'aliment sélectionné est un jambon à haut rendement ayant un taux d'injection de 60% (ex : 60 kg de saumure pour 100 kg de viande). Les produits utilisés lors de cette expérience étaient: du porc haché extra maigre acheté d'une boucherie locale (Métro, Laval, Canada) et le porc était haché le jour même de la préparation de chaque échantillon. Outre de la viande de porc, le jambon contenait de l'érythorbate de sodium (750 ppm), du nitrite de sodium (192 ppm), du tripolyphosphate (0.43%; Les ingrédients alimentaires BSA, St-Léonard, Canada) et de l'eau. La quantité d'eau était réduite d'un aliment à l'autre en fonction de la quantité d'antimicrobiens ajoutée à chaque recette (ex : 1% d'antimicrobiens réduisait 1 % d'eau de la recette originale). Les antimicrobiens utilisés sont l'acétate de sodium, le citrate de sodium, le diacétate de sodium, le lactate de potassium et le propionate de calcium (BSA). Afin de préparer la saumure nécessaire à la recette, tous les ingrédients (incluant les antimicrobiens) ont été mélangés ensemble à l'exception de la viande dans un bécher pour 30 minutes minimum en utilisant un agitateur magnétique. Par la suite la saumure (37.5%) et la viande (62.5%) ont été mélangées pour 2 min. à la vitesse 2 à l'aide d'un mélangeur culinaire de table (A907D, Keenwood, Mississauga, Canada). La viande mélangée ($\approx$ 1 kg) est mise dans un moule d'aluminium rectangulaire jetable (20.3 × 9.8 × 6.3 cm) et est cuite dans un four (Mesallion 850, GE, Missisauga, Canada) à 163°C pour environ 1.25 heure jusqu'à ce que la température interne de l'aliment atteigne 74°C. Après la cuisson, les jambons ont été refroidis dans un réfrigérateur à 4°C pendant 24h. Après la période de refroidissement, le jambon a été divisé en cubes rectangulaires de 20g et chaque cube a été placé dans un tube Falcon de 50 ml. Chaque tube a été conservé à 4°C jusqu'à l'étape de contamination.

Condition de croissance des microorganismes

Cinq souches de *Listeria monocytogenes* (Santé Canada, Health Products and Food Branch, Ottawa, Canada) ont été utilisées pour cette expérience. Les souches ont été isolées d'épisodes de contamination et le numéro, le sérotype et la source de chaque souche sont présentés dans le tableau 2. Avant chaque étape de contamination, les bactéries provenant d'une banque de souches maintenue à -80 °C étaient cultivées séparément dans le TSB (Difco) à 37 °C pendant 24 h.

Tableau 2 : Description des souches de *L. monocytogenes* utilisées dans cette étude

Souches	Sérotype	Source
HPB2558	1/2b	Chien chauds de boeuf
HPB2812	1/2a	Salami fait maison
HPB1043	1/2a	Dinde cuite faite en usine
HPB2569	1/2a	Dinde cuite saumurée
HPB2371	1/2b	Dinde crue

Étape de contamination

Après une période d'incubation initiale de 24h dans le TSB, les bactéries ont été transférées à nouveau dans le TSB et incubées dans les mêmes conditions afin d'obtenir une culture d'environ 10^9 UFC/ml. Le jour de la contamination, les 5 souches ont été mélangées en parts égales. Afin de préparer la solution de contamination, le mélange de souches a été dilué 10^4 fois dans l'eau peptonée (0.1%, Difco) afin de préparer une solution bactérienne contenant entre 10^4 et 10^5 UFC/ml. Un volume de 500 µl de la solution de contamination a été déposé sur le dessus du cube de jambon et par la suite étaler sur la totalité de sa surface en faisant tourner le tube sur son axe pendant 15 secondes. Les tubes ont été conservés à 4°C en position horizontale pour la totalité de l'expérience.

Analyses microbiologiques

Les analyses microbiologiques des échantillons de viande ont été réalisées chaque semaine pour la totalité de l'expérience. Les échantillons choisis au hasard de chaque groupe ont été dénombrés le jour de l'analyse. Les échantillons ont été dilués 1/5 en utilisant de l'eau peptonée (0.1 %), dans un sac à stomacher stérile avec filtre (710 ml, Whirlpak, Nasco, Fort Atkinson, É-U) et en les broyant de façon mécanique pour 1 minute à 260 rpm (400circulator, Seward Laboratory Systems Inc., Port Saint Lucie, É-U). Les échantillons ont été dilués 1/10 dans l'eau peptonée (0.1%) et 100 µl de chaque dilution ont été mis sur une gélose Pacalm (Alpha Bioscience, Baltimore, USA) contenant de l'acryflavine (5 mg/l), de la polymixine B (10 mg/l) et de la ceftazidime (8 mg/l) et les plaques ont été incubées à 37 °C pour 48 h. Après l'incubation, les colonies produisant un précipité noir sur la gélose ont été considérées comme des *L. monocytogenes*. La limite de détection pour ce protocole est de 50 UFC/g.

Analyse des résultats

L'effet des antimicrobiens sélectionnés pour contrôler *L. monocytogenes* dans les échantillons de viande a été évalué sur la base du taux de croissance (µ) des microorganismes. L'équation (1) a été utilisée pour décrire la croissance de *L. monocytogenes* au cours du temps.

$$Y = xe^{\mu t} \quad (1)$$

Le paramètre x signifie la population initiale, µ le taux de croissance en ln UFC/jour/g et t le nombre de jours depuis la contamination. Le jour de la contamination est considéré comme le jour 0. La fonction slope (Excel 2007, Microsoft, Mississauga, Canada) utilisant l'équation (2) a été choisie pour faire une régression linéaire de la partie linéaire de la courbe de croissance afin d'estimer µ.

$$\mu = \frac{\sum(x - \bar{x})(y - \bar{y})}{\sum(x - \bar{x})^2} \quad (2)$$

Le pourcentage d'inhibition présenté dans les différents tableaux a été calculé en utilisant l'équation (3). Le µc signifie le taux de croissance de la formulation contrôle sans les

antimicrobiens et le μa signifie le taux de croissance de la formulation avec les antimicrobiens.

$$\% \text{ inhibition} = \frac{\mu c - \mu a}{\mu a} \times 100 \quad (3)$$

Résultats et discussion

Évaluation des acides organiques

L'objectif de cette étude était d'évaluer l'effet antibactérien des sels d'acides organiques à l'intérieur d'un modèle de charcuterie PAM sur la croissance de *L. monocytogenes* à 4°C. Les effets antibactériens de cinq acides organiques ajoutés dans le jambon, après 35 jours de conservation sont présentés dans le Tableau 3. L'ajout d'un minimum de 0.75% d'acétate est nécessaire pour inhiber complètement la croissance de *L. monocytogenes* pendant 35 jours. Le citrate ne semble pas inhiber la croissance de *L. monocytogenes*. Une concentration de 2.5% n'a pas permis de limiter la croissance du pathogène. Pour le diacétate, 0.25% était suffisant pour inhiber la croissance. Pour le lactate, une concentration supérieure, 3.75% était nécessaire à l'inhibition complète. En ce qui concerne le propionate, une inhibition maximale a été obtenue à 1.58%. Selon l'industriel BSA, les concentrations maximales à utiliser de chaque acide organique sont présentées dans le Tableau 4.

Lors de ces expérimentations le pH de chaque jambon n'a pas été modifié. Ce facteur pourrait avoir influencé les expériences. Un autre concept important est que la plupart des sels d'acide organique sont légèrement alcalins. Toutefois, le diacétate est de l'acide acétique partiellement neutralisé donc il peut diminuer le pH de l'aliment. À titre d'exemple, le diacétate est utilisé pour donner la saveur caractéristique des croustilles au vinaigre. Il est à noter que le jambon contenant le diacétate 0.25% avait un pH de 0.3 unité plus faible que le jambon contrôle. Le jambon fait avec l'acétate, lactate et propionate n'avait pas un pH différent de celui du contrôle. Le pH du jambon avec le citrate 2.5% avait un pH de 0.3 unité plus élevé. Toutefois, un jambon avec le citrate à

2.5% ajusté au pH similaire à celui du contrôle n'a pas démontré d'inhibition de la croissance de *L. monocytogenes*.

Tableau 3: Effet des acides organiques sur la croissance de *L. monocytogenes* dans le jambon après 35 jours

Antimicrobien	Concentration (%)	Inhibition (%)
Acétate	0.25	32
	0.5	64
	0.75	102
	2.5	109
Citrate	2.5	100
	1.5	0
	2.5	0
Diacétate	0.25	103
Lactate	0.45	4
	0.9	35
	2.5	65
	3.75	103
Propionate	0.09	26
	0.18	44
	0.26	54
	1.58	115

Tableau 4: Concentrations maximales devant être utilisées dans les aliments

Acides organiques	Concentrations maximales (%)
Acétate	0.75
Citrate	2.50
Diacétate	0.25
Lactate	3.00
Propionate	0.20

Suite aux résultats obtenus avec les acides organiques seuls, des combinaisons d'acides ont été aussi testées pour en augmenter l'efficacité antimicrobienne et du fait même réduire les concentrations utilisées afin de limiter les impacts sur les qualités organoleptiques et le coût associé à l'ajout de ces antimicrobiens. La combinaison d'acides organiques habituellement utilisée dans l'industrie est le diacétate mélangé avec le lactate dans un mélange 0.1 et 1.44 % respectivement. Les résultats de l'inhibition de la croissance de *L. monocytogenes* en présence d'une combinaison d'acides organiques sont

présentés dans le tableau 5. Les résultats démontrent que pour la combinaison lactate et diacétate la combinaison 1.44 et 0.1 % est la concentration minimale nécessaire pour inhiber la croissance pendant 35 jours. Il est intéressant de noter que la combinaison 0.25 % acétate et 0.9 % lactate est aussi efficace. Cette nouvelle combinaison permet de réduire considérablement la quantité d'antimicrobiens ajoutée dans l'aliment.

Tableau 5: Effet de la combinaison des acides organiques sur la croissance de *L. monocytogenes* dans le jambon après 35 jours

Antimicrobiens	Concentrations (%)	Inhibition (%)
Acétate et lactate	0.25/0.45	70
	0.25/0.9	102
	0.25/1.5	88
Lactate et diacétate	0.86/0.06	14
	1.44/0.1	104
	1.73/0.12	100

Suite aux résultats obtenus avec les différentes combinaisons, un mélange de lactate, acétate et propionate serait une approche permettant le contrôle de la croissance de *L. monocytogenes*. Ainsi l'expérience suivante utilise une concentration constante d'acétate (0.5%) dans toutes les formulations. Les résultats de l'inhibition de la croissance de *L. monocytogenes* en présence d'une combinaison d'acide organique sont présentés dans le tableau 6. Il est important de noter que 0.2% a été utilisé comme concentration maximale de l'acide propionique car cette concentration est le niveau maximal pour ne pas influencer le goût d'un aliment.

Tableau 6: Effet de la combinaison de l'acétate (0.5 %) avec l'acide propionique et lactique sur la croissance de *L. monocytogenes* dans le jambon après 84 jours

Propionique	0	0.05	0.1	0.15	0.2	0	0.05	0.1	0.15	0.2	0	0.05	0.1
Lactique	0	0	0	0	0	0.25	0.25	0.25	0.25	0.25	0.5	0.5	0.5
Inhibition (%)	51	56	59	73	81	40	46	56	66	93	68	62	75
Propionique	0.15	0.2	0	0.05	0.1	0.15	0.2	0	0.05	0.1	0.15	0.2	
Lactique	0.5	0.5	0.75	0.75	0.75	0.75	0.75	1	1	1	1	1	
Inhibition (%)	91	88	72	79	82	95	82	77	63	80	79	92	

Les résultats présentés dans le tableau 6 démontrent que la combinaison de trois sels d'acides organiques (acétate, lactate et propionate) est une solution efficace pour ralentir

le développement de la croissance de *L. monocytogenes* dans les charcuteries. Un ralentissement du taux de croissance pouvant atteindre 95% a été observée.

Effet des nitrites et de l'atmosphère d'emballage

Lors des étapes de standardisation du modèle mathématique, il a été possible d'observer que la présence de nitrite de sodium et de l'emballage sous vide ralentissait la croissance de *L. monocytogenes* dans le jambon. Les Figure 6 et 7 démontrent la croissance de *L. monocytogenes* sous vide et sous air respectivement en présence de concentrations variables de nitrites.

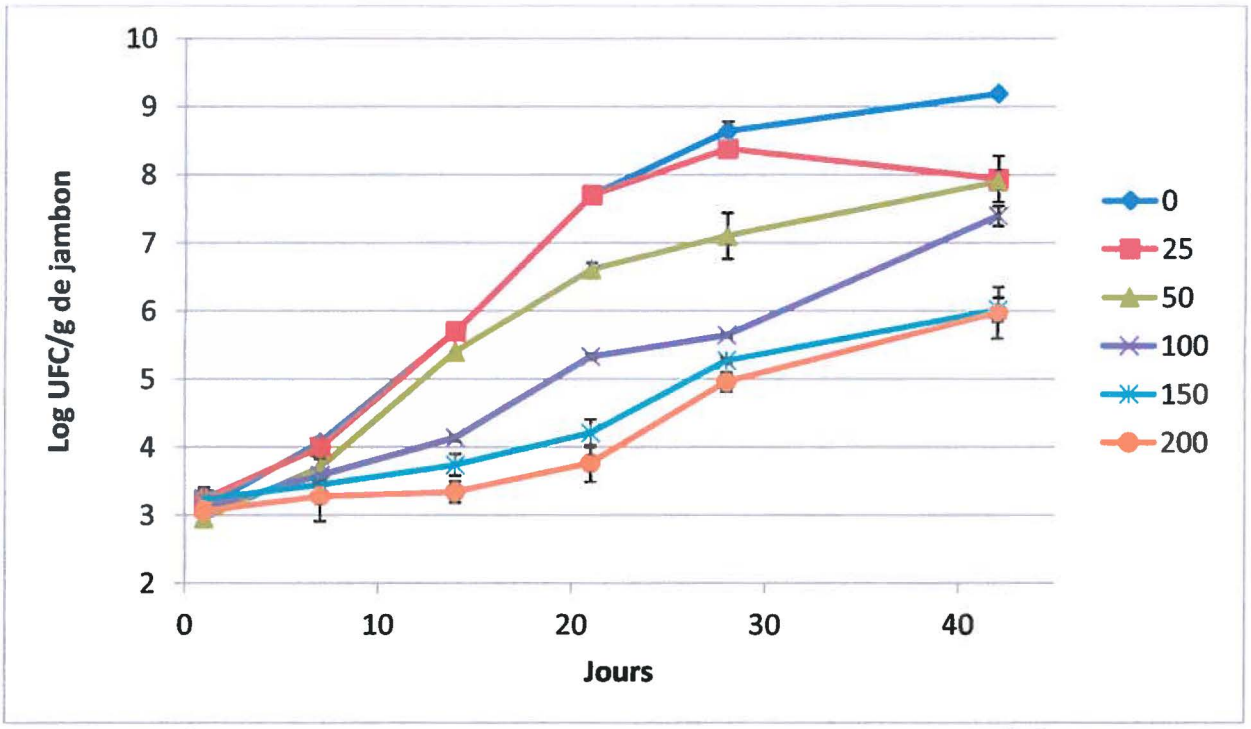


Figure 6: Croissance d'une population mixte de *Listeria monocytogenes* dans le jambon conservé à 4°C emballé sous vide en fonction de la concentration en nitrites de sodium (ppm)

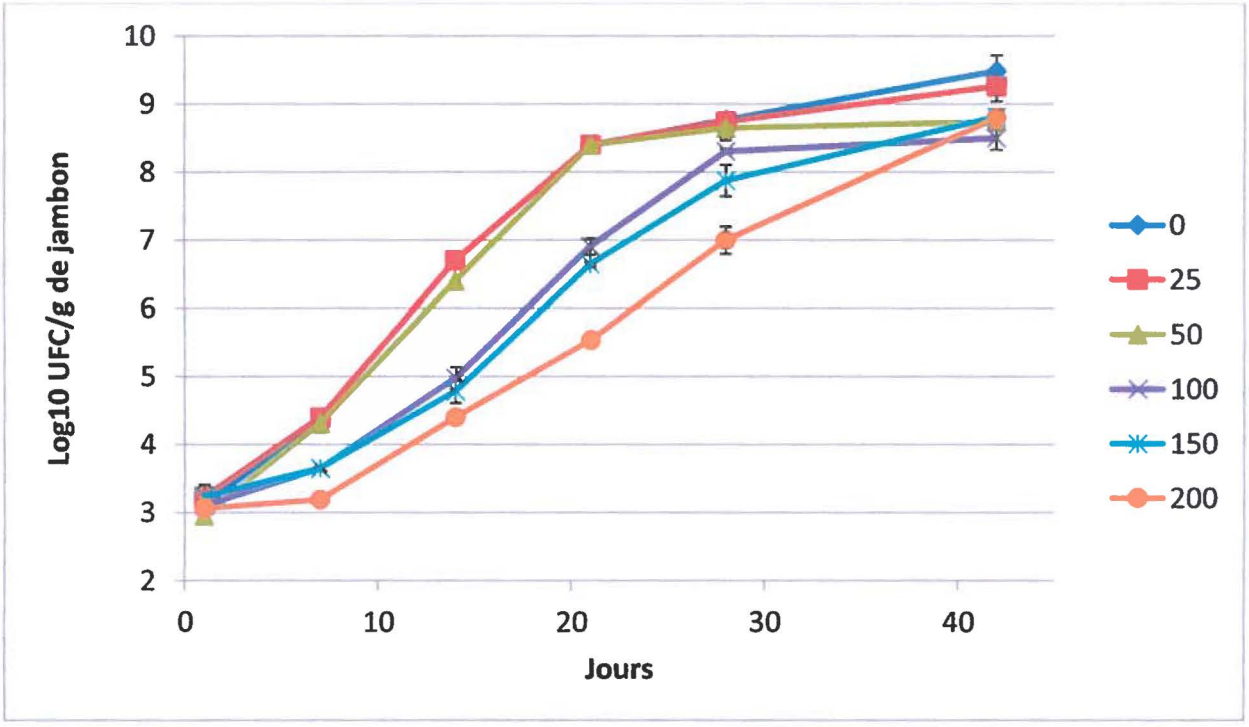


Figure 7: Croissance d'une population mixte de *Listeria monocytogenes* dans le jambon conservé à 4°C emballé sous air en fonction de la concentration en nitrites de sodium (ppm)

Tableau 7: Paramètres de croissance de *L. monocytognes* dans le jambon en fonction des conditions d'emballage et de la concentration en nitrites

	Sous vide				Sous air			
Nitrites (ppm)	Taux de croissance[1]	Δ Taux de croissance[2]	Taux de croissance$_{max}$	↑ log UFC/g[3]	Taux de croissance[1]	Δ Taux de croissance[2]	Taux de croissance$_{max}$	↑ log UFC/g[3]
0	0.370	-	0.658	6.1	0.446	-	0.757	6.4
25	0.301	0.069	0.658	4.7	0.430	0.016	0.757	6
50	0.290	0.081	0.658	4.9	0.419	0.028	0.685	5.8
100	0.244	0.127	0.559	4.3	0.412	0.035	0.633	5.4
150	0.168	0.202	0.391	2.8	0.407	0.039	0.612	5.6
200	0.173	0.198	0.350	2.9	0.396	0.051	0.591	5.7

[1]= moyenne de l'augmentation en ln UFC g^{-1} $jour^{1}$; [2]= variation en comparaison au contrôle en UFC g^{-1} $jour^{-1}$; [3]= variation en comparaison au contrôle en UFC g^{-1} au jour 42

À la lumière de ces résultats, il est possible d'observer que l'emballage sous vide diminue la croissance de *L. monocytogenes*. De plus, l'addition de nitrites de sodium a un effet important sur la croissance du pathogène. Cet effet est d'autant plus important lorsque les nitrites sont combinés à un emballage sous vide.

Conclusion

À la lumière des résultats présentés dans ce chapitre, il est possible de déterminer les paramètres qui devront être évalués lors de la réalisation du modèle mathématique. Puisque dans les conditions présentes dans une charcuterie le citrate ne semble démontrer aucune activité, celui ne sera pas utilisé. En revanche, le lactate, l'acétate et le propionate semblent être des molécules de choix pour le contrôle de *L. monocytogenes*.

Partie 3: Modélisation mathématique

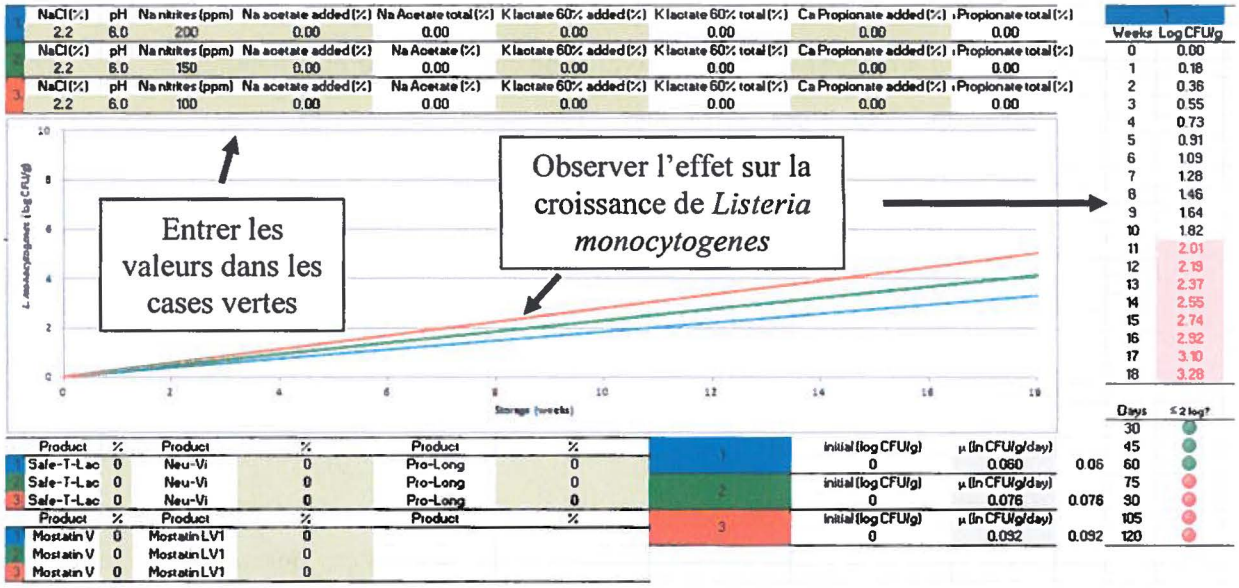
Mise en contexte de la partie 3

La partie 3 présente la création d'un modèle mathématique pouvant prédire la croissance de *Listeria monocytogenes* dans les produits de charcuterie prêt-à-manger.

Suite aux résultats obtenus lors des parties précédentes, il était clair qu'il était possible de limiter la croissance de *Listeria monocytogenes* dans les produits de charcuterie à l'aide de molécules antimicrobiennes. Premièrement, les huiles essentielles n'ont pas été retenues pour le modèle mathématique car les concentrations nécessaires dans le modèle d'aliment choisi étaient trop élevées et influençaient de façon trop importante les qualités organoleptiques de l'aliment. Toutefois, les résultats obtenus démontraient que les acides organiques sont des composés de choix car ils empêchaient la croissance du pathogène, n'influencent pas les qualités organoleptiques et physiques, ont un faible coût, sont potentiellement d'origine naturelle et sont considérés sécuritaires pour la consommation humaine.

La page suivante contient un disque avec le tableur Excel qui permet de visualiser graphiquement l'effet des différents paramètres évalués en se basant sur l'équation mathématique développée.

Programme Informatique



	NaCl(%)	pH	Na nitrites (ppm)	Na acetate added (%)	Na Acetate total (%)	K lactate 60% added (%)	K lactate 60% total (%)	Ca Propionate added (%)	Propionate total (%)
1	2.2	6.0	200	0.00	0.00	0.00	0.00	0.00	0.00
2	NaCl(%)	pH	Na nitrites (ppm)	Na acetate added (%)	Na Acetate (%)	K lactate 60% added (%)	K lactate 60% total (%)	Ca Propionate added (%)	Propionate total (%)
	2.2	6.0	150	0.00	0.00	0.00	0.00	0.00	0.00
3	NaCl(%)	pH	Na nitrites (ppm)	Na acetate added (%)	Na Acetate (%)	K lactate 60% added (%)	K lactate 60% total (%)	Ca Propionate added (%)	Propionate total (%)
	2.2	6.0	100	0.00	0.00	0.00	0.00	0.00	0.00

	Product	%	Product	%	Product	%
1	Safe-T-Lac	0	Neu-Vi	0	Pro-Long	0
2	Safe-T-Lac	0	Neu-Vi	0	Pro-Long	0
3	Safe-T-Lac	0	Neu-Vi	0	Pro-Long	0
	Product	%	Product	%	Product	%
1	Mostatin V	0	Mostatin LV1	0		
2	Mostatin V	0	Mostatin LV1	0		
3	Mostatin V	0	Mostatin LV1	0		

	initial (log CFU/g)	μ (ln CFU/g/day)		Days	≤ 2 log?
1	0	0.060	0.06	30	
2	initial (log CFU/g)	μ (ln CFU/g/day)		45	
	0	0.076	0.076	60	
3	initial (log CFU/g)	μ (ln CFU/g/day)		75	
	0	0.092	0.092	90	
				105	
				120	

Weeks	Log CFU/g
0	0.00
1	0.18
2	0.36
3	0.55
4	0.73
5	0.91
6	1.09
7	1.28
8	1.46
9	1.64
10	1.82
11	2.01
12	2.19
13	2.37
14	2.55
15	2.74
16	2.92
17	3.10
18	3.28

Article 4: Modeling the Growth of *Listeria monocytogenes* in Cured Ready-to-Eat Processed Meat Products containing Various Antimicrobials

Article en preparation pour Meat Science

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Running title: *L. monocytogenes growth in RTE meat products*

Prepared for the Journal of Meat Science

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Contributions des auteurs

J'ai imaginé, mis au point et réalisé toutes les expériences de cette étude. Par la suite, j'ai rédigé le manuscrit scientifique. Khanh Dang Vu m'a assisté lors de la confection du modèle expérimental et de l'analyse statistique des résultats. Khanh Dang Vu a aussi révisé le manuscrit suite à la rédaction. La Prof. Monique Lacroix était la responsable scientifique et la coordinatrice du projet de recherche. De plus, la Prof. Lacroix a supervisé l'élaboration des protocoles, les discussions scientifiques.

Résumé en français

L'objectif de cette étude était d'évaluer l'impact de sept facteurs indépendants (nitrite de sodium, pH, chlorure de sodium, acétate de sodium, lactate de potassium, propionate de calcium et un mélange d'acide alpha du houblon et de nisine) sur la croissance de *Listeria monocytogenes* dans un jambon représentant un modèle de contamination d'une charcuterie prêt-à-manger. Un composé central incluant sept facteurs décris plus haut a été construit et la méthodologie des réponses de surface a été appliquée pour créer un modèle mathématique capable de prédire le taux de croissance de *L. monocytogenes* dans les produits de viande prêt-à-manger. Six paramètres ont démontré une influence significative ($P \leq 0.1$) sur la croissance du microorganisme. Seulement le mélange de houblon et de nisine n'a pas eu d'effet significatif ($P > 0.1$) avec les concentrations utilisées lors de cette étude. Une augmentation de la concentration de chlorure de sodium, de nitrite de sodium, d'acétate de sodium, de lactate de potassium et de propionate de calcium a eu un effet négatif sur la croissance de *L. monocytogenes* tandis qu'une augmentation du pH a eu un effet contraire. L'équation mathématique développée est un outil important afin de réduire le travail nécessaire pour la mise au point de formulations alimentaires ne supportant pas la croissance de *L. monocytogenes* et du même fait assurer la sécurité alimentaire.

Abstract

The objective of this study was to evaluate the impacts of seven independent factors consisting of sodium nitrite, pH, sodium chloride, sodium acetate, sodium lactate syrup, calcium propionate and a blend of nisin and hop alpha acids on the growth rate of *L. monocytogenes* in ham as a model of RTE meat products. A central composite consisted of seven factors mentioned above was designed and the response surface methodology was applied for creating a mathematic model to predict the growth rate of *L. monocytogenes* in RTE meat products. Six parameters showed a significant ($P \leq 0.1$) influence on the growth rate of *L. monocytogenes*. Only the blend of nisin and hop alpha acids did not show any significant effect ($P > 0.1$) in the concentrations used in this study. Increasing concentration of sodium chloride, sodium nitrite, sodium acetate, potassium lactate and calcium propionate showed reduces bacterial growth rate while increasing pH and the inverse effect. The current mathematical equation will be an important tool in order to reduce the required number of challenge studies realized in order to ensure a safe food product.

Keywords: mathematical modeling, *Listeria monocytogenes*, organic acids, deli meat

Introduction

Foods are important sources of diseases of all kind (prions, virus, bacteria, parasites, toxins, heavy metal, etc.). Food contaminants are responsible for various symptoms related to gastroenteritis and can lead to serious consequences such as stomach pain, meningitis, organ failures and even death. In the United-States it was estimated that food consumption causes nearly 48 million illnesses, 128 000 hospitalizations and 3000 deaths every year. Of those alarming numbers, major known pathogens were responsible for 9.4 million illnesses, 56 000 hospitalizations and 1350 deaths and the three most important groups of pathogens are viruses, bacteria and parasites responsible for 59 %, 39 % and 3 % of the total illnesses respectively. *Listeria monocytogenes* is one of the deadliest bacteria found in food related infections. In the United States, L. monocytogenes is responsible for 1455 hospitalizations (94% hospitalization rate) resulting in 255 deaths (16% death rate) every year. *L. monoctygenes* is accountable for 30% of the death related to the illnesses caused by food pathogen bacteria (Scallan, Griffin, Angulo, Tauxe, & Hoekstra, 2011; Scallan, Hoekstra, et al., 2011). *L. monocytogenes* is usually found in soil, water, plants and the intestinal tract of many animals. In addition, 5 to 10 % of healthy humans are asymptomatic transporters of this microorganism. Concerns associated with this microorganism are supported by the fact that this bacterium is ubiquitous to the environment and it can grow at various temperatures (0 to 40 °C) and environmental conditions (Salyers & Whitt, 2002). Although *L. monocytogenes* is a non-spore forming organism and is easily killed by standard cooking techniques (70°C), the growing popularity of the ready-to-eat (RTE) products presents a perfect opportunity for the multiplication and spread of this disease. Therefore, controlling the growth of *L. monocytogenes* in RTE products is of utmost importance for the food industry.

Antimicrobial agents added to food preparations can prevent the growth of *L. monocytogenes* in meat. Current antimicrobial food additives seem only to suppress the growth of the pathogen and not eliminate it in a complex food matrix such as meat.

Antimicrobial additives are mostly organic acids, bacterial metabolites (bacteriocins) and plant extracts. Some organic acids have gained acceptance by the governmental authorities and industry since they were already present in non-negligible quantities in fermented food products, and that their neutralized salt form did not modify the organoleptic properties of the food when used in small quantities. Addition of lactate in combination with diacetate has been widely reported in the literature to be effective in suppressing the growth of this bacteria (Barmpalia, et al., 2005; Lianou, Geornaras, Kendall, Scanga, & Sofos, 2007; Mbandi & Shelef, 2002). Few studies have shown the potential of using propionate in controlling the growth of *L. monocytogenes* in RTE meat products (K. Glass, Preston, & Veesenmeyer, 2007; K. A. Glass, McDonnell, Rassel, & Zierke, 2007). It is also known that the bacteriocin (nisin) produced by the lactic bacteria *Lactococcus lactis* has antilisterial activity in food (De Vuyst & Leroy, 2007). It is important to note that the use of nisin is currently limited by the FDA to 15 ppm in meat. Hops (*Humulus lupulus* L.) are an important source of phenolic compounds in beer and has been found to possess antilisterial activity *in vitro* (Zanoli & Zavatti, 2008). Other factors inherent to the ham basic formulation such as sodium nitrite, sodium chloride and pH will influence the growth of *Listeria* (Karina, Julio, Leda, & Noemi, 2011). Since all these parameter can have crossed related effects, it is important to study their influence in a multiparametric experiment. Response surface methodology (RSM) is a combination of mathematical and statistical techniques for optimization and for development of predictive equation of a process in which interested dependent factors are affected by several independent factors. This method permits one to determine the interactive effects among different independent factors on the dependent factors, which is a very important element in a process consisting of many factors. Some predictive models have been developed by different research groups. For example, Seman et al. (2002) developed an equation to predict the growth of *L. monocytogenes* in ready-to-eat food products using RSM. The authors evaluated the effects of four independent factors consisting of sodium chloride, potassium lactate, sodium diacetate and product moisture content on the growth of *L. monocytogenes*. Mejlholm et al. (2007) also evaluated the growth of *L. monocytogenes* in meat and created some equations that permitted the prediction of the growth of the pathogen in food in presence of specific parameters and antimicrobial

additives. However, this work did not include potential effects of natural antimicrobial agents such as nisin and hop beta acids in these equations. Little is known about the antimicrobial propertires of these factors in combination with other organic acids will have a significant effect in controlling the growth of *L. monocytogenes* in ready-to-eat meat products. The objective of this study was to evaluate the impacts of seven independent factors consisting of sodium nitrite, pH and sodium chloride, sodium acetate, sodium lactate syrup, calcium propionate and a blend of nisin plus hop alpha acids on the growth rate of *L. monocytogenes* in ham as a model of RTE meat products. A central composite consisted of 7 factors mentioned above was designed and the RSM was applied for creating a mathematic model to predict the growth rate of *L. monocytogenes* RTE meat products.

Materials and methods

Sample Preparation

The current methodology is a modified version of the protocol used by (Seman, Borger, Meyer, Hall, & Milkowski, 2002). A central composite design, with 14 star points and 10 replicates of the center point, was used for this study. The factors and levels used are reported in Table 2. Statistica (ver. 10, Statsoft Inc., Tulsa, USA) was used to determine the composition of each run. The 90 products required for the experiment were formulated using extra lean ground pork meat purchased from a local butcher shop (Metro, Laval, Canada) and was ground on the day of preparation of each sample. Products also contained sodium erythorbate (750 ppm), sodium tripolyphosphate (0.43 %; BSA Food Ingredients, St-Leonard, Canada) and water (amounts varied depending on the desired finished-product formulation). Potassium lactate, sodium acetate, calcium propionate, sodium nitrite (BSA Food Ingredients), nisin powder (Profood, IL, USA) and hop alpha acids (Hopsteiner, NewYork, USA) were used in various amounts according to the protocol. All ingredients except meat were mixed together for at least 30 min using a magnetic stirrer in order to prepare the brine solution. Ground meat was then mixed with the brine solution (62.5 and 37.2 % respectively) for 2 min in a tabletop mixer (A907D, Keenwood, Mississauga, Canada) in order to simulate a high yield boneless ham with 60 % injection rate (i.e. 60 kg of brine for 100 kg of meat). The injected meat (≈ 1 kg) was

then poured into a rectangular shape disposable aluminium mold (20.3 × 9.8 × 6.3 cm) and cooked in an oven (Mesallion 850, GE, Missisauga, Canada) at 163°C for 1.25 hours until the internal temperature reached 74°C. Products were chilled for 24h in a refrigerator maintained at 4°C. After chilling, the product was divided into 20g portions using a mechanical slicer (VS250, Omas, Oggiona con Santo Stefano, Italia) and placed into sterile bags (Deli-gold, Winpak, Vaudreuil-Dorion, Canada).

Microorganisms and growth condition

The five *Listeria monocytogenes strains* (Health Canada, Health Products and Food Branch, Ottawa, Canada) used in this study were isolated from foodborne outbreaks and are presented in Table 1. The bacteria were subcultured in tryptic soy broth (TSB, Difco Laboratories, Detroit, USA) at 37 °C for 24 h from the stock culture maintained at −80 °C in TSB containing glycerol (20 %, w/v). Prior to the experiment, 1 mL of culture was incubated through one cycle of growth for 24 h at 37 °C in TSB to obtain a working culture containing approximately 10^9 CFU mL^{-1}. The bacterial culture was centrifuged at 2,000 × g for 15 min at 4 °C and washed with NaCl (0.85 %, w/v) and then resuspended in TSB and incubated for 24 h preceding the experiment.

Contamination procedure

The five *L. monocytogenes* strains were culture separately in TSB for 24h. Prior to inoculation of the ham, 1 ml of each culture were combined. Culture was then diluted 10^4 fold in peptone water (0.1%, Difco) in under to create the contamination solution with a CFU count between 10^4 and 10^5. A volume of 500 µl of contamination solution was then poured on top of each ham slice and spread by applying pressure on the bag surface. Slices were then vacuum packed (97% vacuum and 10 sec vacuum plus; model 350, Spiromac Inc., St-Germain, Canada) and stored at 4°C for the duration of the experiment.

Microbiological analysis

Each formulation was analysed every two weeks. Three random samples were analyzed on each day. Samples were diluted 5-fold using peptone water (0.1%) in a sterile stomacher filter bag (710 ml, Whirlpak, Nasco, Fort Atkinson, USA) and mechanically blended for 1 min. at 260 rpm (400circulator, Seward Laboratory Systems Inc., Port Saint Lucie, USA). Then samples were appropriately diluted 10 fold in peptone water (0.1 %) and 100 µl of each dilution was plated on Palcam agar (Alpha Bioscience, Baltimore, USA) containing acrylflavine (5 mg/L) polymixin B (10 mg/L) and ceftazidime (8 mg/L) and the plates were incubated at 37 °C for 48h. After incubation, colonies producing a black precipitate on the agar plate were considered as *L. monocytogenes*. According to the current protocol the detection limit was 50 CFU/g.

Experimental design and data analysis

A rotatable central composite consisting of 7 independent factors (sodium chloride, pH, sodium nitrite, sodium acetate, sodium lactate syrup, calcium propionate and a mixture of nisin plus hop alpha acids) was designed using STATISTICA 9.0 software (Statsoft Inc., OK, USA.) (Table 2). The design consisted of 64 treatments at cube points, 14 treatments at star points and 12 treatments at center points (total of 90 runs). The radius for the star point was calculated using the formula $2^{(n-1)/4}$, were n is the number of factors used, and therefore it was $2^{(7-1)/4}= 2.83$. The dependent variable was the *L. monocytogenes* growth rate (µ) calculated over a period of 18 weeks. The equation (1) was used to describe the *L. monocytogenes* population over time.

$$Y = xe^{\mu t} \quad (1)$$

Where x is the initial population, µ the growth rate of *L. monocytogenes* in Ln CFU day^{-1} g^{-1}, and t the number of days since the contamination. The day of contamination is considered as day 0. At first CFU counts were transformed using natural logarithm. Then the slope function (Excel 2007, Microsoft, Mississauga, Canada) using equation (2) was used to do a linear regression of the linear part of the growth curve to estimate µ.

Therefore the data points of the stationary phase if observed) were not taken into account for the calculation of the average μ.

$$\mu = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2} \quad (2)$$

When all treatments of the central composite design (Table 2) were conducted, data were used for analysis of variance (ANOVA), regression analysis and response surface methodology. A second-order polynomial equation was built to predict the growth of *L. monocytogenes* in RTE meat products (equation 3):

$$Y = B_0 + \sum_{i=1}^{7} B_i X_i + \sum_{i=1}^{7} B_{ii} X_i^2 + \sum_{i=1}^{6} \sum_{j=i+1}^{7} B_{ij} X_i X_j \quad (3)$$

where Y, predicted response; B_0, the constant coefficient; *Xi* and *Xj*, values of various levels of the independent factor; *Bi*, the linear coefficient of each independent factor; *Bii*, the quadratic coefficient of each factor; and *Bij*, the interactive coefficient between two independent factor. All main effects of the 7 parameters were left in the model even if P > 0.1 since they were the main focus of the experiment. Second order effects were left out of the model if P > 0.1. Only the growth rate was considered in this model since biweekly sampling resolution did not allow usto accurately detect any lag phase.

Results and Discussion

The objective of this study was to evaluate the effects of various antimicrobials and intrinsic parameters on the growth rate of *L. monocytogenes* in a typical ready-to-eat meat product. The growth rate of a population of 5 different strains of *L. monocytogenes* was evaluated over 18 weeks. The equation (1) was used to describe the growth rate of the population. A linear regression of the logarithmic part of the growth curve was obtained describing the growth rate associated with each ham formulation. The values of 0.250 and -0.017 CFU g^{-1} day^{-1} were the maximum and minimum growth rates, respectively.

Analysis of variance

The analysis of variance (ANOVA) is presented in the Table 3. The ANOVA showed that only the mixture of hop alpha acids plus nisin did not influence the growth rate of *L. monocytogenes* significantly ($P > 0.1$). All other main effects showed significant influence on the growth rate: NaCl ($p = 0.0094$), pH ($p = 0.0000$), Nitrite ($p = 0.0000$), Acetate ($p = 0.0000$), Lactate ($p = 0.0000$), propionate ($p = 0.0287$). It was found that NaCl and propionate were less effective as compared to other factors such as pH, nitrite, acetate and lactate. Only the pH demonstrated a significant second order effect ($p = 0.0000$). The absence of impact on the growth rate of the mixture of hop alpha acids plus nisin could be explained by the low concentrations used in this experiment. The maximum applied concentration of this mixture in this study was 20 ppm (Table 2). In the literature, nisin demonstrated bactericidal effects in food products but at higher concentration (Samelis, et al., 2005).

The analysis also discovered 5 significant two-way interactions: NaCl × Nitrite ($p = 0.0087$), NaCl × Acetate ($p = 0.0840$), pH × Nitrite (0.0103), pH × Lactate ($p = 0.0000$), and Nitrite × Lactate ($p = 0.0289$). The influences on the growth rate of the two-way interactions are presented in Figures 1, 2, 3, 4 and 5). Two-way interactions between pH and sodium nitrite and pH and potassium lactate on the growth rate of *L. monocytogenes* were expected. It was found that at lower pH values (from 5.4 to 5.7) and lower concentrations of nitrite (less than 80 ppm) or at higher pH values (from 5.8 to 6.1) and higher concentrations of nitrite (from 180 to 200 ppm), the growth of *L. monocytogenes* was inhibited (Figure 5). Early research done by (Tarr, 1941) showed that the bacteriostactic effect of nitrite in meat was inversely proportional to the pH of the media. Interaction between pH and potassium lactate could be explained by the fact that only the conjugated organic acids are bacteriostatic (Jamilah, Abbas, & Rahman, 2008). Since the equilibrium between the conjugated and disassociated form of a weak acid are dependent of the dissociation constant of that acid (pKa), then the variation of the pH would ultimately affect the concentration of the bacteriostatic acids.

Final equation

The resulting regression equation derived from the current analysis in order to predict the growth rate of *L. monocytogenes* in RTE products stored at 4°C is as follow:

$$5.97887 - [NaCl\ (\%)] \times 0.12096 - pH \times 2.28703 + pH^2 \times 0.22587 + [NaNO_2\ (ppm)] \times 0.00459 - [Sodium\ acetate\ (\%)] \times 0.29216 + [Potassium\ lactate\ (\%)] \times 0.63775 - [Calcium\ propionate\ (\%)] \times 0.15995 + [NaCl\ (\%)] \times [NaNO_2\ (ppm)] \times 0.00061 + [NaCl\ (\%)] \times [Sodium\ acetate\ (\%)] \times 0.10799 - pH \times [NaNO_2\ (ppm)] \times 0.00104 - pH \times [Potassium\ lactate\ (\%)] \times 0.11653 + [NaNO_2\ (ppm)] \times [Potassium\ lactate\ (\%)] \times 0.00032$$

When entered in the final equation, each parameter have the expected effect where the increasing concentrations of NaCl, $NaNO_2$, sodium acetate, potassium lactate and calcium propionate reduce the bacterial growth rate. To the opposite of the other parameters an increased pH value will also increase the growth rate of the bacteria.

Conclusion

The objective of this study was to evaluate the effect of various antimicrobials as solutions to inhibit the growth of *L. monocytogenes* in deli meat. Even if the antimicrobial effects of lactate and acetate have already been studied in previous models, this present study showed the antimicrobial potential of propionate. In addition, interactions were also highlighted between parameters of this model. The equation created with this study will help food produced in making safer food products. The following experiment should determine the boundaries where this particular model should be used. Challenge studies using production samples should be undertaken in order to validate the current model.

Acknowledgements

This research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and BSA Food Ingredients s.e.c/l.p (Montreal, Qc, Canada) under a research contract agreement with the INRS. The NSERC, le Fonds de Recherche sur la Nature et les Technologies (FQRNT) and BSA Food Ingredients s.e.c/l.p supported Dominic Dussault through the Industrial Innovation Scholarships BMP Innovation. The

Armand-Frappier Fondation also supported Dominic Dussault through the doctoral scholarship.

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Table 1 : Source and serotypes of *L. monocytogenes* strains used in this study

Strain	serotype	source
HPB2558	1/2b	Beef hot dogs
HPB2812	1/2a	Homemade salami
HPB1043	1/2a	Turkey frank factory isolate
HPB2569	1/2a	Cooked cured sliced turkey
HPB2371	1/2b	Raw turkey

Table 2: Design matrix of the central composite design for modeling and growth rate of *L. monocytogenes* in processed meat

Run	Na	pH	Ni	Ac	La	Pr	N/A	μ	Run	Na	pH	Ni	Ac	La	Pr	N/A	μ
1	1.15	5.90	65	0.25	0.95	0.07	13.6	0.095	46	1.85	5.90	136	0.51	0.95	0.14	13.6	-0.003
2	1.15	5.90	65	0.25	0.95	0.14	6.5	0.049	47	1.85	5.90	136	0.51	2.05	0.07	13.6	-0.007
3	1.15	5.90	65	0.25	2.05	0.07	6.5	0.000	48	1.85	5.90	136	0.51	2.05	0.14	6.5	-0.004
4	1.15	5.90	65	0.25	2.05	0.14	13.6	0.031	49	1.85	6.30	65	0.25	0.95	0.07	13.6	0.146
5	1.15	5.90	65	0.51	0.95	0.07	6.5	0.059	50	1.85	6.30	65	0.25	0.95	0.14	6.5	0.125
6	1.15	5.90	65	0.51	0.95	0.14	13.6	0.003	51	1.85	6.30	65	0.25	2.05	0.07	6.5	0.084
7	1.15	5.90	65	0.51	2.05	0.07	13.6	0.009	52	1.85	6.30	65	0.25	2.05	0.14	13.6	0.070
8	1.15	5.90	65	0.51	2.05	0.14	6.5	-0.004	53	1.85	6.30	65	0.51	0.95	0.07	6.5	0.076
9	1.15	5.90	136	0.25	0.95	0.07	6.5	0.047	54	1.85	6.30	65	0.51	0.95	0.14	13.6	0.135
10	1.15	5.90	136	0.25	0.95	0.14	13.6	0.016	55	1.85	6.30	65	0.51	2.05	0.07	13.6	0.068
11	1.15	5.90	136	0.25	2.05	0.07	13.6	0.020	56	1.85	6.30	65	0.51	2.05	0.14	6.5	0.064
12	1.15	5.90	136	0.25	2.05	0.14	6.5	0.000	57	1.85	6.30	136	0.25	0.95	0.07	6.5	0.104
13	1.15	5.90	136	0.51	0.95	0.07	13.6	0.006	58	1.85	6.30	136	0.25	0.95	0.14	13.6	0.167
14	1.15	5.90	136	0.51	0.95	0.14	6.5	-0.002	59	1.85	6.30	136	0.25	2.05	0.07	13.6	0.019
15	1.15	5.90	136	0.51	2.05	0.07	6.5	0.001	60	1.85	6.30	136	0.25	2.05	0.14	6.5	0.070
16	1.15	5.90	136	0.51	2.05	0.14	13.6	0.000	61	1.85	6.30	136	0.51	0.95	0.07	13.6	0.119
17	1.15	6.30	65	0.25	0.95	0.07	6.5	0.250	62	1.85	6.30	136	0.51	0.95	0.14	6.5	0.062
18	1.15	6.30	65	0.25	0.95	0.14	13.6	0.187	63	1.85	6.30	136	0.51	2.05	0.07	6.5	0.056
19	1.15	6.30	65	0.25	2.05	0.07	13.6	0.112	64	1.85	6.30	136	0.51	2.05	0.14	13.6	0.022
20	1.15	6.30	65	0.25	2.05	0.14	6.5	0.101	65	0.51	6.10	100	0.38	1.50	0.10	10.0	0.077
21	1.15	6.30	65	0.51	0.95	0.07	13.6	0.193	66	2.49	6.10	100	0.38	1.50	0.10	10.0	0.005
22	1.15	6.30	65	0.51	0.95	0.14	6.5	0.123	67	1.50	5.53	100	0.38	1.50	0.10	10.0	0.005
23	1.15	6.30	65	0.51	2.05	0.07	6.5	0.013	68	1.50	6.67	100	0.38	1.50	0.10	10.0	0.212
24	1.15	6.30	65	0.51	2.05	0.14	13.6	0.008	69	1.50	6.10	0	0.38	1.50	0.10	10.0	0.065
25	1.15	6.30	136	0.25	0.95	0.07	13.6	0.115	70	1.50	6.10	200	0.38	1.50	0.10	10.0	0.012
26	1.15	6.30	136	0.25	0.95	0.14	6.5	0.100	71	1.50	6.10	100	0	1.50	0.10	10.0	0.092
27	1.15	6.30	136	0.25	2.05	0.07	6.5	0.079	72	1.50	6.10	100	0.74	1.50	0.10	10.0	-0.005
28	1.15	6.30	136	0.25	2.05	0.14	13.6	0.037	73	1.50	6.10	100	0.38	0	0.10	10.0	0.088
29	1.15	6.30	136	0.51	0.95	0.07	6.5	0.042	74	1.50	6.10	100	0.38	3.06	0.10	10.0	-0.016
30	1.15	6.30	136	0.51	0.95	0.14	13.6	0.058	75	1.50	6.10	100	0.38	1.50	0	10.0	0.052
31	1.15	6.30	136	0.51	2.05	0.07	13.6	0.011	76	1.50	6.10	100	0.38	1.50	0.20	10.0	0.017
32	1.15	6.30	136	0.51	2.05	0.14	6.5	0.022	77	1.50	6.10	100	0.38	1.50	0.10	0	0.035
33	1.85	5.90	65	0.25	0.95	0.07	6.5	0.044	78	1.50	6.10	100	0.38	1.50	0.10	20.0	0.047
34	1.85	5.90	65	0.25	0.95	0.14	13.6	0.036	79	1.50	6.10	100	0.38	1.50	0.10	10.0	0.022
35	1.85	5.90	65	0.25	2.05	0.07	13.6	0.028	80	1.50	6.10	100	0.38	1.50	0.10	10.0	0.047
36	1.85	5.90	65	0.25	2.05	0.14	6.5	-0.011	81	1.50	6.10	100	0.38	1.50	0.10	10.0	0.046
37	1.85	5.90	65	0.51	0.95	0.07	13.6	-0.011	82	1.50	6.10	100	0.38	1.50	0.10	10.0	0.051
38	1.85	5.90	65	0.51	0.95	0.14	6.5	-0.008	83	1.50	6.10	100	0.38	1.50	0.10	10.0	0.016
39	1.85	5.90	65	0.51	2.05	0.07	6.5	-0.017	84	1.50	6.10	100	0.38	1.50	0.10	10.0	0.031
40	1.85	5.90	65	0.51	2.05	0.14	13.6	-0.011	85	1.50	6.10	100	0.38	1.50	0.10	10.0	0.024
41	1.85	5.90	136	0.25	0.95	0.07	13.6	0.047	86	1.50	6.10	100	0.38	1.50	0.10	10.0	0.030
42	1.85	5.90	136	0.25	0.95	0.14	6.5	0.002	87	1.50	6.10	100	0.38	1.50	0.10	10.0	0.034
43	1.85	5.90	136	0.25	2.05	0.07	6.5	-0.011	88	1.50	6.10	100	0.38	1.50	0.10	10.0	0.048
44	1.85	5.90	136	0.25	2.05	0.14	13.6	-0.006	89	1.50	6.10	100	0.38	1.50	0.10	10.0	0.053
45	1.85	5.90	136	0.51	0.95	0.07	6.5	-0.013	90	1.50	6.10	100	0.38	1.50	0.10	10.0	0.048

Na= NaCl (%), Ni= sodium nitrite (ppm), Ac= sodium acetate (%), La= sodium lactate syrup (%), Pr= calcium propionate, N/A= nisin + hop alpha acid (ppm), μ= growth rate of *L. monocytogenes* (Ln CFU/g/day)

Table 3: Analysis of variance for growth rate constant of *L. monocytogenes*

	Source	df	Sum of square	F ratio	Probability
1	NaCl	1	0.003584	7.1	0.0094
2	pH	1	0.115341	228.7	0.0000
2	pH (Q)	1	0.009868	19.6	0.0000
3	Nitrite	1	0.012992	25.8	0.0000
4	Acetate	1	0.022917	45.4	0.0000
5	Lactate	1	0.040786	80.9	0.0000
6	Propionate	1	0.002507	5.0	0.0287
	NaCl × Nitrite	1	0.003653	7.2	0.0087
	NaCl × Acetate	1	0.001546	3.1	0.0840
2 way	pH × Nitrite	1	0.003486	6.9	0.0103
	pH × Lactate	1	0.010515	20.8	0.0000
	Nitrite × Lactate	1	0.002501	5.0	0.0289
	Total error	77	0.038837		
	Lack of fit	66	0.037003	3.3614	0.015828

Table 4: Regression coefficient for predicting the growth of *L. monocytogenes* in meat

	Factor	Regression coefficient	p	-95% Cnf. Limit	+95% Cnf. Limit
	Constant	5.97887	0.003	2.13545	9.82229
1	NaCl	-0.12096	0.001	-0.18726	-0.05466
2	pH	-2.28703	0.000	-3.53267	-1.04140
	pH (Q)	0.22587	0.000	0.12419	0.32756
3	Nitrite	0.00459	0.064	-0.00028	0.00946
4	Acetate	-0.29216	0.003	-0.48039	-0.10393
5	Lactate	0.63775	0.000	0.32631	0.94920
6	Propionate	-0.15995	0.029	-0.30280	-0.01710
2 way	NaCl xnitrite	0.00061	0.009	0.00016	0.00106
	sNaCl x Acetate	0.10799	0.084	-0.01485	0.23083
	pH x Acetate	-0.00104	0.010	-0.00183	-0.00025
	pH x Lactate	-0.11653	0.000	-0.16735	-0.06571
	Nitrite x Lactate	0.00032	0.029	0.00003	0.00061

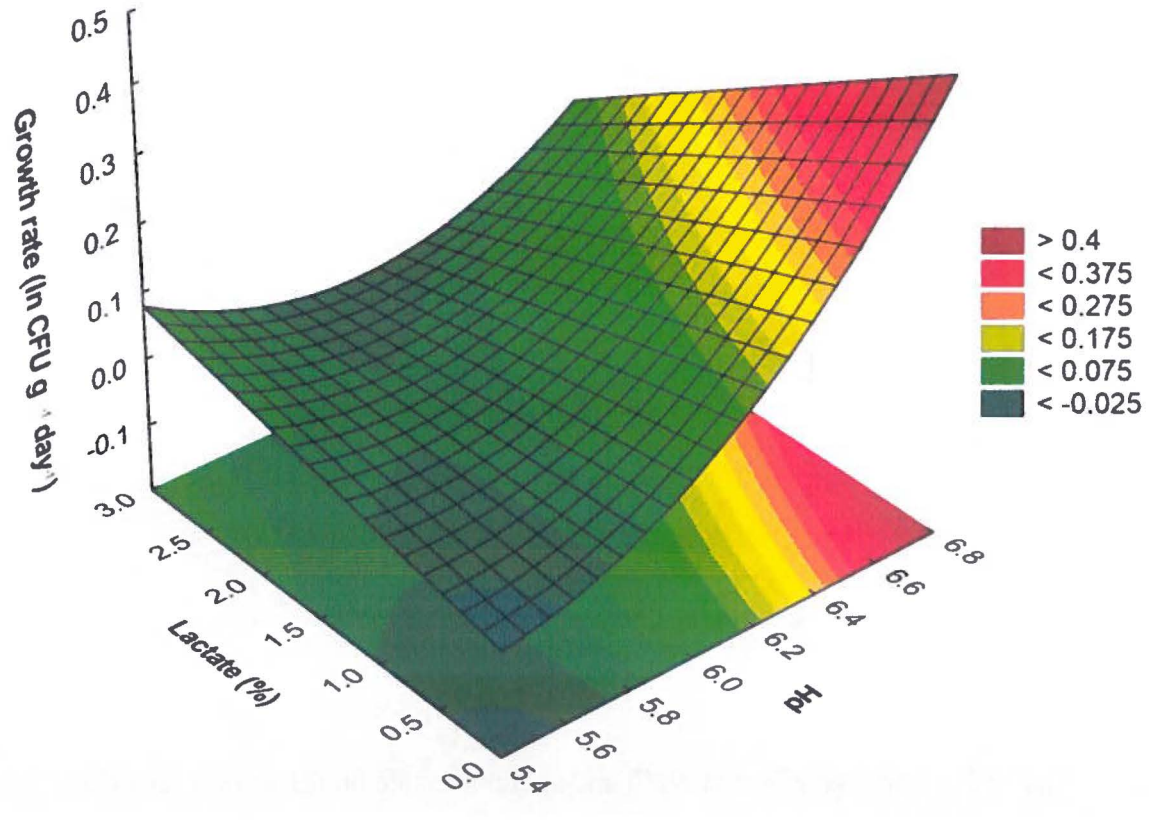

Figure 1: Combined effect of potassium lactate syrup and pH on the growth rate of *L. monocytogenes* in ham

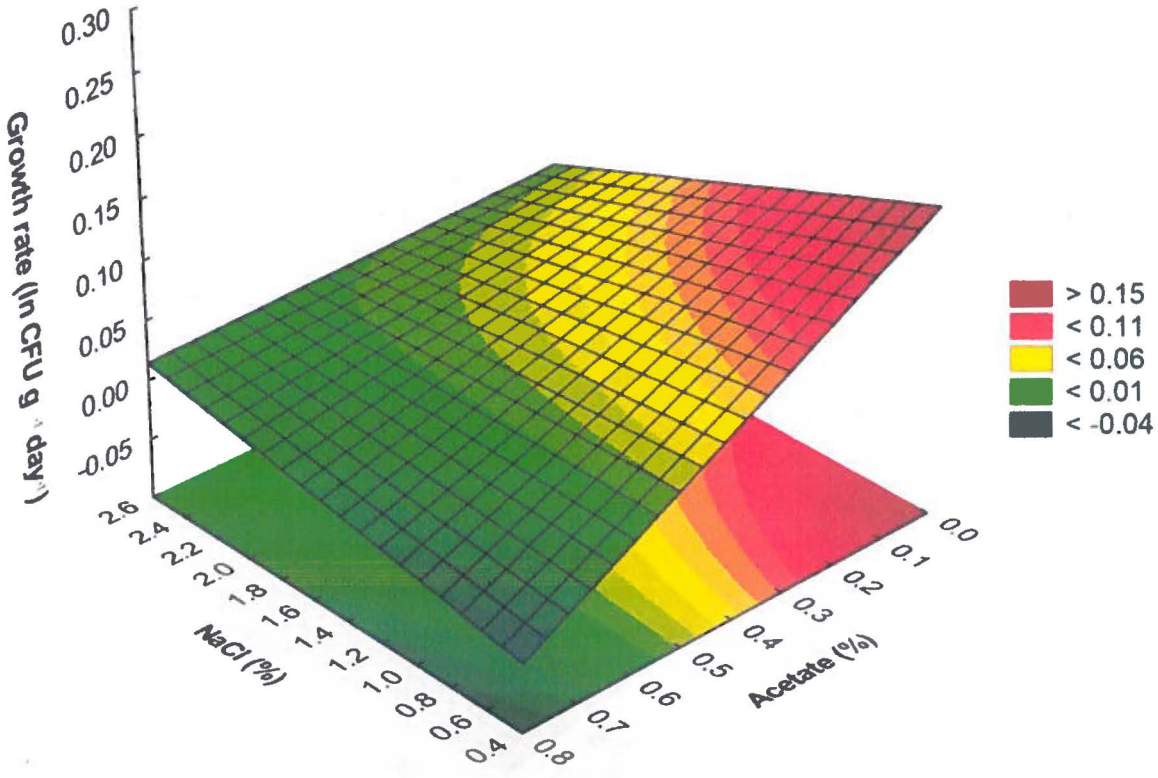

Figure 2: Combined effect of NaCl and sodium acetate on the growth rate of *L. monocytogenes* in ham

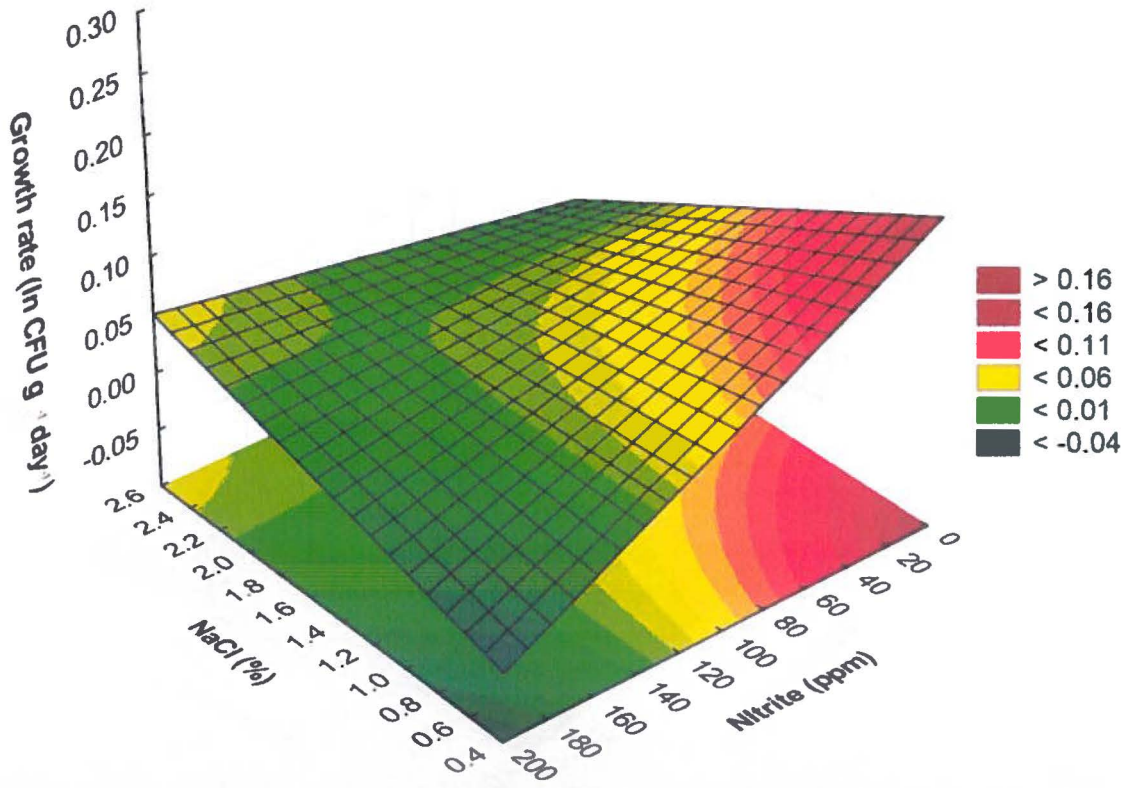

Figure 3: Combined effect of NaCl and sodium nitrite on the growth rate of *L. monocytogenes* in ham

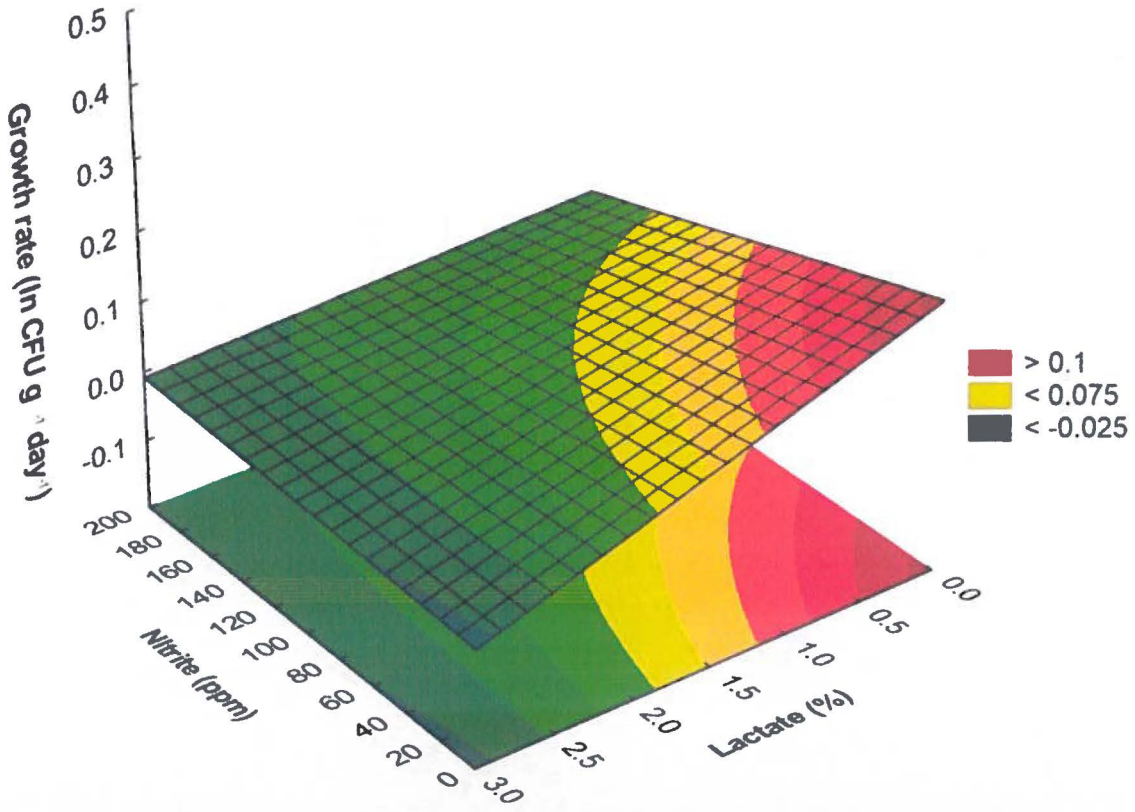

Figure 4: Combined effect of sodium nitrite and potassium lactate syrup on the growth rate of *L. monocytogenes* in ham

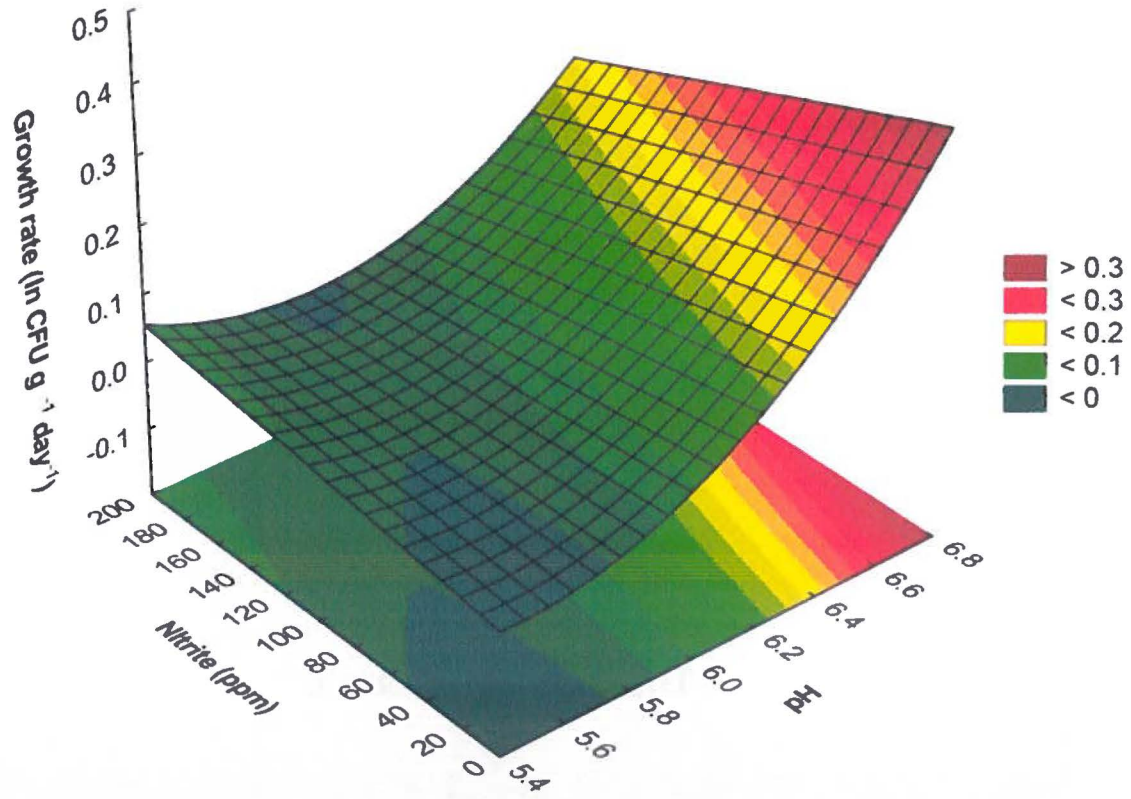

Figure 5: Combined effect of sodium nitrite and pH on the growth rate of *L. monocytogenes* in ham

Discussion globale

Ce prochain chapitre se veut un retour sur les conclusions globales des différents chapitres présentés précedemment.

Huiles Essentielles

L'objectif de ce projet de recherche était de sélectionner des composés d'origine naturelle ayant des propriétés antimicrobiennes dans les produits de charcuterie. Le premier article a permis de caractériser une gamme d'huiles essentielles pour leur pouvoir antimicrobien *in vitro*. Suite à cette première expérience il a été possible de conclure qu'une quantité importante d'huiles essentielles possèdent des propriétés antimicrobiennes. Des 67 huiles essentielles et oléorésines évaluées, 41 ont démontré une activité antimicrobienne. Le spectre d'activité antimicrobienne des huiles essentielles a pu être classé en 3 grands groupes : efficace contre les Gram positifs, efficace contre les Gram positifs et négatifs et efficace contre les Gram positifs, négatifs et *Pseudomonas aeruginosas*. *P. aeruginosas* est une bactérie qui a une résistance accrue aux molécules désinfectantes. La détermination des concentrations minimales inhibitrices de chaque huile essentielle a permis de quantifier cette activité antimicrobienne. La quantification a ainsi permis de sélectionner 4 composés (ail, cannelle chinoise cassi, origan et thym rouge) pour des analyses *in situ*. Les analyses sur modèle alimentaire *in situ* ont permis de démontrer que la croissance de *L. monocytogenes* était réduite au cours du temps de 19 et 10% pour l'huile essentielle d'origan et de cannelle respectivement. Afin de déterminer si les huiles essentielles avaient un potentiel comme agents antimicrobiens dans un aliment lorsqu'elles sont appliquées directement dans le mélange, les concentrations utilisées étaient de 500ppm. Tel que décrit précédemment, la limite d'utilisation des huiles essentielles se trouve entre 50 et 200ppm. Au-delà de cette limite l'impact sur les propriétés organoleptiques est trop important.

La suite des recherches sur ce sujet devrait se concentrer sur la combinaison de traitement. C'est-à-dire, combiner d'autres molécules avec les huiles essentielles afin d'atteindre une concentration d'huile essentielle qui n'aurait pas d'impact sur le goût des produits alimentaires. Une autre approche serait de combiner les huiles essentielles avec des traîtements physiques. Tel que décrit dans le troisième article, il est possible de

multiplier l'effet de molécules antimicrobiennes en les combinant avec des traîtements physiques antimicrobiens.

Extraits d'algues et de cyanobactéries

Ce projet de recherche m'a donné l'opportunité d'évaluer des extraits d'algues et de cyanobactéries provenant de la région d'Hawaii et des Caraïbes. L'environnement marin est une source connue de molécules structurellement complexes et de composés bioactifs. Les résultats ont démontré que certains extraits (*Padina* sp. et *Ulva* sp.) et composés (antillatoxin B, laxaphycins A, B and B3 et malyngamides A, C, I et J) possèdent des activités antimicrobiennes contre les pathogènes Gram positifs (*Listeria monocytogenes*, *Bacillus cereus* et *Staphylococcus aureus*) et ce à de faibles concentrations (≤500 ppm). Deuxièmement, nous avons analysé l'effet des extraits et isolats d'origine marin sur l'induction de la NAD(P)H :quinone réductase sur des cellules d'un hépatome murin Hepa 1c1c7. Les résultats ont démontré que les extraits *de Gracilaria salicornia*, *Ligora* sp. et *Ulva* sp. possèdent un potentiel élevé pour l'induction de NQO1. Cette recherche démontre le potentiel de certains composés marins comme source de substances biologiquement actives pour contrôler les pathogènes alimentaires et la prévention de certains cancers. Suite à la production de ces résultats, il aurait été intéressant de faire une étude *in situ* dans un aliment du même type que celle réalisée sur les huiles essentielles. Toutefois, la faible quantité d'échantillons fournis (1 mg par échantillons) ainsi que le travail nécessaire pour produire ces échantillons par l'Université du Pacifique à Hawaï n'a pas permis la réalisation de cette étude.

Ces résultats démontrent le potentiel de la flore pour deux applications biologiques différentes. La suite de cette recette devrait se concentrer sur la production à plus grande échèle des différents extraits. Ainsi, il serait possible de valider les différents résultats dans un modèle alimentaire.

Acides organiques

Les acides organiques sont des composés de choix pour leur utilisation dans les aliments car sous leur forme de sel neutralisé, ils n'influencent pas les qualités organoleptiques et

physiques, ont un faible coût, sont potentiellement d'origine naturelle (végétal ou microbien) et sont considérés sécuritaires pour la consommation humaine. Suite à l'étude réalisée lors de l'article 3, il a été possible de démontrer qu'un concentré d'acide organique, principalement l'acide propionique, pouvait contrôler la croissance microbienne dans un aliment à base de viande. Avec ce même acide, il a été possible de d'observer que des ingrédients antimicrobiens peuvent avoir des effets synergiques avec des traîtements physique, tel que l'irradiation ionisante, afin de réduire la croissance microbienne. Cette étape à servi de prélude pour la poursuite des expériences sur les bactéries pathogènes. Par la suite, le développement du modèle alimentaire qui est présenté dans le matériel supplémentaire, a permis de démontrer que les sels neutralisés d'acides organiques étaient des candidats de choix pour inhiber la croissance de *L. monocytogenes* dans les charcuteries. En utilisant des concentrations n'affectant pas les propriétés de l'aliment, il était possible d'inhiber la croissance du pathogène sur toute la durée de conservation de l'aliment. Étant donné le mode d'action des acides organiques sur les cellules bactériennes, il n'était toutefois pas possible de réduire le nombre de microorganismes. Cet effet bactériostatique est suffisant afin de limiter le nombre de bactéries sous le seuil de contamination nécessaire au développement de la listériose chez l'humain. À la suite de ces expériences, une problématique subsistait toujours. L'efficacité des mélanges d'acides organiques était influencée par certains paramètres intrinsèques de l'aliment tels, la concentration en chlorure de sodium, en nitrites de sodium, le pH de l'aliment et aussi la présence d'autres acides organiques. Il est donc primordial de caractériser les paramêtres qui influencent l'efficacité antimicrobienne des acides organiques dans une matrice alimentaire.

Modélisation mathématique

La modélisation mathématique est un outil permettant d'évaluer la croissance d'un microorganisme dans des conditions en se basant sur des études de validation antérieures. La modélisation présentée dans l'article 4 a été effectuée pour mesurer l'effet de 3 paramètres intrinsèques des viandes prêtes à manger (chlorure de sodium, nitrite de sodium ainsi que le pH) et 4 solutions antimicrobiennes pouvant être utilisées pour contrôler la croissance de *L. monocytogenes* (lactate de potassium, acétate de sodium,

propionate de calcium et un mélange de nisine et de houblon). Le modèle a permis la création d'une équation mathématique démontrant l'influence de chaque paramètre. Ces résultats sont un pas de plus vers l'avant afin de créer des formulations alimentaires permettant le contrôle de la bactérie *L. monocytogenes* dans les aliments. La feuille de calcul qui a été créé permettra aux formulateurs de l'alimentation de visualiser l'impact de la modification de différents paramètres sur la croissance du pathogène. La création de la feuille de calcul était une étape essentielle afin que la formule puisse devenir un outil simple d'utilisation pour tous et chacun. Toutefois, des études de validation devront être entreprises pour évaluer les limites d'utilisation d'une telle équation.

Conclusion

En conclusion, l'utilisation d'extraits d'origine naturelle est une option valable pour le contrôle de *Listeria monocytogenes* dans les produits de charcuterie. Cet ouvrage démontre que des solutions sont déjà disponibles pour le contrôle des microorganismes dans les aliments. Il est à noter que des recherches sur des antimicrobiens simples ont été entrepris il y a de ça plusieurs années. Certains ouvrages sur les acides organiques remontent à plus de 50 ans. Toutefois, à cette époque, les maladies alimentaires d'importance n'était pas les mêmes qu'aujourd'hui et l'impact des différents microorganismes n'était pas aussi bien compris par la science de l'époque. D'autres problématiques sont en émergence. L'apparition de la problématique de la listériose était un point tournant des années 2000 mais d'autres pathogènes semblent avoir une importance plus grande qu'estimée auparavant. Je crois que la solution à la problématique des maladies d'origine alimentaire se trouve dans la caractérisation des effets antimicrobiens de composés simples déjà utilisés dans l'alimentation. Nous connaissons déjà les effets sur la santé humaine de ces composés et de plus, une caractérisation partielle est déjà disponible. Aussi, l'importance de la matrice alimentaire n'est pas à négliger. Certains paramètres des aliments peuvent augmenter ou diminuer l'efficacité des antimicrobiens. Une meilleure compréhension de ces interactions pourra certes améliorer le contrôle des maladies associées aux aliments.

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Annexe 1: Liste des autres publications

Lors des mes études doctorales, j'ai eu le privilège de participer à différents projets de recherche. Cette participation m'a permis d'ajouter d'autres publications à mon actif. Ici-bas suit la liste des autres contributions n'étant pas directement reliées à mon projet de doctorat.

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