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L'effet antimicrobien des films biodégradables à base d'huiles essentielles et le mécanisme d'action de trois huiles essentielles sur des bactéries Gram-positif et Gram-négatif.

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A mes très chers parents et surtout à ma mère, une dame exceptionnelle.

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Résumé

La concentration minimale inhibitrice (CMI) de 61 huiles essentielles a été déterminée contre une bactérie d'altération: *Pseudomonas putida* et quatre bactéries pathogènes: *Escherichia coli* O157:H7, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Staphylococcus aureus* et *Listeria monocytogenes*. Les huiles essentielles les plus actives ont été choisies pour connaître leur mode d'action sur une bactérie de type Gram-positif, *L. monocytogenes* et une bactérie de type Gram-négatif, *E. coli* O157:H7. De plus, ces huiles ont été immobilisées dans trois types de films et évaluées pour leur pouvoir antimicrobien sur de la viande et deux types de charcuterie.

L'application des films antimicrobiens sur des tranches de bifteck de ronde dans lesquels des huiles essentielles d'origan et de piment ont été immobilisées a permis un contrôle de la croissance de *Pseudomonas* sp. et d'*E. coli* O157:H7. D'autres formulations, plus résistantes à l'eau ont aussi été mises au point afin d'améliorer leurs propriétés physico-chimiques. Ces films contenaient cette fois des extraits d'origan d'Espagne, de cannelle de Chine et/ou de sarriette des montagnes et ont subi deux traitements d'insolubilisation. Ces films ont été appliqués sur trois types de viande (bœuf, jambon ou bologne). Le contrôle d'*E. coli* O157:H7, *S.* Typhimurium et *L. monocytogenes* a été suivi au cours de l'entreposage. Les résultats ont montré que la présence d'origan ou de cannelle avait une efficacité contre la croissance de *S.* Typhimurium sur le boeuf. La présence d'origan a montré une efficacité contre la croissance de *S.* Typhimurium sur de la bologne. Une réduction significative du niveau de *S.* Typhimurium inoculée sur du jambon a aussi été observée après une application de films à base de cannelle et d'origan. La sarriette des montagnes a montré une efficacité contre la croissance de *S.* Typhimurium sur du jambon après 5 jours d'entreposage. L'origan d'Espagne, la cannelle de Chine et la sarriette des montagnes ont permis une diminution significative sur le contenu de *L. monocytogenes* sur de la bologne et du jambon après cinq jours d'entreposage.

Les études de mécanismes d'actions ont montré que l'intégrité des membranes bactériennes a été affectée par la présence des huiles essentielles et ce, quelque soit la concentration d'huile ajoutée (CMI, < CMI ou > CMI). De plus, les huiles essentielles d'origan et de sarriette des montagnes peuvent induire un relargage des composantes

intracellulaires de la cellule. Une diminution significative du pH intracellulaire a aussi été observée chez *E. coli* O157:H7 en présence de cannelle et chez *L. monocytogenes* en présence de cannelle et d'origan ($p \leq 0.05$). Les observations en microscopie électronique ont indiqué que l'enveloppe des deux bactéries (*E. coli* O157:H7 et *L. monocytogenes*) a été sensiblement endommagée en présence des huiles essentielles d'origan et de sarriette des montagnes. Tous ces résultats suggèrent que la principale cible des huiles essentielles demeure au niveau de l'enveloppe cellulaire des bactéries.

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Introduction

L'impact économique et social des maladies d'origine alimentaire ne cesse de croître en faisant à chaque année de plus en plus de victimes. Une pareille problématique pousse les politiques récentes de la santé publique à se baser sur l'analyse et la localisation du risque de contamination. Les sources de contamination sont de réels risques pour la santé. Il faut considérer chacun de ces dangers en termes de fréquence ou de probabilité d'apparition. Il a été signalé que tous les volets alimentaires, que ce soit au niveau de la collecte, de la transformation, du stockage ou de la préparation à domicile doivent appliquer les points de contrôle critiques. Les secteurs agricole et de la santé doivent s'investir plus et remplir leur rôle qui est essentiel pour la santé publique et surtout ne plus dépendre des facteurs économiques. Des problématiques comme la contamination microbienne doivent être maîtrisées par la mise en place d'approche filière plutôt que de généraliser.

Selon statistiques Canada, le nombre de toxi-infections alimentaires reliées à *Salmonella, Staphylococcus aureus, Shigella, Listeria monocytogenes, Campylobacter jejuni* et *Escherichia coli* O157:H7 est estimé respectivement à 630 000, 100 000, 19 000, 2 800, 16 000, et 13 000 cas/an. Les coûts médicaux, les pertes en productivité et les coûts en mortalité engendrés par les toxi-infections alimentaires sont estimés à 6.9 milliards de dollars par an aux Etats Unis (USDA, 2001) et à 500 millions de dollars par an comme dépenses en frais de santé au Canada. Certaines bactéries pathogènes comme *Clostridium perfringens*, *E. coli* O157:H7, *Shigella*, *Campylobacter, Vibrio* sp., sont économiquement importants pour les services de santé publique, de part leur pathogénicité causant des toxi-infections alimentaires et l'altération des produits alimentaires (Todd, 1989/a). Des cas de contamination par *E. coli* O157:H7 ont été identifiés au Québec en 2002. Les symptômes comme la diarrhée et des douleurs abdominales, souvent accompagnées de sang dans les selles ont apparu après une dizaine de jours avec deux de ces cas.

Statistiques Canada a établit qu'en 2004, la production nationale de viande a augmenté d'environ 9,5 % pour atteindre 3,4 millions de tonnes. En plus, le Canada est un important exportateur de viande bovine. Selon les données d'Agriculture et Agroalimentaire Canada, les

exportations de viande sont passées de 1,5 milliards de dollars en 1994 à environ 4,9 milliards de dollars en 2004, ce qui n'empêche pas la faiblesse du secteur des abattoirs, qui réside dans la désuétude des installations qui exigent d'importants investissements. La modernisation de ces installations est inhibée par leur rentabilité marginale. L'industrialisation des mets préparés est aussi à l'origine de nombreux foyers épidémiques enregistrés depuis vingt ans. Il est possible de contrer l'apparition ou la propagation de problèmes de sécurité alimentaire en cherchant les changements survenus dans les pratiques agricoles, industrielles ou de consommation fraîche.

L'exploitation des vertus naturelles des huiles essentielles est un champ très vaste. Une huile essentielle peut agir contre plusieurs microorganismes. Parmi les moyens de conservation utilisés jusque là, ces agents naturels résolvent efficacement et durablement les points critiques du maintien de la sécurité alimentaire. Les huiles essentielles sont de plus en plus étudiées. Il existe même des composants d'huiles essentielles dans des produits cosmétiques, pharmaceutiques et de nettoyage. La composition et la concentration en princips actifs d'une huile essentielle sont des facteurs déterminants pour son efficacité antimicrobienne. La composition d'une huile essentielle est largement influencée par les conditions géographiques (altitude) et terroirs, la variété du plant, le génotype, la période de récolte, le climat et la partie extraite du plant.

L'incorporation de ces huiles essentielles dans des films biodégradables est l'un des moyens de conservation les plus intéressant durant ces deux dernières décennies. En effet, prévenir le changement de la qualité de l'aliment dans le cheminement alimentaire afin de prolonger leur durée de vie est mis en œuvre par l'application de films possédant une barrière sélective aux facteurs physiques et atmosphériques externes.

Chapitre I

Revue de littérature

1. Microbiologie de la viande et des charcuteries

1.1. Définition

La viande et ses produits sont un des quatre groupes alimentaires, qui prend une place importante dans la consommation humaine. La viande est le produit de transformation de tissus généralement stériles. Ces tissus proviennent de l'abattage d'animaux (bovins, ovins) adultes normalement sains (Dickson et Anderson, 1992; Ouattara et al., 1997). En plus d'avoir une activité de l'eau élevée (A_w= 0.99) et un pH proche de la neutralité (6.5 et 7.0), la viande est un milieu très nutritif avec des caractéristiques qui conviennent à la croissance microbienne (Silliker et al., 1980).

Les charcuteries telles que la bologne et le jambon cuit sont des produits transformés par addition d'additifs chimiques tels que le chlorure de sodium, le nitrite de sodium, l'erythrobate de sodium et le phosphate de sodium à la matière première. Cette dernière se trouve être la viande provenant du membre postérieur du porc pour le jambon qui est dénervée et qui subit des étapes de transformation telles que l'injection de la saumure, le barratage, le moulage et enfin la cuisson. La bologne est à son tour fabriquée à partir d'une émulsion d'eau, d'abats tels que le coeur, la panse ou le jarrêt...etc, de la farine, des épices et du sang. Cette émulsion subit alors une opération de poussage dans des boyaux, une cuisson et enfin un refroidissement. Ces produits peuvent être ingérés par le consomateur sans qu'ils subissent de cuisson domestique, ce qui exige un respect plus important des normes bactériennes que celui exigé avec la viande. Cette vigilence est surtout liée à la possibilité de présence de certains microorganismes qui tolèrent l'acidité présente dans ces deux types de substrat.

1.2. Qualité nutritionnelle

Les principaux éléments qui sont nutritifs comme les protéines, les lipides, les vitamines et les minéraux se trouvent dans la viande des ruminants. En effet, selon le Guide alimentaire canadien (2003), la viande fournit une combinaison de nutriments assez équilibrée comme les protéines qui contiennent une forte proportion en acides aminés essentiels tels que la lysine, la méthionine, la cystéine, la thréonine, le tryptophane, l'isoleucine, la leucine, la valine et la phénylalanine. La viande est le tissu musculaire qui regroupe des protéines exocellulaires comme le collagène (responsable de la modification de la texture de la viande après cuisson) et l'élastine et des protéines endocellulaires comme les myofibrilles (ensemble de polymère d'actine et de myosine) responsable de la contraction du muscle, et la myoglobine (chromoprotéine).

Les matières grasses de la viande représentent une source d'énergie pour le corps, elles fournissent les acides gras nécessaires comme l'acide linoléique et l'acide arachidonique et jouent le rôle de transporteur pour les vitamines A, D, E et K (vitamines liposolubles) (Lefebvre, 1990).

De plus, la viande de bœuf contient les vitamines du complexe B dont: la thiamine, la riboflavine, la niacine, la floacine et la vitamine B_{12} (Geay, 2002). La vitamine B_{12} joue un rôle très important au bon fonctionnement du système nerveux. La vitamine B_6 contribue à la prévention de la dermite et des troubles nerveux. La niacine joue un rôle dans la digestion, le système nerveux, la croissance et la reproduction.

Parmi les minéraux importants de la viande, le fer, le zinc, le magnésium ainsi que des traces de sodium, de potassium et de phosphore, qui sont indispensable pour le maintien d'une bonne santé des dents. La viande est une des principales sources de fer, une carence en fer dans l'alimentation humaine peut entraîner une anémie. Le fer contenu dans le bœuf est sous une forme assimilable (hémique). C'est un des composants de l'hémoglobine présent dans les globules rouges et joue un rôle essentiel dans le transport de l'oxygène vers les cellules (Lefebvre, 1990). La viande est aussi riche en zinc. Comme le fer, la biodisponibilité du zinc est aussi plus élevée dans les aliments d'origine animale que végétale. Le zinc joue un rôle très

important dans les réactions enzymatiques et a un impact physiologique sur la synthèse protéique comme l'activation de l'ADN et ARN polymérases, régulation des histones et déclenchement de la lecture du génome (Rock, 2002). Finalement, le phosphore présent dans la viande est un des composants des acides nucléiques et joue un rôle très important dans les réactions métaboliques.

Quant à la qualité nutritionnelle des charcuteries, 100 g de charcuteries apportent plus de 50% des apports en vitamines B1, B2, B3, B6 et B12 (Centre d'information sur les charcuteries). Chez le porc, les lipides intramusculaires présentent un rapport acides gras polyinsaturés / saturés plus élevé que celui observé chez les ruminants, en raison notamment de teneurs relativement élevées en acides gras essentiels linolénique et linoléique (C18:2 et C18:3), conférant à la viande des qualités nutritionnelles intéressantes (Wood et Enser 1996).

1.3. Origine de la contamination

Les opérations d'abattage et de transformation favorisent toutefois la contamination par les micro-organismes de la viande blanche ou rouge initialement stérile (Korkeala et Bjorkroth, 1997). Selon Zhao et al. (2001) la viande crue peut véhiculer des toxi-infections alimentaires et les aliments d'origine animale sont les aliments qui véhiculent le plus de bactéries pathogènes. En effet, une contamination de la carcasse survient déjà au cours de l'abattage. Cette contamination se fait en deux étapes, l'adhérence aux surfaces et la colonisation, celle-ci ne survient que lorsque les conditions sont favorables (Delazari et al., 1998). Mais c'est au cours de l'habillage de la carcasse que survient la première contamination de l'animal lui même, par les bactéries présentes sur sa peau, sur le sol, dans ses matières fécales et dans l'air ambiant (Ouattara et al., 1997). En effet, les microbes présents dans l'air ambiant sont une source potentielle de contamination microbiologique. L'air véhicule les micro-organismes dans les installations et sans les séparations de mur, le taux de bactéries serait encore plus élevé (Rahkio et Korkéala, 1997). De plus, l'absence des mesures d'hygiène engendre la présence de nombreux micro-organismes sur la viande (Lefebvre, 1990).

Selon Rasmussen et al. (1993), les ruminants sont considérés comme un réservoir d'*E. coli* O157:H7 et joue un rôle significatif dans les épidémies d'infections humaines. Il existe des bactéries telles que les *Enterobacteriaceae* et *Salmonella* sp. dans les fèces (partie principale de la dissémination) des animaux vivants qui sont retrouvés postérieurement dans les carcasses. Les analyses sur les changements de la contamination avant et après l'abattage démontrent qu'il y a un lien avec la contamination de la carcasse avec *E. coli* pendant l'abattage (Mc Evoy et al., 2000). En effet, un acheminement fécal des populations génériques d'*E. coli* a lieu par les bêtes eux même et par la peau, ce qui engendre ainsi la contamination de la carcasse (Aslam et al., 2003).

La contamination de la carcasse qui commence dès l'abattage, augmente au cours des procédés de transformation lors de la chaîne alimentaire. Ceci peut être expliqué par les différentes manipulations et le changement de température durant le transport que subissent les carcasses. Lors de l'abattage, la température de la carcasse est autour de 37.5°C et descend jusqu'à approximativement 17.5°C après refroidissement. Généralement, les organismes prédominants sont les *Pseudomonas et les Micrococcus* (Stringer et al., 1969). Un nombre appréciable de *bacillus* est présent juste après l'abattage. Après refroidissement, le taux de *Pseudomonas* baisse alors que celui des *Micrococcus* augmente, et une petite quantité d'*Aspergillus niger* est présente après refroidissement.

Des études faites par Bolton et al. (2002) démontrent qu'une éviscération bien faite réduit l'incidence de la rupture du tube digestif et prévient la contamination des carcasses. Ainsi donc, l'application de mauvaises pratiques engendre un contact entre les carcasses, le sol, les matières fécales et les équipements. Gill et al. (1998) ont observé que la contamination de la viande par les mains et les vêtements des manipulateurs et le non respect des règles d'hygiène dans les abattoirs sont importants. Cette situation pousse les gouvernements à investir dans les services sanitaires, à améliorer les procédés de la chaîne alimentaire et à faire des recherches dans ce sens. Des efforts ont été fournis pour apporter des modifications aux opérations pour corriger l'approche HACCP (Hazard analysis and control point). Ces nouvelles procédures permettront la réduction de la contamination microbienne et la stabilisation du produit afin d'offrir à la population des aliments sains dont l'innocuité et la salubrité sont assurées (Bolton et al., 2002; Hathaway et Mc Kenzie, 1991; Biss et Hathaway, 1995).

Des normes bactériennes doivent être suivies et surtout respectées par les vendeurs et les transformateurs de viande et des produits carnés afin de minimiser les risques de contamination bactérienne. Le tableau 1 exprime les normes gouvernementales impôsées aux utilisateurs de ces produits. Ces normes sont plus sévères envers les produits cuits qu'envers les produits crus, cela est dû au traitement thermique (la cuisson) que vont subir ces derniers et qui permet ainsi de se débarrasser des bactéries déjà présentes. Dans les trois types de produits, la tolérance est de zéro pour *Salmonella* et *L. monocytogenes*. Pour un lot de cinq échantillons de produits crus et saumurés, pas plus que deux échantillons ne doivent présenter un nombre supérieur ou égale à 100 et 50 bactéries d'*E. coli* respectivement et aucun des échantillons du lot ne doit dépasser le nombre de 500 et 100 CFU/gr ou ml, respectivement. Dans les produits cuits, aucune présence des bactéries telles que *S. aureus* et *E. coli* n'est acceptée.

Tableau 1: Lignes directrices des normes bactériennes

Organismes	Echantillons à analyser		Produits cuits		Produits saumurés		Produits crus	
	n	c	m	M	m	M	m	M
Aérobies mésophiles totales	5	2	10^3	10^4	-	-	10^4	10^6
S. aureus	5	2	0	0	10^2	10^4	10^2	10^3
Coliformes totaux	5	2	50	10^2	10^2	500	500	10^3
E. coli	5	2	0	0	50	10^2	10^2	500
Salmonella sp.	5	2	0	0	0	0	-	-
L. monocytogenes	5	2	0	0	0	0	-	-

ACIA, 2003 (Selon le manuel d'hygiène des viandes)

n: nombre d'échantillons analysés.

c: nombre maximal d'échantillons pouvant présenter des valeurs se situant entre m et M.

m: échantillon acceptable si le résultat est égal ou inférieur à cette valeur.

M: seuil limite d'acceptabilité, aucun échantillon ne doit dépasser cette valeur.

Le chiffre 0 signifie absence de bactéries après enrichissement du milieu.

1.4. Les bactéries et les pathologies

Les bactéries responsables des toxi-infections alimentaires représentent 90% des cas contre 6% pour les virus et 1% pour les parasites (Bean et al., 1997; Ouattara et al., 1997). Certaines bactéries impliquées dans les maladies alimentaires et qui ont un tres grand impact sur la santé sont énumérées au tableau 2. Chaque année aux Etats Unis, de 6 à 80 millions de personnes atteintes de maladies alimentaires, 9000 d'entre elles en meurent (Alterkruse et al., 1997)

Tableau 2: Estimation des cas malades et morts par an causés par une infection d'origine alimentaire aux Etats Unis.

Pathogènes	Cas (10^3)	Morts (10^3)	Aliments
Campylobacter jejuni	4000	0.2-1	Poulet, lait, eau non traitée
Salmonella (non typhoïde)	2000	0.5-2	Oeufs, poulet, viande, produits frais, et autres produits crus
E. coli O157:H7	725	0.1-0.2	Bœuf haché, lait, laitue, eau non traitée, jus de pomme non pasteurisés
L. monocytogenes	1.5	0.25-0.5	Viande transformée
Vibrio sp.	10	0.05-0.1	Produits de pêche

Source: Alterkruse et al., 1997.

Il existe trois types de bactéries: des bactéries pathogènes, des bactéries d'altération de l'aliment et des bactéries bénéfiques telles qui existent dans les ferments.

1.4.1. Les bactéries pathogènes

Parmi les bactéries pathogènes les plus souvent responsables des maladies d'origine alimentaire, on retrouve *Staphylococcus aureus, Listeria monocytogenes, Campylobacter jejuni, Vibrio, Yersinia, Clostridium botulinum, Clostridium perfringens* et les Entérobactéries telles que *Salmonella* et *E. coli*. Les conséquences que peuvent avoir ces bactéries sur l'hôte dépendent de l'état de sa santé. En effet, les enfants, les personnes agées, les personnes immuno-déficientes et les femmes enceintes sont les personnes les plus vulnérables. Chaque année aux Etats Unis, on répertorie plus de 76 millions de cas victimes de maladies d'origine alimentaire. Près de 2.4 millions sont causés par *Campylobacter* sp., 1.4 millions sont causés par *Salmonella* sp. et 270 000 cas sont causés par des micro-organismes pathogènes comme *E. coli* (Mead et al., 1999).

La viande crue est souvent contaminée avec *Campylobacter, E. coli* et *Salmonella,* qui colonisent le tractus gastro-intestinal des animaux destinés à la consommation humaine. Certaines de ces bactéries sont concidérées comme étant des bactéries pathogènes comme les *E. coli* entéropathogéniques (EPEC), entéroinvasives (EIEC), entérotoxigéniques (ETEC) et les *E. coli* entérohémorrhagiques (EHEC). Selon Plaut (2000), il suffirait d'une concentration de 100 micro-organismes/ml ou moins *d'E. coli* O157:H7, de *Shigella* sp. et des parasites comme *Cryptosporidium parvum, Giardia lambia* et *Entamoeba histolytica*, des concentrations entre 10^2 et 10^6 de micro-organisms/ml pour *Campylobacter* et des concentrations entre 10^6 et 10^8 de micro-organisms/ml pour *V. cholerae* et *Salmonella* sp. dans un aliment contaminé pour causer une infection entérique chez l'humain.

La prévention d'une contamination causée par la bactérie *Campylobacter* est plus difficile que celle dûe à *Salmonella*, ceci est principalement relié à sa faible dose infectieuse (Daube, 2002). Cette bactérie a la capacité de croître dans des conditions anaérobies strictes. En effet, 25 % des 110 cas de botulisme répertoriés chaque année aux Etats Unis sont d'origine alimentaire, provenant des aliments en conserve (Ouattara et al. 1997).

Une contamination à *S. aureus* est très fréquente (Doyle et al., 1988). De part sa présence chez les humains (nez, peau et cheveux) et chez les animaux (tégument). Ceux-ci sont facilement transportées sur l'aliment surtout si les conditions d'hygiène personnelle ne sont pas respectées. La croissance de *S. aureus* est confirmée dans les aliments riches en protéines comme les viandes et les produits carnés (Millette, 2001). La maladie engendrée provient d'une toxi-infection alimentaire. *S. aureus* produit une toxine lorsque la viande est entreposée à une température intermédiaire, qui est résistante à la cuisson. Parmi les agents responsables des toxi-infections, il y a: *Cryptosporidium* sp., *L. monocytogenes, Archobacter* sp., *Aeromonas hydrophila*...etc.

L'évolution des micro-organismes est la cause d'émergence de nouvelles pathologies chez l'homme et *E. coli* O157:H7 en est un exemple. Cette bactérie a été mise en évidence en 1982 aux Etats Unis après avoir déclenché une épidémie suite à la consommation d'hamburgers contaminés (Daube, 2002; Altekruse et al., 1997; Riley et al., 1983).

La *Salmonella* se développe là où les pratiques d'hygiènes sont insuffisantes. Des sérotypes comme Enteritidis ou Typhimurium sont hébergés par nos animaux domestiques. Il est à rappeler que la dose infectieuse est élevée et qu'une bonne cuisson les détruit. Cette bactérie cause des infections gastro-intestinales qui peuvent durer jusqu'à quelques semaines. En dépit des efforts fournis pour réduire les risques de contamination, la salmonellose persiste. En Espagne, par exemple, de 1984 à 1994: *Salmonella entiridis* était impliquée dans 80% des maladies alimentaires (toxi-infection). Sur six serotypes de *Salmonella* identifiés, *Salmonella entiridis* représentait 77.6% dans les produits éviscérés, c'est ce qui confirme sa pathogénécité (Carraminana et al., 1997).

Il est important de prendre en considération aussi la résistance microbienne aux antibiotiques observée chez certaines bactéries comme *Salmonella* Typhimurium DT104 qui a émergé en Angleterre en 1995, ou *Clostridium jejuni* qui a fait ravage en Europe dans les débuts des années 90 (Altekruse et al., 1997).

Quant à *Listeria*, cette dernière se multiplie plus librement dans les produits conditionnés sous vide ou sous une atmosphère modifiée, que sur une viande fraîche (Daube, 2002). Aux Etats

Unis, de 1989 à 1993, beaucoup d'efforts ont été fournis pour réduire la contamination par *Listeria*, qui ont permis jusqu'à 40 % de réduction de (Altekruse et al., 1997). La listeriose affecte surtout les femmes enceintes, plus précisément le fœtus, les nouveaux nés et les adultes à faible immunité (CDC, 2003).

Il existe des antibiotiques efficaces pour certaines maladies engendrées par des bactéries, toutefois ces composés sont bénéfiques pour certaines maladies comme: la listériose, la fièvre thyphoïde ou l'infection hemoragique, la shigellose, le cholera ou la campylobacteriose au premier stade de la maladie. Cependant, pour la gastroenterite causée par *E. coli* entérohémorrhagique ou à une salmonellose non thyphoïdale au premier stade de la maladie, des traitements thérapeutiques par des réhydratations suffisent (Gill et Hamer, 2001).

1.4.2. Les syndromes cliniques des maladies d'origine alimentaire

Les bactéries pathogènes peuvent causer des gastro-entérites, des maladies invasives et des complications peuvent survenir lors des cas sévères. Les bactéries qui causent les symptômes cliniques les plus souvent retrouvés sont citées ci-dessous.

1.4.2.1. *Campylobacter jejuni*

Cette bactérie a été identifiée comme la cause prédominante du syndrome Guillain barré (Blaser, 1997; Zhao et al., 2001) ou dans des cas extrêmes, cette bactérie peut causer de l'arthrite ou des méningites. C'est un micro-organisme pathogène important présent dans la voie intestinale de plusieurs animaux comme le poulet et le bœuf (Radomyski et al., 1994).

1.4.2.2. *Shigella, E. coli, Clostridium jejuni* et *Salmonella*

Ce sont des bactéries retrouvées majoritairement dans la viande, les ragoûts de viande, les sauces au jus de poulet et le poulet. *Salmonella* est aussi retrouvée dans les oeufs, le lait et ses dérivés (USDA, 2001). De fréquentes diarrhées, des évacuations pénibles, des douleurs abdominales dans le bas de l'abdomen, des étourdissements, une hypotension et une irruption cutanée ont été observés (Plaut, 2000).

Les produits de charcuterie véhiculent toujours un grand nombre de *Salmonella* dont les sérovars Typhimurium et Derby qui représentent respectivement 31,3 % et 15,3 % des souches isolées. Le nombre de souches de *Salmonella* isolées des viandes de boeuf décroît modérément avec en tête le sérovar Typhimurium (21,2 %): 1364 souches ont été isolées des viandes de porc dont les principaux sérovars sont Typhimurium (35,5 %), Derby (22,8 %) et Bredenev (7,4 %) (Peiffer, 1999).

1.4.2.3. *Staphylococcus aureus* et *Bacillus cereus*

De fréquentes nausées sans vomissement, pas de diarrhée avec possibilité de constipation et dans certains cas, des douleurs au niveau de l'intestin grêle. Pour peu de temps, les nausées sont substituées aux vomis. Ces symptômes apparaissent rapidement et sont dues aux toxines produites dans l'aliment (intoxication alimentaire) (Plaut, 2000). *S. aureus* est restrouvé dans le poulet et dans la viande crue et *B. cereus* est plus présente dans les aliments embalés dans des conditions aérobiques.

1.4.2.4. *Escherichia coli*

Cette bactérie est retrouvée principalement dans le boeuf haché (USDA, 2001). *E. coli* permet d'indiquer la présence de d'autres bactéries entériques pathogènes. Selon Fukushima et Seki. (2004), un grand nombre de serotypes d'*E. coli* verotoxigenic est lié aux infections intestinales humaines. Certains de ces sérotypes peuvent aussi causer des diarrhées sanguinolantes aiguës ainsi que le syndrome urémique hémolytique qui engendre une insuffisance rénale.

1.4.2.5. *Listeria moncytogenes*

Cette bactérie, présente principalement dans les viandes transformées comme les charcuteries est responsable de la listériose, engendrant ainsi des cas sévères tels que la méningite et la septicémie (Prescott et al., 1995). Deux importants facteurs peuvent favoriser la multiplication de cette bactérie dans les charcuteries, la réduction de la compétition bactérienne qui a lieu après les traitements thermique et de refrigération et sa tolérance aux teneurs élevés au sel (Doyle et al., 2001). Du fait que cette bactérie soit un contaminant de post-transformation,

capable de résister à un stress environnemental sévère, comme les températures de réfrigération et les concentrations élevées de sel, le niveau de tolérance imposé par l'USDA (United States Département of Agriculture) est de zéro sur les viandes transformées (Foong et al., 2004).

1.4.2.6. *Clostridium botulinum*

Clostridium botulinum est une bactérie qui sporule et dont les spores sont thermorésistants (Prescott *et al.*, 1995). Cette bactérie est présente majoritairement dans les produits alimentaires en conserve et cause des troubles de la vision, une bouche sèche, une faiblesse des muscles, de la constipation ainsi que des léthargies seulement après 18 à 36 h d'ingestion (CDC, 2003).

1.4.3. Les bactéries d'altération

L'altération bactérienne survient dans n'importe quel aliment, d'origine animale ou végétale. Des bactéries telles que *Pseudomonas fluorescens* et *Pseudomonas marginalis* sont responsables de la putréfaction du céleri, qui se manifeste en une décoloration (à 5 ou 25°C). *Leuconostoc mesenteroides* est aussi responsable de la putréfaction du céleri en formant de la boue (Robbs et al., 1996; Prakash et al., 2000 b). Les principales bactéries responsables de l'altération de la viande sont: *Brochothrix thermosphacta, Carnobacterium* sp. *(Carnobacterium divergens, Carnobacterium maltaromaticum), Enterobacteriaceae, Lactobacillus* sp. *(Lactobacillus curvatus, Lactobacillus sake, Lactobacillus plantarum), Leuconostoc* sp., *Pseudomonas* sp., *Shewanella putrefaciens* et *Weissella* sp. Les bactéries fluorescentes telles que *Pseudomonas fluorescens* et *P. putida* sont ubiquitaires. De ce fait, ces bactéries se trouvent dans l'environnement et les aliments. Néanmoins, *P. putida* se trouve plus dans l'environnement (Gennari et al., 1992). Cette souche bactérienne est le principal facteur de contamination des viandes. Malgré la décontamination qui survient après l'abattage sur les carcasses, il reste une microflore qui est transférée sur les équipements (Borch, 1996; Gill et Jones, 2000; Midelet et Carpentier, 2002), pouvant former des biofilms. Ces derniers contaminent à leur tour les aliments (Bell, 1997; Yu *et al.*, 1999; Midelet et Carpentier, 2002). *P. putida* et *L. moncytogenes* sont des bactéries qui ont la capacité de produire des exopolysccharides, ce qui est indispensable pour former des microcolonies (Allison et Sutherland, 1987). La densité de ces biofilms qui dépend de

la nature du matériaux joue un grand rôle dans la quantité en UFC (Unité Formant une Colonie) transférée à la viande (Midelet et Carpentier, 2002). C'est pourquoi, il est recommandé d'utiliser des surfaces de travail en stainless afin de réduire l'adhésion des bactéries à la surface des milieux de travail.

I.4.3.1. Les indicateurs du phénomène d'altération

Le phénomène d'altération est une problématique importante qui perturbe les projets des industries de la viande. Il existe des indicateurs chimiques qui peuvent nous renseigner sur la présence d'une contamination comme:

1. Les esters éthylés sont produits par *P. fragi.*
2. Les sulfides d'hydrogène sont produits par les *Enterobacteriaceae.*
3. Les sulfides dimethyl sont produits par les *Pseudomonas* sp.
4. 3-methylbutanol et acetone/diactyl sont produits par *Enterobactiaceae, Brochothrix et Lactobacillus* sp.
5. Les acide lactique et acétique sont produits par les bactéries lactiques.

Sous des atmosphères aérobes, il y a la formation du sulphymyoglobine, qui donne la couleur verdâtre à la viande et qui résulte de la transformation des pigments du muscle par les sulfites d'hydrogène (Borch et al., 1996). Toutefois, selon Borch et al. (1996), les produits résultant d'une altération de viande emballée sous des conditions anaérobes sont moins dangereux que les produits provenant d'une altération de viande emballée sous air.

Un des indicateurs d'altération par les bactéries lactiques et par *Clostridium* sp. dans les emballages est l'augmentation de la concentration en CO_2. En plus, *Clostridium* sp. produit des odeurs indésirables en présence de protéines (Borch et al., 1996). Il y a aussi la production d'un liquide visqueux et blanchâtre, produit par les bactéries lactiques (Korkeala et al., 1997). Les produits formés dépendent de la quantité et du type de bactéries présents. Par exemple, la formation de D lactate et acétate indiquent un nombre important de *Lactobacillus* sp. Une formation aussi de D lactate et éthanol induisent également la présence d'un nombre important de *Leuconostoc* (Borch et al., 1996). La durée nécessaire pour la mise en évidence d'une viande

altérée dans des conditions anaérobes est entre 19-30 jours à 4°C et à 2°C respectivement (Korkéala et al., 1989). Des indicateurs bactériens sont alors révélés par des odeurs indésirables. Ces odeurs peuvent être détectées après une semaine lorsque le niveau bactérien atteint 10^7 UFC/cm^2 et à une température de 4°C (Borch et al., 1996). Ceci est dû à la dégradation des protéines provoquée par *Pseudomonas* qui donne la couleur marron à la viande.

2. Conservation de la viande

De tout temps, l'homme a cherché à conserver sa nourriture, des méthodes primitives consistaient à sécher de la viande au soleil, à les faire fumer, à déshydrater la viande. Plusieurs moyens de conservation ont été développés avec l'avancement de la technologie, comme la conservation par le froid, qui est un des moyens le plus utilisé, l'irradiation par les rayons gamma, l'ajout de substances bactériostatiques ou bactéricides comme les additifs chimiques, les bactériocines produites par les bactéries lactiques (probiotiques), les molécules actives (les phénols) ou encore l'utilisation d'emballages biodégradables à base d'huiles essentielles à effet anti-microbien, qui est en voie d'optimisation à usage commercial.

2.1. Méthodes de contrôle de la croissance microbienne

La présence de bactéries d'altération est inévitable. Ces dernières sont présentes dans tous les produits alimentaires et dans presque tous les procédés de conservation. Chaque groupe de bactérie présent dans un procédé précis est remplacé par un autre groupe dans un autre procédé de conservation. La microflore mésophile initiale de la viande se situe entre 10^2 et 10^3 UFC/cm^2 et provient de la contamination initiale (Borch et al., 1996). Seulement 10% des bactéries peuvent croître à des températures de réfrigération.

Les travaux de Borch et al. (1996) ont démontré que la durée de conservation du bœuf est de 5 à 6 jours, soit la période nécessaire pour atteindre 10^7 UFC/cm^2. Une conservation prolongée conduit à une croissance lente des bactéries qui produisent des acides lactiques et des entérobactéries et même à une croissance rapide des *Pseudomonas* sp., un genre chimiohétérotrophe, capable de dégrader les sucres par oxydation en produisant du CO_2 (Prescott et al., 1995).

Il faut respecter le rapport entre le temps de conservation et la température. Plus la température est élevée, plus le temps est réduit. Toutefois, la refrigération ne permet pas un contrôle des micro-organismes pathogènes psychotrophes responsables de maladies d'origine alimentaire tels que *L. moncytogenes*, *A. hydrophila* et *Y. enterocolitica* (Prakash et al., 2000a). Il est reconnu que les bactéries responsables des phénomènes d'altération des viandes sont des bactéries psychrotrophes et mésophiles. Ces bactéries réduisent la durée de vie de l'aliment pendant la réfrigération (Lefebvre, 1990). Il est à noter aussi que la diminution de température conduit à une diminution de la croissance bactérienne. On atteint une croissance bactérienne de 10^7 UFC/cm^2 après quatorze semaines à –1.5°C et après trois semaines à 4°C (Borch et al., 1996). Les produits de viande transformée sont à leur tour décontaminés au cours de la cuisson. Toutefois, une contamination secondaire peut survenir au cours de l'emballage par le personnel et les équipements (Korkéala et al., 1997). Les bactéries comme *Aeromonas* sp., *Brochothrix thermosphacta* et les *Enterobacteriaceae* peuvent croître dépendamment de la température de conservation et du pH (McMullen et Stiles, 1993). Plus la température est basse et plus les bactéries comme les *Enterobacteriaceae* et *Brochothrix* sont inhibées (Greer et Dilts, 1992).

En fonction des méthodes de conservation, différentes espèces microbiennes sont rencontrées. A titre d'exemple, les environnements gazeux influencent la croissance bactérienne. L'emballage sous une atmosphère modifiée retarde la maturation des aliments (Prakash et al., 2000a). Dans des produits emballés sous vide ou sous MAP (emballage sous atmosphères modifiées), les bactéries lactiques forment la flore dominante comme *Carnobactérium* sp., *Lactobacillus* sp. *et Leuconostoc* sp. (Borch et al., 1996). Les bactéries qui peuvent croître dans une viande emballée sous vide avec un pH élevé sont: *Brochothrix thermosphacta*, les *Enterobacteriaceae* (*Enterobacter* sp.), *Lactobacillus* sp. et *S. putrefaciens* (Broch et al., 1996).

Une viande emballée sous vide peut atteindre une durée de conservation allant jusqu'à 10 à 12 semaines à 0°C (Borch et al., 1996). Un ennui est que la concentration en oxygène diminue et la concentration en CO_2 augmente au cours de l'entreposage, favorisant ainsi la croissance des bactéries qui tolèrent le CO_2. Pour les produits carnés, on utilise souvent des emballages sous

vide. Certaines conditions favorisent la croissance des bactéries psychotrophes comme les bactéries lactiques (Borch et al., 1996).

2.2. Méthodes de conservation de la viande

2.2.1. L'irradiation

L'irradiation des aliments est un des moyens de conservation à froid et non chimique, qui pasteurise l'aliment en éliminant les bactéries pathogènes par les rayons gamma (Lefebvre, 1990). Les services de sécurité alimentaire autorisent l'utilisation des radiations ionisantes pour la conservation alimentaire, comme les rayons gamma du Co^{60} et le Cs^{137}. L'emploi d'isotopes radioactifs actifs comme le Co^{60} et Cs^{137} est exempt de risque de radioactivité puisque leur énergie qui est respectivement de 1.17 à 1.33 Mev pour le cobalt et de 0.66 Mev pour le césium, est trop faible et inférieure à 10 Mev pour rendre radioactif n'importe quelle matière.
Le $cobalt^{60}$ est le radionucléide le plus utilisé comme source de rayons gamma pour l'irradiation des aliments. Il est obtenu par l'addition d'un neutron au cobalt 59 dans un réacteur nucléaire. L'énergie émise par les radiations ionisantes est absorbée par l'aliment, engendrant ainsi une ionisation. Cette ionisation conduit à une excitation des atomes des molécules. Le photon libéré par le cobalt 60 lors du passage du noyau excité à son état normal engendre la libération d'ondes électromagnétiques, qui sont les rayons gamma. Le Co^{60} cède ainsi une partie de son énergie en éjectant un électron de la couche périphérique de l'atome, cet électron ayant à son tour une quantité importante d'énergie ne peut être accepté par un autre atome, cet électron est alors éjecté par un autre atome à son tour, jusqu'à ce que l'énergie résiduelle soit égale à l'énergie de rupture des liaisons covalentes conduisant ainsi à la formation de radicaux libres.

L'eau absorbe l'énergie des radiations ionisantes et subit la radiolyse, en produisant des radicaux libres (Thakur et Singh, 1994). L'eau a une importance extrême dans la radiochimie des aliments, vu qu'elle est présente en grande quantité. Ces produits résultant vont soit réagir entre eux ou avec d'autres constituants, soit par addition, par abstraction ou par oxydo-réduction. C'est ce qui représente les réactions secondaires de l'irradiation. Des facteurs peuvent être contrôlés pour diminuer les produits de la radiolyse comme la diminution de la température, qui a un effet

direct sur la viscosité du milieu et qui permet la réduction de la formation des produits secondaires (Sain-lebe et Raffi, 1985). La présence d'oxygène dans le milieu est aussi un facteur à contrôler car elle engendre la formation d'ozone et de peroxyde d'hydrogène au cours de l'irradiation (Thakur et Singh, 1994).

L'irradiation est un traitement de conservation qui permet la prolongation de la durée de vie de l'aliment sans l'altérer. L'action sur les protéines et les glucides est très mineure. L'effet de l'irradiation sur les protéines et les lipides peut engendrer des odeurs de composés soufrés engendrés par l'oxydation des acides aminés soufrés qui se dissipent avec le temps et une odeur de rancidité causée par l'oxydation des lipides, respectivement. Ces réactions sont causées essentiellement par des catalyseurs comme les radicaux libres (Shahidi, 1998; Gomes et al., 2003) et le fer (Paquin, 1985; Pollonio, 1994; Gomes et al., 2003). Malgré celà, les résultats de plusieurs études prouvent que les échantillons irradiés ont une durée de conservation supérieure aux échantillons non irradiés.

2.2.1.1. La dose

Lors de l'irradiation d'un aliment, la dose appliquée est la quantité d'énergie absorbée par celui-ci. La dose appliquée influence le niveau de conservation des aliments irradiés. Une dose supérieure à 10 kGy correspond à une radappertisation, ce traitement rend l'aliment stérile. Une dose entre 2.5 et 6 kGy correspond à une radicidation, qui nous permet d'éliminer les bactéries viables non sporulantes, et une dose entre 1 et 5 kGy correspond à une radurisation. A cette dose, les aliments ne sont pas conservés à long terme (Takhur et Singh, 1994; Giroux, 1999).
Selon le comité de la salubrité des aliments irradiés, les doses d'irradiation utilisées doivent être inférieures à 10 kGy pour contrôler la croissance des bactéries pathogènes et d'altération dans la viande et les produits carnés comme *L. monocytogenes*, *S.* Typhimurium (Fu et al., 1995), *E. coli* O157:H7 et *Y. enterocolitica* (Fu et al., 1995) (Giroux, 1999).

2.2.1.2. L'irradiation et les micro-organismes

Les rayons ionisants gamma pénètrent dans l'aliment et détruisent les micro-organismes qui se trouvent en surface ou en profondeur. Les rayonnements ionisants entraînent principalement des lésions au niveau de l'ADN, par une rupture des brins, une hydroxylation des bases, notamment la thymine, la dégradation des sucres et des pontages anormaux entre les bases. Ainsi ces rayonnements sont responsables de la mort immédiate ou des dysfonctionnements métaboliques et ces dysfonctionnements se traduisent à court terme par la mort différée ou perte du potentiel reproductif ou de la réduction de la radiorésistance qui engendre la formation d'espèces de micro-organismes mutants (Vasseur, 1991).

Des doses d'irradiation de 0 kGy, 3 kGy et 4 kGy ont permis respectivement de refrigérer du poulet pendant une durée de 4, 6 et 10 jours (Gomes et al., 2003) où la flore psychrotrophe des échantillons non irradiés était nettement supérieure à celle des échantillons irradiés. Cependant, les lipides sont plus sensibles et peuvent engendrer la formation d'hydroperoxydes en présence d'oxygène (Vasseur, 1991).

L'irradiation de tranches de pâté de boeuf à une dose de 1 kGy a permis d'atteindre une concentration des coliformes totaux et des bactéries aérobes en dessous du seuil de détection pendant 5 jours de conservation à 4 °C (Giroux et al., 2001). Une dose d'irradiation de 1 kGy appliquée sur des endives a permis une réduction de la population bactérienne (Langerak et al., 1978; Fan et al., 2003). Cette même dose appliquée sur des poivrons ou des carottes a permis une réduction des bactéries d'altération et ainsi une stabilisation de la qualité sensorielle de l'aliment (Farkas et al., 1997). Une prolongation de la durée de conservation du céleri de 7 à 29 jours a pu être obtenue avec une dose d'irradiation de 1kGy, éliminant ainsi la présence de *Listeria monocytogenes* et *E. coli* avec une réduction de 5 log sans altérer les propriétés sensorielles de l'aliment (Prakash et al., 2000b). Aussi une diminution de 1.5 log de la flore aérobe a été observée sur de la laitue romaine irradiée à une dose de 0.35 kGy (Prakash et al., 2000a; Fan et al., 2003). Les travaux de Giroux (1999) ont démontré que de faibles doses d'irradiation permettent une diminution de la flore aérobe mésophile et une dose de 2 kGy détruit les Entérobactéries.

Une autre technique d'irradiation existe, c'est l'utilisation d'un accélérateur d'électrons. Cette dernière consiste à bombarder avec des électrons des aliments. Une irradiation avec des accélérateurs d'électrons à une dose de 1.1 kGy sur du riz avec enveloppe et une dose de 7.5 kGy sur du riz sans enveloppe ont permis la destruction des coliformes, *E. coli*, *B. cereus*, *Clostridium* sulfito réducteur et la réduction des comptes aérobes mésophiles (Sarrias et al., 2003).

A une dose de 2 kGy, la qualité sensorielle du coriandre n'a pas été affectée, en plus de celà, comparé à un témoin, une diminution des comptes aérobes totaux a été observé après 14 jours de conservation (Fan et al., 2003). La croissance de *L. monocytogenes* a pu être contrôlée par l'irradiation à une dose dépendante de l'inoculum. En effet, une inhibition de la croissance est obtenue à une dose de 2 kGy sur une viande transformée et conservée à 4°C pendant cinq semaines avec une charge microbienne de 5 log (Foong et al., 2004).

La radiosensibilisation d'*E. coli* et de *Salmonella* Typhi présentes dans la viande hachée a été nettement augmentée lorsque une combinaison entre l'addition du thym et de ses principaux constituants (carvacrol et thymol) et l'irradiation a été effectuée (Chiasson et al., 2004)

2.2.2. Les bactéries lactiques et les probiotiques

L'appellation de bactérie lactique est donnée à toute bactérie Gram-positif qui produit de l'acide lactique. Les bactéries lactiques sont principalement utilisées comme constituants majeurs de l'utilisation du glucose lors de la fermentation. Une sélection de souches est faite par rapport aux conditions prédéfinies, pour être adaptées aux fabrications.

Il est très rare que des bactéries puissent croître à des valeurs de pH inférieures à celles des bactéries lactiques. Une acidification par les bactéries lactiques entraîne une inhibition d'*E. coli*, de *Pseudomonas* sp., de *Salmonella* sp., de *Clostridia* sp. ou de *L. monocytogenes* (Desmazeaud, 1996).

Certaines bactéries lactiques synthétisent des bactériocines, des peptides antimicrobiennes, des acides organiques, du diacétyle, de l'acétoïne, du peroxyde d'hydrogène etc et sont ainsi capables d'agir contre d'autres bactéries pathogènes. Les acides organiques

générés par ces bactéries tels que l'acide acétique et l'acide lactique sont très toxiques pour les bactéries. Greer et Dilts. (1992) ont déjà étudié cette question sur des bactéries pathogènes comme *L. monocytogenes*, *Y. enterocolitica*, des bactéries pathogènes mésophiles comme *S. typhimirium, E. coli, Campylobacter jejuni* et *S. aureus*, et sur des bactéries d'altération. Ces auteurs ont observé que les bactéries étaient sensibles aux deux acides. Toutefois, la concentration en acide, la température appliquée et la concentration initiale de la culture bactérienne sont des facteurs influençant l'efficacité du traitement. Une réduction des comptes bactériens pathogènes et d'altération a été observée par Senne et Gilliland. (2003) sur de la viande fraîche, entreposée à 5°C pendant six jours. Cette diminution a été imputée au peroxyde d'hydrogène, produit par une bactérie lactique: *Lactobacillus delbrueckii, souche, lactis RM2-5.* Les bactéries lactiques offrent une excellente préservation pour les produits alimentaires sans les altérer. Cependant, leur effet antagoniste peut être amoindri par la présence de catalases dans le peroxysome des fibres musculaires, qui agissent sur les peroxydes d'hydrogène. Ce qui explique l'obtention de meilleures résultats sur des carcasses, qui ont une couche de gras comme couche supérieure protectrice que sur des tranches de steak. Ces catalases sont plus actives dans des conditions aérobes que dans des conditions anaérobes (Senne et Gilliland, 2003).

L'une des bactériocines la plus connue et produite par les bactéries qui produisent de l'acide lactique (BAL) est la nisine, une protéine antimicrobienne. Cutter et Siragusa (1996) ont démontré que la nisine permettait la conservation du bœuf pendant sept jours et l'inhibition de la croissance de *B. thermosphacta.*

L'effet des bactéries lactiques dans un traitement est mieux optimisé par l'addition d'agents chimiques comme l'acétate de sodium ou le sorbate de potassium (Millette, 2003). Diverses bactériocines ont été découvertes, mais l'emploi de certaines est limité par leur solubilité, la non-stabilité à la chaleur et leurs effets secondaires sur la santé (O'sullivan et al., 2002). Selon Al Sheddy et al. (1999) une synergie entre les BAL comme *Bifidobactérium* et des acides organiques comme l'acétate de sodium conduit à la prolongation de la durée de conservation de la viande de chameau à 4°C de plus de six jours.

L'efficacité antimicrobienne des bactériocines a été prouvée par divers chercheurs plus contre des bactéries Gram-positif que Gram-négatif, cela est dû aux protéines de type porine qui se trouvent dans la membrane externe lipopolysaccharidique des bactéries Gram-négatif. Ces protéines ne laissent passer que des molécules dont la masse moléculaire ne dépassant pas 600 Da alors que la bactériocine la plus petite a une masse moléculaire qui s'approcherait de 3000 Da (Abee et al., 1995). Les bactériocines agissent en inhibant la synthèse des membranes cellulaires ou en causant des lésions membranaires (pores) induisant ainsi la destruction léthale des bactéries.

2.2.3. Les acides organiques

Des acides organiques comme l'acide acétique, l'acide ascorbique, l'acide citrique, l'acide formique, l'acide gluconique, l'acide lactique et l'acide propionique sont considérés comme des substances antimicrobiennes. Ouattara et al. (1997) ont démontré que l'acide acétique, lactique et propionique sont les plus performants. L'acide propionique et acétique sont considérés comme bactéricides spécialement contre *Salmonella* sp. Cette bactérie est pourtant reconnue pour sa tolérance à l'acidité (Doyle et al., 1997). Selon Doyle et al. (1997), lorsque des acides organiques sont ajoutés, plusieurs processus surviennent tels que la réduction du pH du substrat et la dépression du pH intracellulaire. Ceci pourrait être expliqué par le fait que l'acide non dissocié se dissocie à l'interieur du cytoplasme en produisant des protons qui sont toxiques pour la cellule. Cette libération de protons acidifie le milieu et désorganise les processus de synthèse de la cellule (pH intracellulaire basique) ainsi que le transfert membranaire par l'altération de la perméabilité. En effet, la membrane n'étant pas perméable aux protons, dépense et gaspille plus d'énergie pour les expulser vers l'exterieur. Cette interférence réduit la production d'ATP et réduit aussi le transport actif des nutriments à l'intérieur de la cellule. L'action des acides organiques dépend du pH du milieu. Plus le milieu est acide et plus l'acide est sous sa forme non dissociée, soit la forme responsable de l'activité antimicrobienne (Doyle et al., 1988).

Un lavage avec des désinfectants comme le peroxyde d'hydrogène, le chlorhexidine ou l'acide acétique ont permis une diminution respective de 4 à 5 log et moins de 1 log la flore endogène lorsque le bœuf est contaminé avec *E. coli* O157:H7 (Delazari et al., 1998). Ces auteurs ont conclu que dépendamment des tissus contaminés, des types des micro-organismes et des concentrations de désinfectants, une décontamination chimique est meilleure qu'une décontamination physique.

Ouattara et al. (1997) ont montré que les acides organiques ne sont pas efficaces avec toutes les bactéries. Il existe des degrés de résistance comme le genre *Lactobacillus* qui est considéré comme le plus résistant, suivi de *Pseudomonas fluorescens*. Les bactéries d'altération sont moins résistantes que les bactéries pathogènes (Greer et Dilts, 1992; Ouattara et al., 1997). Greer et Dilts (1992) ont testé l'activité antimicrobienne de deux acides: l'acide acétique et l'acide lactique et ont démontré que des bactéries pathogènes telles que *S. aureus* était la plus sensible et *S.* Typhimurium était la moins sensible à ces acides.

L'association des acides organiques avec d'autres traitements améliore leur effet. Solorzano et al. (2002) ont procédé par un rinçage des carcasses de boeuf avec de l'eau chaude suivi d'un rinçage avec des acides organiques, il y a eu amélioration de la qualité microbiologique de ces carcasses. Toutefois, il est à remarquer que l'effet des acides organiques n'est pas bien exploitée dans les abattoirs, puisque la vaporisation d'eau est utilisée pour la prévention de la dessiccation des carcasses et ces traitements diluent les concentrations d'acide, réduisant ainsi leur effet antimicrobien.

2.2.4. Autres applications

Le lysozyme, un composant naturel antimicrobien souvent utilisé dans la préservation des aliments, en raison de sa capacité de couper la liaison entre le NAG et le NAM (Nicklin et al., 1999), permettant ainsi l'hydrolyse du peptidoglycan (Branen et Davidson, 2004). Le lysozyme n'est pas efficace contre toutes les bactéries Gram-positif, ceci est dû à la présence de l'acide teichoïque et à la proportion des liaisons polysaccharidiques de type 1-6 qui sont plus résistantes que les liaisons de type 1-4 (Doyle et al., 1997). Son efficacité est inférieure avec les bactéries

Gram-négatif, cela est dû à la présence des lipopolysaccharides et des lipoprotéines (Doyle et al., 1997). Divers chercheurs sont arrivés à démontrer que l'addition d'un agent chimique comme l'EDTA (Acide Ethylenediaminetetraacetic), à une enzyme telle que la lysozyme ou le fait d'agir sur un facteur extrinsèque comme le pH et la température permet d'avoir un effet synergique sur des bactéries Gram-négatif, celà est expliqué par le fait que les ions divalents de magnésium (Mg^{+2}) et de calcium (Ca^{+2}) qui sont des ponts entre les phosphates de la membrane (Millette, 2003) et qui maintiennent l'intégrité de la couche lipopolysaccharidique soient chélatés par l'EDTA (Doyle et al., 1997), permettant ainsi une réduction des répulsions électrostatiques et une sensibilité de la membrane lipopolysacharidique (Millette, 2003).

La lactoferrine, une glycoprotéine trouvée dans les liquides physiologiques (lait, salive et fluide séminal) et les leucocytes polymorphynucléaires (Farnaud et Evans, 2003), possède aussi une activité antimicrobienne. La lactoferrine a la capacité de chélater le fer en réduisant le fer ferrique en fer ferreux (Fernaud et al., 2003), rendant ainsi ce dernier déficient dans l'environnement microbien. Cette protéine est aussi capable de chélater les cations (magnésium et calcium), responsables de la stabilité des lipopolysaccharides de la membrane microbienne externe des Gram-négatif (Ellison et al., 1988; Doyle et al., 1997). Cette glycoprotéine a aussi la capacité de se lier au lipide A des LPS, permettant ainsi une libération des LPS et une interaction avec les protéines porines (Farnaud et Evans, 2003). Et enfin, cette dernière permet d'inhiber l'adhésion des micro-organismes à la surface des muqueuses (Naidu et al., 1993; Doyle et al., 1997; Taylor et al., 2004) et joue un rôle synergétique avec d'autres agents antibactériens tels que les antibiotiques et le lysozyme (Ellison et al., 1988; Ellison et Giehl, 1991; Farnaud et Evans, 2003).

Des composés naturels et antimicrobiens tels que les isothiocyanates (allyl, ethyl, phenyl, benzyl) résultent de l'action des myrosinases, action déclenchée lors de la lyse tissulaire. Ces enzymes agissent sur des substrats comme les glucosinolates, substances des cellules des plantes herbacées. Les bactéries Gram-positif y sont moins sensibles que les bactéries Gram-négatif (Doyle et al., 1997).

3. Les huiles essentielles

Depuis les anciens temps, les épices sont présentes dans l'alimentation humaine, pour leur saveur, leurs propriétés nutraceutiques et physiologiques. Les épices jouent un rôle très important dans l'alimentation humaine. Elles sont utilisées par frictionnement sur la viande rouge ou blanche avant la cuisson pour donner de l'arôme.

Les huiles essentielles sont les produits secondaires du métabolisme de la plante, chaque variété a une forme physiologique et biochimique différente avec un équipement enzymatique spécial, génétiquement codifié, qui oriente la biosynthèse par une formation préférentielle de leurs composantes (Russo et al., 1998).

3.1. La composition des huiles essentielles

Différentes compositions d'huiles peuvent être obtenues de la même plante, dépendamment de la partie de laquelle elle est extraite. Les conditions géographiques (altitude) du terroir, la variété du plant, le génotype, la période de récolte, le climat, la partie extraite sur le plant influencent la composition d'une huile essentielle (Arrebola et al., 1994; Juliano et al., 2000; McGimpsey et Douglas., 1994; Rhyu, 1979; Salgueiro et al., 1997; Venskutonis, 1996). Tous ces paramètres de différence qualitative influencent fortement l'effet antimicrobien d'une huile, par la différence de la composition et de la variété des principes actifs présents dans une variété ou dans une partie précise et absents dans une autre. La différence de proportion d'un principe actif dans les différentes espèces d'une même plante représente un paramètre de différence quantitatif. Ce facteur est influencé par le génotype, le développement ontogénique et les conditions environnementales de croissance (Rassoli et al., 2003).Les huiles essentielles sont composées généralement de monoterpènes phénoliques et de terpènes hydrocarbonés.

3.1.1. Les monoterpènes phénoliques

Les composés phénoliques les plus retrouvés dans les huiles essentielles sont le carvacrol, le thymol, l'eugenol, geraniol et le terpineol. Selon Freidman et al. (2002), il existe une relation entre la structure des composés phénoliques et leur activité antimicrobienne. La position de la double liaison sur les phénols semble être la principale cause de leur efficacité. Par exemple, l'eugénol est treize fois plus efficace que son isomère l'iso-eugénol contre *L. monocytogenes* et *C. jejuni*. L'estragole a un meilleur pouvoir antimicrobien que l'iso-estragole anéthol, qui est trente fois moins efficace que l'estragole.

Selon Dorman et Deans (2000), l'activité antimicrobienne des composés à structure phénolique est reliée à la présence de groupements hydroxyles, à la position du groupement à l'intérieur du noyau phénolique et à la présence d'une moitié acétate.

Le métabolisme des composés phénoliques se fait après une conversion auto-oxydative du γ-terpinène en P-cymène qui est suivie par une hydroxylation du P-cymène en thymol, carvacrol, P-cymène-8-ol ou en alcool cuminyl (Russo et al., 1998).

3.1.2. Les terpènes hydrocarbonés

Les terpènes hydrocarbonés sont moins actifs que les terpènes phénoliques en raison de leur limite d'hydrogénation et de leur solubilité dans l'eau (Griffin et al., 2000; Skaltsa et al., 2003). Cependant les terpènes oxygénés tel que les aldéhydes: citronnellal, cinnamaldéhyde et le citral ont une importante activité antifongique (Knoblock et al., 1987; Jansen et al., 1987; Panizzi et al., 1993; Adam et al., 1998; Skaltsa et al., 2003). Les alcools terpénoïdes hydrocarbonés comme le linalool confèrent leur activité antimicrobienne par la dénaturation des protéines et de leurs propriétés désydratantes. L'activité antimicrobienne des terpénoides peut être également améliorée par carbonylation de celles-ci par la fonction cétone (fonction oxygène) (Dorman et Deans, 2000).

3.2. Composition et vertus de certaines huiles essentielles

3.2.1. Le clou de girofle

Le clou de girofle provient de la dessiccation des fleurs non ouvertes du *Syzygium aromaticum* (Pszczola, 2001). Ce dernier est composé de 14% à 24% d'huiles volatiles, dont 95 % d'eugénol (Doyle et al., 1997). L'eugénol, qui se trouve essentiellement dans le clou de girofle est utilisé pour ses vertus antiseptiques, préservatives, et anesthésiques. Il est considéré comme l'épice le plus aromatique. Des composés actifs tel que l'eugénol sont considérés comme des inhibiteurs de croissance microbienne pour des bactéries Gram-négatif telles qu'*E. coli, Enterobacter sakazaki*, des bactéries résistantes à des antibiotiques tel que la pénicilline, la bacitracine, la gentamycine, l'érythromycine et la sulphamethizole. Ce composé est aussi efficace contre la croissance de *Klebsiella pneumoniae* et des bactéries Gram-positif telles que *S. aureus*, *Micrococcus* sp. et *Bacillus* sp. (Suresh et al., 1992).

3.2.2. La cannelle

L'huile de la fleur de *Cinnamomum zeylanicum* contient du cinnamyl acétate et la feuille contient de l'eugénol (Jayaprakasha et al., 2000). L'écorce et la racine contiennent du cinnamaldéhyde et du camphre (Jayaprakasha et al., 2000). Selon Freidman et al. (2000), l'huile de cannelle commerciale qui se trouve dans les aliments contient des terpènes hydrocarbonés tel que le linalool, le carvone, le cinnamaldéhyde et un composé phénolique tel que l'eugénol. Le cinnamaldéhyde, le principal composant actif de la cannelle est responsable de l'odeur et du goût caractéristiques de cette épice. Ce composé, à une concentration de 150 ppm, inhibe la croissance et la production de toxine par *Aspergillus parasiticus* (Kim et al., 1995). Klapthor (1999) a démontré qu'une concentration de 0.3% d'huile essentielle de cannelle ajoutée au jus de pomme non pasteurisé, conservé pendant trois jours à 25 °C a permis de détruire 99.5% d'*E. coli* O157:H7. Selon le même auteur, l'efficacité de la cannelle est plus importante dans des substrats liquides que dans des substrats solides. Ceci est dû à une meilleure surface de contact entre la bactérie et le composé actif. Selon Chang et al. (2002), l'addition de 10 mg/g de deux huiles essentielles provenant de deux variétés différentes de cannelle avait suffi pour entrainer 100 % de mortalité après sept jours chez un des termites les plus problématiques (*Coptotermes*

formosanus), un termite responsable de nombreux dommages sur le bois et les textures à base de cellulose. A une dose inférieure, la variété de cannelle qui contenait un pourcentage de 76% de cinnamaldéhyde donnait de meilleurs résultats que la variété qui avait un pourcentage de 8.35%.

Les recherches de Chan et al. (2002) prouvent que l'activité des principes actifs ayant un groupement aldéhyde est toujours meilleure. Une concentration de 5 mg/g de cinnamaldéhyde, de benzaldehyde et de 3-phenylpropionaldehyde a suffi pour exercer une activité anti-termitique après seulement une journée avec un pourcentage de 100%. A une dose de 1 mg/g, il n'y a que le cinnamaldéhyde qui reste efficace avec un pourcentage de 96%. La présence d'une longue chaîne hydrocarbonée (CH) et d'une double liaison serait responsables de ce phénomène.

3.2.3. Le cumin

Le cumin est le produit séché de la graine de la plante *Cuminum cyminum*, le composé responsable de l'odeur et du goût du cumin est le cumin aldéhyde. Différentes recherches sont en cours pour évaluer ses vertus contre le stress (Pszczola, 2001).

3.2.4. Le basilic

Le basilic provient des feuilles desséchées de la plante *Ocimum basilicum*, le composé responsable de l'odeur et du goût est le méthyle chavicole (un terpène hydrocarboné) (Pszczola, 2001). Des concentrations minimale inhibitrice (CMI) de 0.125% et 0.2% contre respectivement *A. hydrophila* et *P. fluorescens*, ont été déterminées par Wan et al. (1998) avec du basilic à base de methyl chavicol, montrant un effet bactéricide sur des cellules en phase stationnaire. L'utilisation de l'huile essentielle de basilic à des concentrations de 0.1% ou de 1% pourrait remplacer la chlorine, utilisée lors du lavage de la salade à une concentration de 125 ppm. La chlorine engendre la production de produits indésirables tels que les chloramines et les trihalométhanes, deux composés cancérigènes. L'huile essentielle de basilic a un large spectre d'inhibition contre des bactéries Gram-positif et Gram-négatif, à l'exception de certaines espèces de *Clostridium* (*Clostridium sporogenes*) et des souches de *Pseudomonas* sp. (Wan et al., 1998).

3.2.5. Le capsicum

Des épices utilisées dans des pays tropicaux comme l'Amérique latine, où la consommation du poivre rouge, poivre blanc, poivre noir et le paprika est importante. Le poivre rouge par exemple, provient des fruits mûrs de la plante *Capsicum frutescens*. Les épices comme le poivre rouge et le paprika contiennent de la capsaicine, substance cristalline, très piquante (Pszczola, 2001), cette substance est reconnue pour ses propriétés antimicrobiennes (Nakatani, 1994; Ejechi et al., 1999).

3.2.6. Le romarin

Ce produit provient de la dessiccation des feuilles de la plante *Rosmarinus officinalis*. Le romarin a des propriétés antioxydantes par la prévention de l'oxydation des lipides qui est responsable de l'altération du goût des aliments. Le 1,8-cineol, le camphène, le camphre, le bornéol, le bornyl acétate et l'alpha pinène (des terpènes hydrocarbonés) sont les composants primaires de l'huile essentielle du romarin (Doyle et al., 1997; Pszczola, 2001).

3.2.7. Le thym

Le thym est une épice obtenue par les feuilles séchées du *Thymus vulgaris*, il est très utilisé dans diverses recettes et a des vertus médicinales, des propriétés antibactériennes et antioxydantes. Le composé actif de l'huile essentielle de thym est la carminative, un composé antispasmodique et un apaisant contre les irritations. Certains traitements pharmaceutiques contiennent de l'huile de thym, qui traite les toux et qui se trouve dans des préparations anti-fongiques et antiseptiques pour la bouche (Pszczola, 2001). L'huile de thym contient du méthyle benzoate (un terpène hydrocarboné), du thymol, du linalool et du carvacrol (trois terpènes phénoliques) (Freidman et al., 2000). Selon De feo et al. (2003), le thym spinulosis par exemple a un faible pourcentage en phénols, mais élevé en précurseurs biogénétiques comme gamma-terpinène et p-cymène, des terpènes hydrocarbonés.

3.2.8. La graine de sésame

La graine de sésame provient de la plante *Sesame indicum.* Cette graine contient 25 % de protéines qui sont riches en méthionine et en tryptophane. Les acides gras présents dans les huiles essentielles sont composés de 45 à 50 % d'acide linoléique. L'huile essentielle de la graine de sésame a été considérée comme une excellente huile pouvant réduire les risques du cancer et qui a de bonnes propriétés antioxydantes (Pszczola, 2001).

3.2.9. L'origan

L'huile essentielle d'origan contient du méthyl benzoate, du carvacrol et du thymol (Freidman et al., 2000). L'origan a une activité antimicrobienne très élevée. Cette huile contient contient 80-90% de composés phénoliques tel que le thymol et le carvacrol et des terpènes hydrocarbonés tel que le gamma-terpinène, le P-cymène, le myrcène, le thymyl, le methyl ether, le carvacryl methyl ether-trans-B-caryophyllene et le B-bis abolène.

3.3. L'activité antimicrobienne

Beaucoup de produits naturels ont été approuvés et sont utilisés comme traitements alternatifs médicaux antibactériens (Jantova et al., 2000). Les huiles essentielles sont considérées comme des agents conservateurs en alimentation, mais leur emploi est limité dans certains aliments à cause de leurs propriétés gustatives assez fortes. Les recherches actuelles sont axées sur la détermination de la CMI afin de joindre l'effet antimicrobien à la prolongation de la durée de conservation.

Selon Kim et al. (1995), les huiles essentielles sont efficaces et reconnues comme étant antibactériennes avec des bactéries de type Gram-positif et Gram-négatif. Des extraits de plantes tels que *Exochorda* et *Holodiscus* de la famille des rosacées sont composés principalement de flavonoïdes et de triterpènes. Ils ont un plus grand spectre d'inhibition contre les bactéries de type Gram-positif telles que *S. aureus* et *Enterococcus faecalis* que sur les Gram-négatif (Jantova et al., 2000). Les résultats de cette recherche démontrent aussi que l'extrait de géranium odorante à une concentration de 250 µg/ml, dont la composition est principalement constituée de

flavonoïdes, coumarins et de terpènes, a permis une inhibition de la croissance de *Pseudomonas aeruginosa*. Des épices provenant de l'ail, du clou de girofle, de la cannelle, de l'origan et de la sauge détruisent 99% de la bactérie *E. coli* dans du bœuf haché (Pszczola, 2001). Selon de Fao et al. (2003), le *Thymus spinulosus* est plus efficace que certains antibiotiques tels que la gentamycine et la tétracycline contre *S. aureus, Enterococcus faecalis, B. cereus, E. coli, S. thyphimurium, P. aeruginosa,* avec de plus grandes zones d'inhibition.

Les concentrations efficaces des huiles diffèrent d'un microorganisme à un autre. Il a été démontré par exemple que le thym inhibe la croissance d'*Aspergillus niger* et *Aspergillus flavus* à une concentration de 700 µg alors qu'une concentration de 400 µg d'extrait d'origan est nécessaire pour inhiber ces moisissures (Paster et al., 1990). Il est à noter que l'origan et le thym sont très efficaces contre *C. jejuni*. L'origan est aussi efficace contre *C. sporogenes* (Paster et al., 1990). Paster et al. (1990) démontrent que l'emploi des huiles essentielles dans des conditions anaérobes ou microaérobiques donne un meilleur effet antimicrobien. Ceci peut être expliqué par la réduction des changements d'oxydation que subissent les principes actifs des huiles et la diminution de la production d'énergie par les bactéries aérobes.

Une combinaison entre une utilisation d'huiles essentielles de poivre à des concentrations au-dessous de leurs concentrations inhibitrices en présence de sel avec un traitement thermique de 80°C pendant une minute a permis une prolongation de la conservation de la tomate pendant un mois sans qu'il y est croissance de champignons, ce temps de conservation a même pu être prolongé sans l'utilisation du traitement thermique jusqu'à trois mois (Ejechi et al., 1999).

D'après Shelef et al. (1984), les bactéries Gram-positif telles que *B. cereus* et *S. aureus* sont plus sensibles à la sauge que les bactéries Gram-négatif tel que *Salmonella* Typhimurium et *Pseudomonas* sp. Une réduction de la production de spores a été observée à une concentration de sauge égale à 1%, additionné à un milieu nutritif. Cette concentration a permis de réduire de 2 log la production de spores (Shelef et al., 1984). Cependant, la croissance n'a pas été influencée, contrairement à la formation des spores. Aussi, la sauge à des concentrations respectives de 2% et de 0.5%, additionné à du bœuf a permis une réduction de 2.1 log et de 2.6 log le contenu en *Bacillus cereus* dans du riz (Shelef et al., 1984). Il est à noter que l'utilisation de la sauge dans

des aliments riches en protéines et en lipides est moins efficace que dans des aliments riches en eau ou en sel à cause de l'effet protecteur.

Contre certaines bactéries, il existe une certaine proportionnalité entre la concentration et la zone d'inhibition des composés actifs (Tableau 3). Ce tableau interprète les résultats de Kim et al. (1995). Pour les signes négatifs, ll existe un effet inhibiteur non proportionnel entre la dose et la concentration.

Tableau 3: Effets de divers composés actifs sur les bactéries.

Bactéries/composés actifs	carvacrol	géraniol	citral	terpinéol	perillaldéhyde	eugénol	linalool	Citronellal
E. coli	+	+	-	+	+	+	+	-
E. coli 0157:H7	+	+	+	+	+	+	+	-
Salmonella Typhimurium	+	+	+	+	+	+	+	-
Listeria monocytogenes	+	+	+	+	+	+	-	+
Vibrio vlunificus	+	+	+	+	+	+	+	+

L'ordre décroissant de classement par rapport à l'activité bactéricide des composés est comme suit: Carvacrol et citral, géraniol, perillaldehyde et eugénol, linalool, terpinéol et citronellal.

Une importante variété de bactéries d'altération est inhibée par les huiles essentielles de citron et d'orange, qui ont un plus large effet par rapport à l'origan, le clou de girofle, l'oignon, l'ail et le thym (Subba et al., 1967; Kim et al., 1995).

Un mélange de trois extraits d'épices de ciboulette chinoise, de cannelle et de corni fructus a un meilleur spectre antimicrobien que celui de leur utilisation individuelle ou même que l'utilisation du sorbate de potassium. Plus la concentration du mélange des trois extraits est élevée et plus l'effet inhibiteur contre *E. coli* est efficace. Ce résultat a été obtenu avec une concentration de 5 mg/ml du mélange et de 10 mg/ml de sorbate de potassium (Mau et al., 2001). Ces extraits ont aussi montré des effets positifs sur des aliments tel que le jus d'orange, le porc et le lait. Ce mélange n'étant pas affecté par le traitement thermique pourrait même être utilisé comme agent de conservation alimentaire durant les procédés à hautes températures.

Des recherches ont été effectuées par Basilico et al. (1999) sur le fléau de la production d'ochratoxine métabolisée pas des genres comme *Aspergillus* et *Penicillium*, qui contaminent entre autres les denrées alimentaires et qui causent des états néphrotoxiques, hépatotoxiques, tératogéniques et immunosuppressifs. Des huiles essentielles d'origan et de menthe inhibent la croissance mycélienne et la production de l'Ochratoxine A, jusqu'à une durée de conservation de 21 jours, à une concentration de 1000 ppm. L'huile essentielle de basilic agit ainsi, jusqu'à une durée de conservation de sept jours. À une concentration de 500 ppm, aucune de ces huiles ne montre un effet antibactérien contre ces moisissures.

Aligiannis et al. (2001) ont testé deux espèces d'origan sur différentes bactéries. L'huile essentielle de l'*origanum scabrum* à une concentration de carvacrol de 74.86 % possède une très importante activité antimicrobienne, avec une CMI entre 0.28 et 1.27 mg/ml sur *S. aureus*, *Staphylococcus epidermitis, Klebsiella pneumoniae* et *E. coli*. Alors que l'huile essentielle de l'*Origanum microphyllum*, qui est dépourvu de carvacrol a une activité antimicrobienne plus faible avec des valeurs de CMI plus élevées entre 1.81 et 8.85 mg/ml. Les travaux de Lambert et al. (2001) prouvent que la majeure partie de l'effet inhibiteur de l'origan provient de l'effet additif du thymol et du carvacrol. Selon Kelander et al. (1998), ces deux derniers permettent de désintégrer la membrane de ces deux bactéries: *E. coli* et *S.* Typhimurium à des concentrations

proches de la CMI. Burt et Reinders (2003) ont aussi observé un effet bactéricide de l'origan avec *E. coli* O157:H7. Une concentration de 625 µl/l a permis cet effet après une minute d'incubation, à des températures de 10, 20 et 37°C. Même que des concentrations inférieures (312 et 156 µl) ont montré des effets bactéricides après cinq minutes. Alors que le thym, le piment et le clou de girofle ont montré un effet bactéricide et bactériostatique à des concentrations plus élevées.

Toutes les huiles essentielles riches en carvacrol ont une activité antimicrobienne élevée contre un bon nombre de micro-organismes à l'exception de *P. aeruginosa,* qui est la bactérie la moins sensible parmi celles analysées (Aligiannis et al., 2001). Cette bactérie a montré aussi une résistance avec le thym. Le *Thymus kotschyanus* a un effet statique alors que le *Thymus persicus* n'a eu aucun effet contre *P. aeruginosa* (Rasooli et al., 2003). Néanmoins, ces deux variétés ont montré des activités bactéricides avec des micro-organismes comme: *E. coli, S. aureus* et les spores de *Bacillus subtilis.* D'autres variétés de thym comme le *Thymus pubescens* et le *Thymus serpyllum* ont montré des effets bactéricides contre 50 % de la population bactérienne testée telle que *E. coli* et *S. aureus*, à l'exception de *P. aeruginosa*, en 15 minutes (Rassoli et al., 2002). Cette dernière est moins sensible au mélange de carvacrol et de thymol que *S. aureus* (Lambert et al., 1991).

Le procymidone, un fongicide chimique possède une IC50 (Concentration Inhibitrice de croissance de 50% de la population bactérienne) de 0.2%. Cette concentration est supérieure à celle retrouvée chez les huiles essentielles d'*Origanum compactum* (35.1 ppm) et de *Thymus glandulosus* (79.2 ppm) sur la moisissure *Botrytis cinerea.* Ces deux huiles contiennent respectivement et essentiellement du carvacrol (58.1%) et du thymol (43.2%) (Chebli et al., 2004). Les résultats de cette étude montrent cependant que la capacité d'inhibition de croissance de cette moisissure dépend de la concentration de ces dernières. Un autre champignon, *Candida* sp. a montré une sensibilité envers des huiles essentielles de thym tel que *Thymus vulgaris* et *Thymus zygis,* à des concentrations sous inhibitrices, permettant ainsi l'inhibition de la germination de ce champignon. A des concentrations minimales inhibitrices (CMI) entre 0.16 et 0.35 µl/ ml (v/v), une intense lésion de la membrane a été observée (Pina-vaz et al., 2004).

Des concentrations entre 250 et 400 µg/ml des huiles essentielles de *Thymus vulgaris* (Thym), d'*Origanum dictamus* (Dictamus), d'*O. vulgare* (Origan) et d'*O. majorana* (Marjoram) permettent d'inhiber la croissance et la production de conidies de *Penicillium digitatum*, un champignon de postrécolte qui est difficilement vaincu par des fongicides synthétiques (Daferera et al., 2000).

Des activités bactéricides ont été allouées aux huiles essentielles de jasmin, de gardénia, de graine de carotte, de patchouli, de céleri, d'épinards et d'orange contre *C. jejuni*. Les principaux composés actifs de ces huiles comme les terpènes hydrocarbonés tels que le cinnamaldéhyde, le benzaldehyde, l'estragole, le citral, le perillaldehyde, le carvone R et le géranyl acétate et les terpènes phénoliques tel que le carvacrol, le thymol, et l'eugenol ont aussi été bactéricides contre *C. jejuni*. Des huiles essentielles d'origan, de cumin, de thym, de palmarosa, de clou de girofle, de citronnelle et de piment ont été bactéricides contre *Salmonella enterica*. Les principaux composés actifs de ces huiles comme les terpènes hydrocarbonés tel que le cinnamaldéhyde, le salicylaldéhyde, le citral, le périllaldéhyde et l'estragole et les terpènes phénoliques tel que le carvacrol, le thymol, l'eugénol, le géraniol, et l'isoeugénol ont aussi été bactéricides contre *S. enterica* (Friedman et al., 2002).

Des recherches effectuées par Mahmoud (1999) prouvent que des espèces comme *Lupinus albus, Ammi visaga* et *Xanthium pungens*, ont un pouvoir inhibiteur contre la croissance et la production d'aflatoxine produite par *A. flavus* à des concentrations maximales inhibitrices de 10 mg/ml (réduction de croissance mycélienne de 45.3% et de production d'aflatoxine de 60%).

Les sesquiterpènes hydrocarbonés sont les principaux composants des huiles essentielles du *Stachys* sp. entre autres *Stachys scardica*, communément appelé Epiaire annuelle, qui possède une activité antimicrobienne contre certaines bactéries telles que *B. cereus, B. subtilis, E. coli, Micrococcus flavus* et *S. epidermitis* et des champignons comme *Aspegillus niger, Trichophyton* sp., *Pennicillium ochrochloron* et *Candida albicans* (Skaltsa et al., 2003).

L'huile essentielle d'*Achillea clavennae L*, communément appelée l'achillée contient 29.5% de camphre et a une activité antimicrobienne maximale contre des bactéries pathogènes Gram-négatif comme *K. pneumoniae* et *P. aeruginosa*, deux bactéries pouvant être les agents causales de la toux et de la fièvre, *E.coli* et contre des bactéries Gram-positif comme *S. aureus*. Néanmoins les bactéries Gram-négatif sont plus résistantes à cette huile que les Gram-positif. En plus d'être antibactérienne, cette huile permet aussi l'inhibition d'organismes filamenteux tel que *C. albicans* (Bezic et al., 2003).

L'huile essentielle de Palmarosa, qui contient essentiellement du géraniol (65%) et de l'acétate géranyl (20%), est fongistatique contre les champignons filamenteux tel que *A. niger* et *Penicillium funiculosum* (Delespaul et al., 2000; Prashar et al., 2003). Cette huile permet également d'inhiber la croissance du champignon *Cryptococcus neoformans*, agent qui cause l'infection au cours du dernier stade de la maladie du SIDA (Prashar et al., 2003).

P. aeruginosa est plus résistante à des antibiotiques comme l'ampicilline ou la céfoxitine qu'à des huiles essentielles de *Micromeria* sp., connu sous le nom de sarriette. En effet, une concentration de 10µl/disque de *Micromeria albanica* et *M. dalmatica*, des huiles riches en oxyde pipéritenone permet l'obtention d'une zone d'inhibition de 12 mm contre *P. aeruginosa* et des concentrations inférieures de ces huiles ont permis une inhibition de croissance chez *E. coli*, *B. subtilis*, *Micrococcus luteus* et *S. aureus*. Ces huiles essentielles ont eu aussi un effet antifongique contre *A. niger*, *A. ochraceus* et *Cladosporium cladosporioides*. Un effet plus important a été observé chez *M. albanica* qui contient en plus du carvacrol. Des CMI de 0.2 à 0.4 µl/ml nettement inférieures à celles d'un antifongique tel que le bifonazole avec des CMI de 2 à 4 µl/ml ont été obtenues (Marinkovic et al., 2002).

3.4. Mode d'action

Un classement en ordre décroissant est établi pour les principes actifs des huiles essentielles, ce classement est principalement basé sur leur activité antimicrobienne. Les phénols sont en tête de liste suivis par les alcools, les aldéhydes, les cétones, les éthers et enfin les hydrocarbones (Faid et al., 1996; Daferera et al., 2000).

Chaque bactérie possède une réponse différente face aux composés actifs des huiles essentielles car les modes d'action de ces derniers diffèrent. Selon Kim et al. (1995), il y a trois principales réactions: l'interférence avec les phospholipides membranaires des bactéries qui augmente la perméabilité de la membrane, la perte en nutriments et la réduction des processus enzymatiques, la diminution de l'énergie cellulaire (activité de l'ATPase) et du métabolisme cellulaire et enfin la destruction du matériel génétique. Selon Lambert et al. (1991) en présence de certains composés des huiles essentielles, un endommagement de la membrane plasmique des bactéries et une dispersion de deux éléments faisant partie de la force proton-motrice (FPM), qui sont le gradient pH et le potentiel électrique, surviennent conduisant ainsi à une fuite des protons, des ions de phosphates et de potassium (Lambert et al., 1991).

Les composés électronégatifs comme les aldéhydes contenus dans certaines huiles essentielles interfèrent avec le processus biologique, troublant le transfert d'électron et réagissent avec les protéines et les acides nucléiques. (Dorman et Deans, 2000).

Les huiles essentielles d'origan ou de clou de girofle permettent un endommagement plus important de l'enveloppe externe de la bactérie *E. coli* que celui de la Polymixine B, ce qui leur confère une plus importante activité antibactérienne (Rhayour et al., 2003). En effet, les résultats de cette recherche montrent qu'avec les huiles essentielles, la viabilité bactérienne diminue rapidement dès les dix premières minutes et après une vingtaine de minutes pour l'antibiotique.

Des recherches effectuées sur l'huile essentielle de thé, ont montré son activité bactéricide sur *E. coli* en phase exponentielle qui était plus importante qu'en phase stationnaire. Ce qui a conduit Gustafson et al. (1998) à émettre l'hypothèse que cette huile affectait les processus de synthèse. Carson et al (2002) a aussi démontré que *S. aureus* en phase exponentielle était sensible à l'huile de thé.

Contrairement à plusieurs antibiotiques, les composés hydrophobes des huiles essentielles peuvent atteindre l'espace périplasmique des bactéries Gram-négatif à travers les protéines porines (Lambert et al., 1991; Helander et al., 1998). Les composés phénoliques des huiles essentielles inhibent le transport direct des protéines cellulaires et aussi le transport actif des

nutriments en interférant avec les éléments qui jouent un rôle dans la FPM (Doyle et al., 1997). Selon Friedman et al. (2002), cela pourrait être expliqué par l'inhibition d'enzymes essentielles au métabolisme et la chélation d'éléments essentielles qui se trouvent sous forme de traces comme le fer. Les propriétés bipôphiliques des terpénoides, solubles dans l'eau, agissent aussi au niveau de la barrière phospholipidique qui inhibe le transport d'électron, la translocation des protéines et l'inhibition des réactions enzymatiques (Dorman et Deans, 2000).

Le carvacrol, un terpène phénolique attaque la membrane, en la rendant perméable aux ions de potassium et aux protons, acidifiant ainsi le cytoplasme de la bactérie, rendant impossible la synthèse de l'ATP et diminuant la quantité d'ATP intracellulaire (Ultee et al., 2000) car l'augmentation du flux passif des ions affecte la transduction de l'énergie (Sikkema et al, 1995; Prashar et al., 2003).

Les composés actifs de certaines épices comme le clou de girofle, la cannelle, la sauge et la muscade ont un pouvoir inhibiteur, surtout lié au groupement phénolique de l'eugénol et le groupement carbonyle du cinnammaldéhyde contre les enzymes responsables de la putréfaction présents chez certaines bactéries tel que la lysine, l'histidine, et l'ornithine décarboxylases. Wendakoon et Sakaguchi (1995) ont montré que la présence des huiles affectaient l'activité de ces enzymes chez *Enterobacter aerogenes.* Ces enzymes sont responsables de la putréfaction en formant des amines biogènes comme l'histamine qui est le produit de la décarboxylation de l'histidine, la putrescine qui est le produit de la décarboxylation de trois acides aminés comme: la glutamine, l'arginine et l'agmatine, et la cadaverine qui est le produit de la décarboxylation de la lysine (Den Brinker et al., 1995). Il est très difficile d'éliminer ces composés par les traitements physiques tel que le froid ou la chaleur.

Avant que l'effet bactéricide de l'huile essentielle des feuilles de l'*Ocimum gratissimum L* ne soit atteint contre la bactérie *Shigella*, des concentrations sub-inhibitrices (0.75 et 1µl) permettent de diminuer ses facteurs de virulence, comme l'activité de la protéase extracellulaire responsable de l'héma-agglutination, la quantité en sucre rhamnose des O-lipopolysaccharides responsable des diarrhées et la keratoconjonctivite responsable de la diminution de la pigmentation rouge des yeux (Iwalokun et al., 2003). Cela semble être très utile, permettant ainsi

de diminuer les CMI des antibiotiques employés contre cette bactérie tels que la tétracycline et l'aminopénicilline. Le spectre d'action de cette plante est encore plus large, allant même jusqu'à l'inhibition du glutathion S transférase avec une IC50 de 10 µl /ml (Fakae et al., 2000), chargé de la défense des nématodes (Ascaris) contre la réponse immunitaire de l'hôte (Brophy et al., 1989; Fakae et al., 2000) et qui est aussi la première cible des agents chimiques.

Des huiles essentielles de thym possèdent une importante activité antifongique, permettant ainsi de traiter des candidoses, par destruction directe et primaire de la membrane cellulaire et non résultante de la perturbation métabolique (Pina-Vaz et al., 2004). Un antifongique tel que l'huile essentielle de Palmarosa qui s'installe et s'accumule dans la membrane plasmique attaque l'intégrité de la membrane cellulaire, induit ainsi le relargage des ions potassium et de magnésium, ce qui modifie entre autre la composition de la membrane, la rendant plus fluide, en augmentant la proportion en acides gras saturés et en diminuant celle des acides gras insaturés, augmentant ainsi les canaux de la membrane. Cependant ce n'est pas par ce moyen que la dissipation des ions de potassium se fait (Prashar et al., 2003). L'activité antifongique des huiles essentielles qui sont composées essentiellement de composés phénoliques pourrait provenir des ponts hydrogènes qui se forment entre les groupements phénoxyles et les sites actifs des enzymes (Farag et al., 1989; Daferera et al., 2000).

3.5. Autres utilisations

3.5.1. Effet immunostimulant

En médecine traditionnelle, des extraits des feuilles du Plantin (*Plantago major*) sont considérés comme étant anti-inflammatoires, antihelminthique, antihistaminique, expectorants, diurétique et hypotensifs (Grigorescu et al., 1973; Matev et al., 1982; Franca et al., 1996; Gomez-Flores et al., 2000). Le fluide intracellulaire possède une activité prophylactique contre les tumeurs des glandes mammaires (Lithander, 1992; Gomez-Flores et al., 2000). Les extraits de feuilles de cette plante permettent l'immunomodultaion et jouent le même rôle que l'INF Y et les LPS en stimulant la production du NO et des TNF-α par les macrophages et augmentent la prolifération des cellules lymphocytes en association avec les mitogènes tel que le Concanavalin

(Gajewski et al., 1989; Gomez-Flores et al., 2000). D'autres recherches ont permis de confirmer cette fonction d'immunomodulation donnée aux extraits d'herbes, telle que l'*Echinacea* et la *Glodenseal* qui permettent une production plus accélérée des immunoglobulines et une augmentation significative des réponses immunes (Rehman et al., 1999). Le virus de l'hépatite B reste aussi une problématique non résolue dans le monde de la recherche. Toutefois, des extraits d'espèces de *Phyllanthus* ont permis l'inhibition de la réplication, de l'activité de la polymérase et de la transcrption de l'ARNm chez ce virus (Calixto et al., 1998).

3.5.2. L'activité antioxydante

La respiration normale et aérobe des cellules eucaryotes qui implique diverses réactions métaboliques engendre des ROS (espèces réagissant à l'oxygène), tel l'oxydation enzymatique de l'acide linoléique, qui à leur tour causent des dommages cellulaires comme le cancer ou les maladies cardiovasculaires. Pour cela, des antioxydants sont utilisés pour protéger les cellules. Il en existe plusieurs mais qui sont synthétiques, instables, volatils, toxiques et qui ont un effet anormal sur le système enzymatique (Inatani et al., 1983; Choi et al., 2000). Environ 0.4 à 4% de l'oxygène inhalé est converti par réduction monoélectrique au lieu de quatre électrons suite à une imperfection mitochondriale qui engendre une fuite d'électrons, formant ainsi des substances oxygénées activées (EOA) comme les radicaux libres. Malgré le rôle physiologique des radicaux libres à faible concentration qui est de réguler l'apoptose et moduler l'expression de gènes qui codent pour les enzymes antioxydantes, leur effet est controversé à une concentration élevée d'ou l'utilité de l'utilisation des antioxydants (Pincemail, 2004).

En industrie alimentaire, les antioxydants sont très utilisés afin de conserver la qualité organoleptique de l'aliment qui risque d'être altéré du fait de l'oxydation des lipides. Aussi, un régime alimentaire à base d'aliments riches en antioxydants permet de rééquilibrer le stress oxydant de l'organisme. Il existe quatre types d'action antioxydante: inhibition de radicaux libres, chélation de métal, décomposition des peroxydes et nettoyeur d'oxygène (Choi et al., 2000).

L'oxydation du LDL (low density lipoproteins) engendre des dysfonctionnements comme l'athérosclérose ou des crises cardiaques, qui peuvent être évités en utilisant des antioxydants qui

contrôlent l'état pathophysiologique de l'organisme (Grassmann *et al.*, 2003). En effet, des maladies sont causées par les radicaux libres (Choi et al., 2000) tel que les maladies cardio et cérébrovasculaires, cancer, inflammation ou des maladies chroniques (Khanum et al., 1999; Choi et al., 2000).

Certaines épices possèdent un pouvoir antioxydant, ce dernier confère une certaine protection aux bactéries. Ainsi, la décontamination d'un aliment par l'irradiation qui contient certaines épices ne donne pas toujours l'effet désiré. La présence d'extraits de poivre rouge ou de poivre noir et de turmeric permet de protéger l'ADN bactérien et ainsi diminuer l'effet de l'irradiation sur des bactéries comme *E. coli, Bacillus megaterium* et *Bacillus pumilus* (Sharma et al., 2000). L'activité antioxydante de ces trois extraits provient respectivement de leurs principaux composés actifs tel que la capsanthine, la pipérine et le curcumin (Srinivas et al., 1992; Noguchi et al., 1994; Tipsrisukond, 1998; Sharma et al., 2000).

Les extraits de *Satureja hortensis* ont une activité antioxydante aussi comparable aux antioxydants synthétiques tel que le BHT (Hydroxytoluene butyle), du à la présence de γ–terpinène et des composés phénoliques tel que le thymol, le carvacrol et l'acide rosmarinique (Güllüce et al., 2003). Des terpènes hydrocarbonés retrouvés dans des huiles essentielles de plantes tel que le carveol, le limonène, le sobrérol et l'alcool périllylique sont très efficaces dans les traitements de cancer du sein ou du foie (Choi et al., 2000). L'huile essentielle du Citrus possède une importante activité antioxydante en protégeant l'organisme contre les radicaux libres. Ceci est dû à la présence de ses constituants majeurs comme le géraniol, le terpinolène et le δ- terpinène (Choi et al., 2000).

L'eugénol, principe actif de certaines épices, est utilisé pour stabiliser le trans-cinnamaldéhyde, qui au delà d'un chauffage à 60 °C, s'oxyde et se transforme en benzaldéhyde et en glyoxal (Freidman et al., 2000). Selon Jovanovic (2001), le cumin a une activité antioxydante élevée, capable d'inhiber la croissance cellulaire maligne et les enzymes COX I et COX II, médiateurs des syndromes inflammatoires, stimuler le glutathion, un anti-oxydant naturel, et surtout de neutraliser les radicaux libres cancérigènes comme les ROS (radicaux hydroxy, peroxy, superoxide), des composés aussi formés par des neutrophiles contre les micro-

organismes pathogènes (Grassmann et al., 2003) et les NOS (oxyde nitrique et peroxonitrique).

Les monoterpènes responsables de l'activité antioxydante du pin (*Pinus mugo*) sont le δ–terpinène et le terpinolène. Ces composés possèdent des doubles liaisons et des atomes de carbone bisallylique, qui ont une grande capacité à céder des électrons pour les radicaux libres (Grassmann et al., 2003).

Des suppléments alimentaires tel que l'huile essentielle d'origan à une concentration de 100 mg/kG mélangé à l'alimentation de la volaille a permis de protéger la viande blanche contre l'oxydation des gras au cours de l'entreposage (Botsoglou et al., 2002), des viandes riches en acides gras polyinsaturés qui présentent une plus grande sensibilité à l'oxydation (Botsoglou et al., 2002).

Des métaux tel que le fer libre sont d'importants catalyseurs dans la formation des EOA (espèces oxygénées actives) (Pincemail, 2004). Ces derniers peuvent être bloquées par l'utilisation de composés phénoliques qui sont considérés comme étant des accepteurs de radicaux libres et de chélateurs de métaux (Afanas'ev et al., 1989; Xin et al., 1990; Medina et al., 1999).

En effet, des composés phénoliques extraits d'une huile d'olive extra vierge ajoutés à de la saumure de thon permet l'obtention d'un effet antioxydant meilleur que celui obtenu avec des agents synthétiques, par une inhibition de la formation des hydroperoxydes et de la décomposition de ces derniers en hexanal (Medina *et al.*, 1999).

4. Immobilisation des huiles essentielles dans des films biodégradables

Lors de ces deux dernières décennies, la biotechnologie s'est beaucoup intéressée au recyclage des sous produits industriels et à la rémédiation des emballages synthétiques qui polluent l'environnement. Pour cela, des recherches ont été effectuées afin de valoriser entre autres les polysaccharides indigestibles comme la pectine et la cellulose. En les utilisant comme matrice de films biodégradables. Dans cette optique, les scientifiques se sont intéressés aussi à la

fabrication des films biodégradables. Les films organiques peuvent en plus être recyclés dans les autres applications. Jusqu'à présent, différentes formulations ont été élaborées. En plus, un emballage doit protéger les produits (comme les aliments) contre les facteurs physiques et les facteurs environnementaux atmosphériques comme la vapeur d'eau, les gaz et les odeurs (Hong et Krochta, 2003). Le mécanisme de formation des films affecte essentiellement les propriétés structurelles des films. L'efficacité de la barrière à l'humidité dépend de la polarité des composants et de la distribution homogène des ingrédients hydrophobes (Debeaufort et al., 1993; Pérez-Gago et Krochta, 1999).

Les films à base de polymères consommables permettent de fournir une barrière au transfert de masse des matières, de protéger les ingrédients alimentaires et d'améliorer l'intégrité mécanique des aliments en conservant leur état (Pérez-Gago et al., 1999). Parmi les films biodégradables, il existe le film à base d'un mélange de PVC et d'amidon. Il a été démontré par Ishigaki et al. (1999) que ce dernier est dégradé plus rapidement avec une solution d'enzymes qu'avec un consortium bactérien. Selon Jiancai et Chan (2000), en plus de la propriété de se biodégrader, les films sont considérés comme barrière à l'oxygène, au dioxyde de carbone et au transfert des lipides. Parmi les biopolymères utilisés, il y a le lactosérum du lait qui est très efficace. Ce dernier comporte bon nombre de propriétés, comme une excellente valeur nutritive, absence d'odeur ou de retenir les ingrédients volatils.

Les films biodégradables possèdent une matrice, un support sur lequel se base le film. Cette matrice peut être de nature polysaccharidique ou de nature protéique. Chacune peut provenir d'une source végétale ou animale. Pour les polysaccharides, la pectine, l'alginate, l'agarose ou la cellulose proviennent des végétaux. Le chitosane provient d'une source animale qui est la carapace des crustacés. Pour les protéines, le collagène et la caséine proviennent d'une source animale. Le gluten et la zéine proviennent d'une source végétale (LeTien, 2004). Néanmoins, ces matrices, de nature hydrophile ne représentent pas de barrière à l'humidité. Il est important que le film ne perde pas son eau et ne soit pas perméable à l'humidité du matériau ou à l'oxygène. La cellulose, un polymère qui possède une structure cristalline et de fortes liaisons hydrogènes est insoluble et difficile à dégrader. Pour cela, des modifications chimiques peuvent

être effectuées en le transformant entre autres en carboxymethyl cellulose (CMC).

Cependant, diverses recherches ont été effectuées pour l'utilisation de films à base de polymères naturels qui ont la caractéristique de contrôler la libération des agents bioactifs. Ces derniers représentent un support fiable pour des agents antimicrobiens, des agents antioxydants. Ces polymères naturels sont biodégradables, du fait qu'ils contiennent des groupements hydrolysables dans leur chaîne qui se dégradent en des composés à faible poids moléculaire et non toxiques (Anon, 1999). L'hydrophilicité affecte fortement les interactions avec le matériau à emballer (Anon, 1999).

Selon Mezgheni et al. (1998), les liens intermoléculaires sont responsables de la rigidité ou de la flexibilité d'un matériau. Un nombre élevé de ces liens conduit à un matériau plus rigide. Un agent plastifiant est souvent employé pour réduire les forces intermoléculaires et améliorer la flexibilité et l'extensibilité du film. Aussi, un plastifiant diminue le transfert de chaleur, évitant ainsi la dégradation thermique des films (Irissin-Mangata et al., 2001). Un effet plastifiant peut être obtenu par des composés hydrophiles comme les polyols (glycérol, sorbitol) et l'acide lactique. L'utilisation du glycérol doit être contrôlée du fait qu'il augmente la perméabilité à la vapeur d'eau. Les films ayant du glycérol comme plastifiant perdent leurs propriétés mécaniques avec le temps, cela est imputé à sa migration. Des agents plastifiants (dont le groupement hydroxyle des polyols est remplacé par un groupement amine) comme le diethanolamine et le triethanolamine ont permis une meilleure extensibilité, élasticité et augmentation de la durée de vie des films à base de gluten, par rapport aux films avec du glycérol comme plastifiant (Irissin-mangata et al., 2001). Le poids moléculaire des amines est plus élevé que celui des polyols, un autre avantage à considérer, évitant ainsi la libération du plastifiant du film.

Afin d'éviter la perméabilité des films aux gaz et à l'eau, les matières premières telles que les protéines et les polysacharides sont réticulées pour obtenir des liens inter et intramoléculaires et ainsi une matière condensée.

4.1. Films à base de protéines

Les protéines sont des hétéropolymères thermoplastiques d'acides aminés polaires et non polaires, qui sont capables de former des liaisons intermoléculaires et diverses interactions qui leur confèrent un potentiel en propriétés fonctionnelles (Guilbert et al., 1994; Marquie et al., 1995; Irissin-Mangata et al., 2001).

Bradenburg et al. (1993) ont synthétisés des films à base de protéines de soya à différents pH. A un pH de 6, le film n'était pas transparent, ce qui induisait une incomplète solubilisation des protéines. Le film avait une faible barrière à l'eau et de faibles propriétés mécaniques.

Oussalah et al. (2004) ont démontré qu'un film biodégradable à base de protéines laitières telles que la caséine et le WPI (Isolat de protéines du lactosérum) a permis d'obtenir une base qui a supporté des huiles essentielles antimicrobiennes. Ce film alors considéré comme étant antimicrobien a été utilisé comme un moyen de conservation de la viande. Toutefois, ces films avaient l'inconvénient d'absorber de l'eau au fil des jours.

4.1.1. La réticulation

Les protéines possèdent de larges propriétés fonctionnelles pour former un film du fait des interactions qui surviennent entre les liaisons intermoléculaires des acides aminés. Cependant, les propriétés filmogènes des protéines ne sont pas suffisantes pour contrer leur perméabilité aux gaz et à l'eau. Dans cette optique, différentes recherches ont été réalisées afin d'améliorer cet important critère. Des réactions de réticulation ont été developpés. Ces réactions engendrent la polycondensation des protéines. Dans les films à base de protéines laitières comme la caséinate de calcium et le WPI, la réticulation est différente pour chaque composé.

4.1.1.1. Réticulation du caséinate de calcium

Une réticulation de la solution de caséinate de calcium est effectuée par irradiation aux rayons gamma. Ce traitement permet entre autres d'augmenter les liens hydrogène, les interactions électrostatiques et hydrophobes. L'irradiation engendre la formation des radicaux

hydroxyles, issus de la radiolyse de l'eau. Ce groupement va alors réagir avec la tyrosine (acide aminé aromatique, à groupement hydroxyle), donnant ainsi une molécule d'eau et une molécule tyrosyle (groupement cétonique). Une dimérisation survient suite à un réarrangement céto-énolique, formant ainsi la bityrosine (Chen, 1995; Ressouany et al., 1998A; Mezgheni et al., 1998A; Sabato et al., 2001; Oussalah et al., 2004).

4.1.1.2. Réticulation du WPI

La réticulation des WPI se fait par un traitement thermique, qui est appelé alors la thermocondensation (Vachon et al., 2000). Le principe est basé sur l'exposition et l'oxydation des groupements thiols internes des cystéines. La chaleur permet la formation de liens intermoléculaires de type disulfure (Shimada et Cheftel, 1998). La perméabilité à la vapeur d'eau des films à base de WPI est plus importante dans les pH acides. Après atteinte du point isoélectrique, leur perméabilité à la vapeur d'eau est au maximum. Ceci est causé par l'agrégation des protéines. Cette dernière pourrait aussi de diminuer la mobilité d'éventuelles substances hydrophobes (Pérez-Gago et Krochta, 1999).

4.2. Films à base de polysaccharides

Le chitosane est un polysaccharide obtenu suite à une déacétylation de la chitine extraite des carapaces de crustacés marins et des insectes (Poncelet et al., 1999). Ce dernier est très utilisé en milieu pharmaceutique comme excipient humide de granulation, agent mouillant et enfin dans la synthèse des films. Le chitosane a un haut poids moléculaire, c'est ce qui lui confère de bonnes propriétés pour former un film tel que la présence des liaisons hydrogène inter et intra-moléculaires (Cervera et al., 2004). Un polysaccharide tel que le chitosane permet en plus de contrôler la croissance des moisissures et des levures (Shaban et al., 2002), ainsi que celles des bactéries comme *Aeromonas hydrophila* à une concentration minimale inhibitrice de 0.04% (Shaban et al., 2002), effet proportionnel au traitement à l'irradiation du polymère. En effet, une dose d'irradiation de 150 kGy appliquée au chitosane permet une décontamination plus élevée qu'une dose de 100 kGy. Cette dernière appliquée sur du chitosane a néanmoins permis

une décontamination maximale sur *E. coli* (Matsuhashi et Kume, 1997).

L'alginate est un autre polymère de nature polysaccharidique provenant de diverses algues qui est très utilisé pour faire des gels ou des films. Cet hétéropolysaccharide est constitué d'unités d'acide L-guluronique et d'acide D-mannuronique. Des billes d'alginate ont été synthétisées par Le tien et al. (2004) pour encapsuler des bactéries lactiques afin de les protéger lors du transit gastrique.

4.2.1. Gélification ionotropique de l'alginate

Des gels peuvent être obtenus à partir de polymères chargés, capables de capter des protons, immergés dans une solution qui contient des ions multivalents. La gélification de l'alginate est de type ionotropique et se produit en présence d'ions multivalents tel que le calcium. Plus la concentration en groupements guluronique dans la molécule d'alginate est élevé et plus le gel formé sera résistant car c'est à cette partie que le calcium se lie (Poncelet et al., 1999).

Hypothèse

L'hypothèse de ce travail est: l'immobilisation d'huiles essentielles à large spectre antimicrobien dans des films biodégradables réticulés à base de caséinate de calcium et d'alginate pourrait permettre un meilleur contrôle sur la croissance de bactéries d'altération ou pathogènes tel que *Pseudomonas* sp., *E. coli* O157:H7, *S. enterica* subsp. *enterica* serovar Typhimurium, *S. aureus* et *L. monocytogenes* inoculés sur du biftek de ronde, de la bologne ou du jambon, entreposés à 4°C.

Objectifs

Les objectifs de cette recherche sont:

1- Sélectionner les huiles essentielles ayant des propriétés inhibitrices contre une bactérie d'altération et contre quatre bactéries pathogènes les plus souvent responsables des toxi-infections alimentaires.

2- Mettre au point différentes formulations de film à base de polymères naturels offrant de bonnes propriétés mécaniques une fois déposés sur la viande et permettant une libération contrôlée des huiles essentielles.

3- Mettre au point une méthode d'immobilisation des huiles essentielles dans des films biodégradables afin de contrôler la diffusion de ces huiles du film vers le substrat au cours de l'entreposage.

4- Vérifier le pouvoir antimicrobien et la diffusion contrôlée des huiles essentielles immobilisés dans des films biodégradables à base de caséinate de calcium sur la croissance de *Pseudomonas* sp. et d'*E. coli* O157:H7 inoculés sur du biftek de ronde.

5- Vérifier le pouvoir antimicrobien et la diffusion contrôlée des huiles essentielles immobilisés dans des films à base d'alginate sur la croissance de *L. monocytogenes* et *S.* Typhimurium inoculés sur du jambon et *E. coli* O157:H7 et *S.* Typhimurium inoculés sur de la bologne.

6- Élucider le mécanisme d'action de certaines huiles essentielles sur deux types de bactéries, une de type Gram-positif et une autre de type Gram-négatif et enfin effectuer une comparaison entre ces deux modes d'action.

Chapitres II: Articles

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Antimicrobial and antioxidant effects of milk protein-based film containing essential oils for the preservation of beef round

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Résumé

Des films comestibles à base de protéines laitières contenant 1.0% (wt/vol) d'huile essentielle d'origan, 1.0% (wt/v) d'huile essentielle de piment ou 1.0% (wt/vol) d'huiles essentielles d'origan et de piment (1:1) ont été déposés sur des tranches de boeuf afin de contrôler la croissance des bactéries pathogènes et prolonger durée de conservation pendant l'entreposage à 4°C. La viande et les films ont été périodiquement prélevés pendant sept jours pour effectuer une analyse microbienne et biochimique. Le potentiel d'oxydation de lipide présents dans la viande a été évalué par la détermination des substances réactives thiobarbituriques (TBARS). La disponibilité des composés phénoliques des huiles essentielles a été évaluée par la détermination des composés phénoliques totaux présents dans les films pendant le stockage. Les propriétés antioxydantes des films ont été également évaluées pendant la conservation après un procédé modifié de la méthode colorimétrique de DPD (N, N-diéthylique-p-phénylènediamine). L'application des films bioactifs sur une viande qui contenait 10^3 UFC/cm^2 d'*E. coli* O157:H7 ou de *Pseudomonas* sp. a prouvé que le film qui contenait de l'huile essentielle d'origan était le plus efficace contre les deux bactéries tandis que le film qui contenait l'huile essentielle de piment semblait être moins efficace contre ces deux bactéries. Une réduction de 0.95 log UFC/cm^2 de *Pseudomonas* sp. a été observée à la fin de la période d'entreposage avec les échantillons qui contenaient les films à base d'huile essentielle d'origan. Une réduction de 1.12 log d'*E. coli* O157:H7 a aussi été notée avec les échantillons enduits des films à base d'huile essentielle d'origan.

Abstract

Milk protein-based edible films containing oregano 1.0% (w/v), pimento 1.0% or oregano-pimento (1:1) essential oils mix 1.0% were applied on beef muscle slices in order to control the growth of pathogenic bacteria and increase the shelf life during storage at 4°C. Meat and film were periodically tested during seven days for microbial and biochemical analysis. Lipid oxidation potential of meat was evaluated by the determination of thiobarbituric reactive substances (TBARS). The availability of phenolic compounds from essential oils was evaluated by the determination of total phenolic compounds present in the films during storage. Antioxidant properties of films during storage were also evaluated following a modified procedure of the DPD (N,N-diethyl-p-phenylenediamine) colorimetric method. Oregano based films stabilized lipid oxidation in beef *muscle* samples, whereas pimento based films presented the highest antioxidant activity. The application of bioactive films on meat surface containing 10^3 CFU/cm^2 of *E. coli* O157:H7 or *Pseudomonas* spp. showed that film containing oregano was the most effective against both bacteria while film containing pimento oils seems to be the least effective against these two bacteria. A 0.95 log reduction of *Pseudomonas* sp. level as compared to samples without film was observed at the end of storage in presence of films containing oregano extracts. A 1.12 log reduction of *E. coli* O157:H7 level was noted in samples coated with oregano based films.

Keywords: whole muscle meat, antimicrobial, antioxidant, edible film, essential oils, lipid oxidation, phenolic compounds, *E. coli*, *Pseudomonas*

Introduction

Useful shelf life is the time require for a food to become unaccepable from a sensory, nutritional, microbiological, or safety perspective (*1*). Carved beef has a shelf life that varies between 3 and 5 days when kept at 4°C (*2*). *Pseudomonas, Enterobacteria* and lactic acid bacteria are responsible for meat deterioration (*3*). Depending on the region, over one quarter to one third of the world wide production of meat is lost every year because of this deterioration (*4*). Moreover, meat and meat products can be contaminated by pathogenic bacteria such as *Listeria monocytogenes*, *Salmonella* Typhimurium, *Escherichia coli* O157:H7 and *Yersinia enterocolitica*, responsible of foodborne illness and deaths (*5*). Controlling the numbers and growth of pathogenic bacteria therefore remains an important objective for sectors of the meat production industry.

Spices are rich in phenolic compounds, such as flavonoid and phenolic acids, which exhibit a wide range of biological effects, including antioxidant (*6*) and antimicrobial (*7,8*) properties. The antimicrobial activity of phenolic antioxidants has been studied in meat and its products (*9*). Many spices and herb and their extracts possess antimicrobial activity, which is almost invariably due to their essential oil fraction (*10*). For example, essential oil fractions of oregano and pimento are efficient against various foodborne bacteria such as *Salmonella* (*11-13*). Recent studies have also shown that essential oils of oregano and pimento are among the most active in this respect against strains of *E. coli* including *E. coli* O157:H7 (*14-17*). Spices extracts from oregano, sage, rosemary, thyme and pimento, are also reported to possess antioxidant properties comparable or greater than BHA and BHT (*18-21*). Antioxidant properties of these extracts have been mostly attributed to phenolic compounds present in their essential oil fractions (*22*).

In order to control food contamination and quality loss, edible coating or biodegradable packaging has been recently introduced in food processing. Several applications for meat, poultry, and seafoods have been reviewed by Gennadios et al. (*23*) with particular emphasis on the reduction of lipid oxidation, weight loss, moisture loss, microbial load, and volatile flavor

loss. The coating can serve as a carrier for antimicrobial compounds and/or antioxidant compounds in order to maintain high concentrations of preservatives on the food surfaces. Selected antimicrobial and antioxidant compounds including organic acids or essential oils have been incorporated in edible films and coating in order to control bacterial growth during storage of meat (*24,25*) and shrimps (*26*). Immobilization of organic acids in edible coatings based on calcium alginate gel or whey protein has also been used to control *Listeria monocytogenes* on beef tissue (*27,28*).

According of Papadokostaki et al. (*29*) a relationship between polymer structure and the transport of active molecules has been reported. From recent studies done in our laboratories, heat and gamma irradiation have been shown to produce cross-linking and improved physical and functional properties of edible film coatings via the production of cross-links between protein molecules (*30-32*). It can be hypothesized that structure modification induced by gamma irradiation could increase the capacity of edible and cross-linked films to control the release of immobilized active compounds. Also, the application of polymers on solid or semi-solid foods could increase the antimicrobial and antioxidant efficiency by maintaining high concentrations of active molecules on the food surface, where microbial growth occurs mostly. The objectives of the present study were to 1) evaluate the ability of milk protein-based edible films containing 1.0% essential oils of oregano, pimento or a oregano-pimento mixture in a ratio 1:1 (w/w) to control *Pseudomonas* sp. and *E. coli* O157:H7 growth on surface-inoculated beef muscle ; 2) determine the antioxidant properties of films and to determinate the level of 2-thiobarbituric acid (TBA) values production in beef muscle ; 3) evaluate the availability of phenolic compounds present in films during storage at 4°C.

Materials and Methods

Preparation of film

Protein based films were prepared with milk proteins according to a method developed in our laboratory (*33-35*). Calcium caseinate (93.0% w/w protein) and Whey Protein Isolate (WPI, 93.0% w/w protein) were provided by New Zealand Milk Product Inc. (Santa Rosa, CA, USA)

and by Davisco Foods International Inc. (Eden Prairies, MN , USA), respectively. Calcium caseinate and WPI were mixed in a ratio of 1:1 (w/w) and slowly solubilized at room temperature for 1 h in distilled water containing 5.0 % (w/v) glycerol (Mat laboratory, Beauport, QC, Canada) and 0.25 % (w/v) of carboxymethyl cellulose (CMC, low viscosity; Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) for a total protein concentration of 5.0 % (w/v) in the film forming solution. Carboxymethylcellulose (CMC) was added as protein stabilizer and glycerol as role of a plasticizer (*31*). The protein-based solution was treated by thermal treatment (90°C for 30 min), followed by gamma irradiation treatment at a dose of 32 kGy and at a dose rate of 22.928 kGy/h, using a ^{60}Co source UC-15A (MDS-Nordion International Inc., Kanata, ON, Canada) according to Vachon *et al.* (*32*). Heating WPI was essential for the formation of the intermolecular disulfide bonds. This process is necessary to obtain a flexible film with good mechanical properties (*32,36*). Irradiation of calcium caseinate was used to allow the formation of the intermolecular bityrosine bonds (*37*) in order to improve the functional and rheological properties of the film (*30*) and for immobilization of active compounds (*29*). Immediately after irradiation, four emulsions corresponding to four different experimental groups were prepared: without essential oils, oregano, pimento and pimento-oregano mixture. For each emulsion, modified starch (Purity gum BE, National Starch and Chemical Compagny, Bridgewater, NJ, USA) and L-α phosphatidylcholine (soya lecithin, 18% (w/w) purity, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) were mixed and solubilized at room temperature in distilled water for a final modified starch concentration of 0.5 % (w/v) and a final lecithin concentration of 0.2 % (w/v) in the film forming solution. Oregano and pimento oil (Cedarome Canada Inc, Brossard, QC, Canada) or a mixture of the two in a 1:1 (w/w) ratio were added to the starch-lecithin solutions for a final oil concentration of 1.0 % (w/v) in the film forming solution. The film forming solution was homogenized at room temperature for 2 min using a Homogenizator Ultra-Turrax TP18/1059 (Janke & Kunkel, Staufen, Germany) at 20000 rpm. Lecithin was essential for the formation of micellae and the presence of modified starch improved emulsion stability. Five (5) ml of each film formulation were placed into small petri dishes (50 x 11 mm) and dried under a laminar flow hood for 24 h at room temperature and 40-50% relative humidity (RH).

Preparation of inocula

Three *Pseudomonas* strains (*Pseudomonas putida* CRDA V372, *Pseudomonas fragi* CRDA V378 and *Pseudomonas fluorescens* V491) and *E. coli* O157:H7 strain (EDL933) were provided by Agriculture and Agri-Food Canada (Lacombe Research Station, Lacombe, AB, Canada) and CRDA (Food Research and development Centre, St-Hyacinthe, QC, Canada). Strains were individually subcultured (1.0% (v/v)) in Brain Heart Infusion (BHI, Difco Laboratories, Detroit, MI, USA) at 35 ± 1°C from the stock cultures maintained at –80 ± 1°C in BHI containing 20% glycerol. Prior to the experiment, 1 ml of each culture was incubated through two incubations of 24 h at 35 ± 1°C in BHI broth (9 ml). After 48 h, the 10 ml *Pseudomonas* cultures were mixed and placed into a 50-ml tube. The *E. coli* O157:H7 and mixed *Pseudomonas* sp. culture were centrifuged at 1300 x g for 15 min and washed with sterile NaCl 0.85% (w/v) and then resuspended in the same NaCl solution.

Samples preparation

Beef from the semimembranosus (inside round) (15 kg) was purchased at a local grocery store (IGA, Laval, QC) and transported to INRS-Institut Armand Frappier-Canadian Irradiation Center (CIC) under refrigerated conditions in an ice filled cooler container. Meat was cut out into 225 pieces (150 pieces for microbiological analysis and 75 pieces for biochemical analysis) of equal thickness (7.5 mm) and diameter (47 mm) using a pastry cutter. The 225 beef muscle pieces (10 pieces of beef/package) were placed in 0.5 mm metallized polyester/2 mm EVA copolymer sterile bag (305 mm x 210 mm, Winpak, St-Léonard, QC, Canada) and sealed under vacuum. The packages were stored overnight at – 80 ± 1°C until sterilization treatment by irradiation. The irradiation treatment was done at a dose of 25 kGy and a dose rate of 22.928 kGy/h, using a ^{60}Co source UC-15A (MDS-Nordion International Inc., Kanata, ON, Canada). After irradiation, 150 pieces of beef muscle were inoculated with either *E. coli* O157:H7 or the *Pseudomonas* sp. mixture to obtain a final concentration of 10^3 CFU/cm^2 of meat. Each beef muscle sample was inoculated with a volume of 50 µl which was spread out over all upper surface of meat. The 75 pieces of meat for the biochemical analysis were not inoculated. Samples of meat was divided into five separate groups: i) the control group without film (**M**); ii)

a protein-based film coating without essential oils (**C**); iii) a protein-based film coating containing oregano oil (**O**); iv) a protein-based film coating containing pimento oil (**P**); v) a protein-based film coating containing oregano and oil-pimento oil in a 1:1 (w/w) ratio (**P/O**). Meat samples of each group were placed individually into sterile petri dishes (50 x 11 mm) and except for the control, all samples were covered on either side with one of the corresponding films. Petri dishes were sealed hermetically and stored at 4 ± 1°C and samples were tested periodically (days 4, 5, 6 and 7) for microbiological and biochemical analysis. Day 1 corresponded to the day of samples preparation.

Bacterial enumeration

Each meat sample was homogenized for 2 min in 77 ml of sterile peptone water (0.1% (w/v), Difco Laboratories, Detroit, MI) using a Lab-blender 400 stomacher (Seward medical, London, UK). From this homogenate, serial dilutions were prepared, and appropriate ones were spread plated on sterile petri plates containing McConkey and BHI agar (Difco Laboratories, Detroit, MI, USA) for *E. coli* O157:H7 and *Pseudomonas* sp., respectively. Then, BHI plates were incubated for 24 h and McConkey plates were incubated for 48 h at 35 ± 1°C, and results were expressed as log CFU per cm^2 of meat.

Lipid oxidation of meat

Lipid oxidation potential of meat was evaluated by the determination of thiobarbituric acid reactive substances (TBARS). A 10 g portion of each meat sample was blended with 50 ml of distilled-deionized water and 10 ml of trichloroacetic acid (15%, final concentration ; TCA, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) using a Lab-blender 400 stomacher (Laboratory Equipment, London, UK) for 2 min. The homogenate was centrifuged at 5900g for 5 min and the supernatant was filtered through a Whatman 0.45 µm filter paper (Whatman International Ltd., Maidstone, UK). To 8 ml of the filtrate, 2 ml of 0.06 M thiobarbituric acid (TBA, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) was added. The solution was mixed using a vortex for 15 s and placed into a 80°C water bath for 90 min and then cooled on ice. Absorbance was read at 520 nm on a DMS 200 spectrophotometer (Varian Techtron Pty. Ltd,

Mulgrade, Australia), and concentration of malonaldehyde was calculated based on a standard curve obtained using serial dilutions of a solution of 1,1,3,3-tetramethoxypropane (97% (v/v)) TMP; Sigma-Aldrich Canada Ltd, Oakville, ON, Canada). The TBA values were expressed as mg of malonaldehyde per kg of meat.

Phenolic compounds availability

To evaluate the availability of phenolic compounds in the packaging, film samples (1 g each) were cut using a bistoury; the obtained thin strips were placed in 50 ml of acetone solution and kept under agitation with a magnetic stir bar at room temperature for 30 min. Then the extract was filtered through a Whatman 0.45 µm filter paper (Whatman International Ltd., Maidstone, UK). Total phenolic compound content in each film extract was determined according to the Folin-Ciocalteu procedure (*38*), and results were expressed as mg of gallic acid equivalent (GAE) per mL of extract.

Antioxidant properties of films

Antioxidant properties were evaluated by their antiradical capacity following a modified procedure of the DPD (N,N-diethyl-p-phenylenediamine) colorimetric method (*39*), as reported by Le Tien *et al.* (*40*). A piece of film weighing 25 mg was added in a cell containing 3 mL of 0.15 M NaCl and subjected to an electrolysis for 1 min (continuous current, 400 Volts, 10 mA) using a generator (model 1000/500, Bio-Rad, Mississauga, ON, Canada). After electrolysis, 200 µL were sampled and added to 2 mL of DPD solution (25 mg/ml ; Sigma-Aldrich Canada Ltd, Oakville, ON, Canada). The oxidative species released during the electrolysis reacted instantaneously with DPD, producing a red coloration and the intensity of the color was measured at 515 nm using a DMS 200 spectrophotometer (Varian Techtron Pty. Ltd, Mulgrade, Australia). The colorimetric reaction was calibrated with ascorbic acid. The antioxidant capacity describes the film capacity to inhibit the accumulation of oxidative species (able oxidize DPD) such as free radicals and consequently to prevent the appearance of the red coloration at 515 nm. The scavenging percentage is calculated according to the following equation:

Scavenging (%) = $100-[(OD_{sample}/OD_{control})x100]$

where $OD_{control}$ represents the OD of electrolyzed solution in the absence of film. In fact, OD is directly related to the degree of oxidation of DPD reagent by the oxidative species. Thus, films able to reduce completely the level of reactive oxidative species will have a 100% scavenging capacity.

Experimental design and statistical analysis

Experiments for microbiological analysis were done using a 3 x 5 x 2 x 5 factorial design (3 replicates, 5 treatments (control, film without essential oils, oregano, pimento and oregano-pimento mix of essential oils), 2 bacteria (*Pseudomonas* sp. and *E. coli*) and 5 storage periods (1, 4, 5, 6 and 7 days)).

Biochemical analysis were done using a 3 x 5 x 5 factorial design (3 replicates, 5 treatments (control, film without essential oils, oregano, pimento and oregano-pimento mix of essential oils), and 5 storage periods (1, 4, 5, 6 and 7 days)).

Analysis of variance and Duncan's multiple-range tests were employed to perform statistical analysis on all results. Differences between means were considered significant when $P \leq 0.05$. Stat-Packets Statistical Analysis software (Walonick Associates, Inc. MN. USA) was used for the analysis. For each measurement, three samples in each repetition were tested.

Results and Discussion

Microbiology

Effect of films on *Pseudomonas* sp. and *E. coli* O157:H7 counts during storage at 4°C are presented in **Tables 1** and **2**. Results showed that the use of film containing essential oils reduced significantly ($P \leq 0.05$) the microorganism level in meat as compared to meat samples coated with **C** film and **M** samples during seven days of storage. Moreover, results showed that **O** film was the most effective against the growth of both bacteria. **P/O** based films were significantly ($P \leq 0.05$) more efficient against the growth of *E. coli* O157:H7 than **P** based film. However **P/O**

and **P** based films have a similar effectiveness against the growth of *Pseudomonas* sp. Results also showed that the application of **C** films enhanced the growth of both bacteria on meat.

Results on *Pseudomonas* content presented in **Table 1** showed that after 4 days of storage, the application of **O**, **P** and **P/O** based films showed a significant 0.50, 0.25 and 0.60-log decrease ($P \leq 0.05$), respectively. At day 4, *Pseudomonas* content was respectively 2.53 ± 0.21, 2.77 ± 0.28 and 2.45 ± 0.12 log CFU/cm^2 in meat coated with **O**, **P** and **P/O** based films. However, the *Pseudomonas* content was stable in **M** and **C** meat samples during the 4 first days showing a level of 3.03 ± 0.30 and 3.09 ± 0.29 log CFU/cm^2, respectively. Also, the level of microorganisms in meat coated with **O** based film was stable until the end of the storage. Between day 4 and 7, the content of *Pseudomonas* increased significantly ($P \leq 0.05$) in **M**, **C**, **P** and **P/O** samples. At the end of storage the content of *Pseudomonas* in **M**, **C**, **P** and **P/O** samples was respectively 3.33 ± 0.11, 3.67 ± 0.10, 3.04 ± 0.14 and 2.87 ± 0.12 log CFU/cm^2 as compared to 2.38 ± 0.09 log CFU/cm^2 in **O** samples. These results showed a 0.95-log reduction in **O** samples after 7 days of storage as compared to 0.30-log and 0.45-log reduction in **P** and **P/O** samples, respectively.

The determination of *E. coli* O157:H7 counts is presented in **Table 2**. Results showed that the content of *E. coli* O157:H7 was stable in **M** and **P** samples during the 4 first days of storage showing a level of 2.80 ± 0.18 and 2.73 ± 0.21 log CFU/cm^2, respectively. During the same period, a significant 0.68 log increase ($P \leq 0.05$) was noted in **C** samples to reach a value of 3.50 ± 0.24 log CFU/cm^2 while a significant decrease ($P \leq 0.05$) of 0.40 and 0.38-log was respectively observed in **O** and **P/O** samples to reach a respective value of 2.40 ± 0.02 and 2.42 ± 0.09 log CFU/cm^2. Between day 4 and 7, the content of *E. coli* O157:H7 in **O** samples was stable while a significant increase ($P \leq 0.05$) was observed in all other samples. The *E. coli* O157:H7 counts in **M**, **C**, **P** and **P/O** samples at day 7 was respectively 3.52 ± 0.30, 5.02 ± 0.40, 3.58 ± 0.21 and 2.92 ± 0.32 log CFU/cm^2. These results showed that **O** and **P/O** films were able to reduce by 1.12 and 0.60 log, respectively, the content of *E. coli* O157:H7 during the seven days of storage. However **P** film did not have any effect ($P > 0.05$) on the content of *E. coli* O157:H7 during storage. At the end of the storage period, the level of *E. coli* O157:H7 in **C** samples was significantly higher ($P \leq 0.05$) compared to all the other samples.

Our study showed that the incorporation of essential oils into the cross-linked coating film formulation significantly decreased ($P \leq 0.05$) the level of *E. coli* O157:H7 and *Pseudomonas* spp. contents on meat samples. However, our data suggest also that both bacteria seem to use milk protein-based film in absence of essential oils as a substrate to sustain their growth. Bagamboula et al. (*41*) have observed that, when oregano and pimento extracts were added at a concentration of 1 % (w/v) in MH agar, these spice extracts inhibited the growth of five strains such as *E. coli* LMG 8223. Also, Burt and Reinders (*16*) have shown *in vitro* results in agreement with those obtained in our study where oregano essential oil is more active against strain of *E. coli* O157:H7 than pimento essential oil. In addition, our results showed that an oil concentration of 1% (w/v) was effective against *Pseudomonas* sp. and *E. coli* O157:H7 growth. Bagamboula et al. (*41*) showed that the minimal inhibitory concentration (MIC) of oregano was 0.5% (w/v) for five bacterial strains (*Shigella sonnei* strain 6, *S. sonnei* CIP 82.49, *S. flexneri* strain1, *S. flexneri* CIP 82.48 and *E. coli* LMG 8223). These results are in agreement with those obtained by Paster et al. (*11*) who found that below 0.25%, the oregano essential oil was ineffective or very slightly effective against five tested bacteria (*Pseudomonas aeruginosa, Salmonella* Typhimurium, *Staphylococcus aureus*, *Campylobacter jejuni* and *Clostridium sporogenes*). However, Gangrade et al. (*42*) examined the antimicrobial properties of the different essential oils, in their pure state at four dilutions (1:10, 1:100, 1:1000 and 1:10000) against *E. coli*. They found that essential oils had a significant inhibitory effect ($P \leq 0.05$) against bacterial strain for all dilutions tested.

Chemical analysis of essential oils provided by the manufacturer has indicated that the major compounds identified in oregano essential oil are phenolic monoterpenes *i.e.*, carvacrol (61.75%) and thymol (4.32%), and nonoterpenic hydrocarbons such as *p*-cimene (10.73%), γ-terpinene (7.42%), caryophyllene (3.20%) and myrcene (1.94%). Tested pimento essential oil is rich in phenolic monoterpenes *i.e.*, eugenol (74.40%) and methyl eugenol (5.98%), and monoterpenic hydrocarbon caryophyllene (6.81%).

It is known that phenolic compounds such as carvacrol, thymol, and eugenol exhibit a major antimicrobial effect (*43-50*). According to Farag et al. (*51*), the antimicrobial activities of essential oils are predominantly related to their main components. Diluted by 100-fold, the

essentials oils inhibited the tested meat spoilage organisms, and a relationship between the inhibitory effect of essentials oils and the presence of eugenol was found (*52*). Kim et al. (*48*) found that eugenol possesses a potent inhibitory activity against five bacterial strains including *E. coli* O157:H7 and noted a dose-related increase of the inhibition zone against all tested microorganisms. The antimicrobial potential of oregano essential oils is related to their high phenolic contents, particularly carvacrol and thymol (*12*). According to Helander et al. (*13*), carvacrol and thymol are able to inhibit *E. coli* O157:H7 at a concentration between 1-3 mM by disintegration of the outer membrane and by the release of outer membrane-associated materials from the cells to the external medium. Moreover according to these authors, these components decrease the intracellular ATP pool of *E. coli* O157:H7 and also increase extracellular ATP pool, indicating a disruptive action on the cytoplasmic membrane. Besides, substances with similar properties, such as eugenol and carvacrol, may have additive or cummulative effects. Güllüce et al. (*53*) have reported that the combination of these compounds showed a synergistic antimicrobial effect on microorganisms. The synergistic effect of these chemical compounds could also account for the activity of the essential oils (*54*). Our results, however, failed to demonstrate a synergistic antimicrobial effect of oregano oil in combination with pimento oil.

Lipid oxidation

Results of the TBARS concentration in meat, which represents the malonaldehyde concentration, are presented in **Table 3.** The initial concentration of TBARS was 1.19 ± 0.16 mg/kg at day one of storage. The concentration was stable at in **M** samples during 5 days of storage, followed by a significant increase ($P \leq 0.05$) to reach a value of 1.77 ± 0.05 mg/kg at day 6. At day 4, no significant difference ($P > 0.05$) was observed between all treated samples and **M** samples, except for **O** samples where the TBARS content was significantly ($P \leq 0.05$) higher with a value of 1.94 ± 0.15 mg/kg. The TBARS value in **C**, **P** and **P/O** samples was respectively 1.54 ± 0.19, 1.64 ± 0.21 and 1.65 ± 0.21 mg/kg at day 4 as compared to 1.26 ± 0.37 mg/kg in M samples. However, the TBARS concentration in **O** samples remained stable from day 4 to day 7. During the same period of time, TBARS content increased significantly ($P \leq 0.05$) in **M**, **P** and **P/O** samples. TBARS content in **C** samples increased significantly ($P \leq 0.05$) between day 4 and 5 to reach a value of 1.98 ± 0.15 mg/kg, followed by a stabilization until the

end of storage. At day 7, no significant difference ($P > 0.05$) was noted between **M**, **C** and **O** samples. Moreover, TBARS values were significantly ($P \leq 0.05$) higher in **P** (2.81 ± 0.30 mg/kg) and **P/O** (2.67 ± 0.28 mg/kg) samples than in **M** (1.60 ± 0.24 mg/kg), **C** (2.02 ± 0.12 mg/kg) and **O** (1.76 ± 0.18 mg/kg) samples.

These results showed that the incorporation of essential oils in the films did not improve the protection of the meat samples against lipid oxidation. **P** and **P/O** based films increased significantly ($P \leq 0.05$) lipid oxidation in beef muscle samples, in spite the fact that these oils contained compounds which were identified as potential antioxidants. These results seem apparently inconsistent with previous studies. Lee and Anh (*55*) showed that phenolic compounds, such as gallate and sesamol, were effective antioxidants in reducing thiobarbituric acid reactive substances in turkey breast during 5 days of storage. Nam et al., (*56*) observed a similar trend in ground beef storage at 4 °C during 7 days. Phenolic compounds such as quercetin were also effective in preventing lipid oxidation in both raw and cooked turkey during 7 days of storage (*57*). Nevertheless, it is known that pimento oil is rich in phenolic compounds such as eugenol and these compounds are unstable. The degradation pathway of some phenolic compounds could generate phenolic aldehydes. These aldehydes could provoke a similar reaction to malonaldehyde reaction when lipid oxidation of meat was evaluated by the determination of thiobarbituric reactive substances. It would probably result in an increase of TBARS values which would mask the antioxidant effect of essential oils. This hypothesis could explain also the difference between our results and those presented in the literature. Indeed, all previous authors used phenolic compounds at concentrations of 0.01% (w/v) in meat homogenates as compared to 1% in our study. The results indicated that **C** and **O** based films stablized lipid oxidation in beef muscle samples. The results also showed that the ability of oregano oil to inhibit lipid peroxidation is more important than pimento oil which is the richest in phenolic content. A similar trend was observed by Martinez-Tome et al. (*58*). Results obtained with **C** film are probably related to the presence of some active compounds showing scavenging properties in film. Effectively, according to Le Tien et al. (*40*) milk proteins and CMC present in film formulations possess good antiradical properties. These authors showed that whey protein and CMC were the most effective compounds.

Availability of phenolic compounds

The availability of total phenolic compounds in **O**, **P** and **P/O** based films was determined during the whole storage period at 4 °C (**Table 4**). Initial concentration of phenolic compounds was 2.08 ± 0.05, 4.46 ± 0.07 and 2.92 ± 0.12 mg/g in **O**, **P** and **P/O** films, respectively. Between day 1 and 4, a significant increase ($P \leq 0.05$) of phenolic compounds availability was noted in all films evaluated. At day 4, total phenolic concentrations were 7.02 ± 0.23, 11.90 ± 0.34 and 10.22 ± 0.50 mg/g in **O**, **P** and **P/O** based films, respectively. From day 4 to 7 a significant decrease ($P \leq 0.05$) of the phenolic compounds availability was observed in **P** and **P/O** based film to reach a respective level of 10.39 ± 0.28 and 7.53 ± 0.71 mg/g at day 7. However, during the same period of time, the phenolic compounds availability in **O** based films remained stable until the end of the storage time with a value of 6.60 ± 0.62 mg/g at day 7. The results also indicated that at day 1, the phenolic compounds availability in **P** based films is twice (4.46 ± 0.07 mg/g versus 2.08 ± 0.05 mg/g) that of **O** based films. However, the average release rate of phenolic compounds between day 1 and 4 are respectively 1.64, 2.48 and 2.43 mg/g/day for **O**, **P** and **P/O** based films. At day 4 and 7, the phenolic compounds availability in the **P** based films is respectively 1.70 (11.90 ± 0.34 mg/g versus 7.02 ± 0.23 mg/g) and 1.57 (10.39 ± 0.28 mg/g versus 6.60 ± 0.62 mg/g) times higher than the phenolic compounds availability in the **O** based films. According to these results, it seems that the phenolic compounds migration in oregano is higher than in pimento based films. Chemical analysis of essential oils provided by the manufacturer has shown that tested pimento and oregano essential oils have, respectively, an average concentration of phenolic monoterpenes of 80 and 68% (w/w). The major phenolic monoterpenes of these essentials oils (eugenol and methyl eugenol in pimento; and carvacrol and thymol in oregano) have comparable molecular weights. On the other hand, the hydrophobicity of these compounds, which is related to their structure, could have an influence on their migration (*59, 60*). According to Zhao and Singh (*61*), eugenol is a highly hydrophobic compound. Indeed, the increase of the phenolic compounds availability in all films at day 4 is probably due to the increase of film hydration obtained in presence of meat, making easier the extraction of phenolic compounds. This hypothesis is consistent with the fact that the high level of film hydration could increase the diffusion rate of phenolic compounds from the film to the meat, and could consequently decrease the phenolic compounds availability in the films.

Antiradical properties of films

The antiradical properties of films are presented in **Table 5**. The antiradical properties of **C**, **O**, **P** and **P/O** based films were respectively 58.78 ± 3.45, 70.60 ± 5.56, 132.46 ± 11.33 and 111.22 ± 9.21 USP/g at day 1. The antiradical properties increased significantly ($P \leq 0.05$) in **C** and **O** based films to reach a respective value of 80.22 ± 7.64 and 89.33 ± 8.92 USP/g after 4 days of storage, followed by a stablization and a respective value of 74.98 ± 7.51 and 84.37 ± 8.59 USP/g was reached at day 7. On the other hand, the antiradical properties remained stable in **P** and **P/O** based films from 1 day to 6 days of storage, followed by a significant decrease ($P \leq 0.05$) at day 7 to reach a respective value of 109.37 ± 10.42 and 98.77 ± 9.01 USP/g. The antiradical properties reported on the basis of phenolic compounds availability in films are presented **Table 6**. The antiradical properties of **O**, **P** and **P/O** based films were respectively 33.94 ± 2.67, 29.70 ± 2.54, and 38.09 ± 3.15 USP/mg at day 1. The antiradical properties decreased significantly ($P \leq 0.05$) in and **O, P** and **P/O** based films to reach a respective value of 12.73 ± 1.27, 10.53 ± 0.96 and 11.23 ± 0.99 USP/mg at day 4, followed by a stablization and a respective value of 12.78 ± 1.30, 10.53 ± 1.00 and 13.12 ± 1.20 USP/mg was reached at day 7. This result suggests a release of phenolic compounds between day 4 and 7.

The results of **Table 5** showed that the pimento essential oils addition permitted a significant increase ($P \leq 0.05$) of the antiradical properties. On the other hand, no significant ($P > 0.05$) difference was observed between **C** and **O** during the whole storage period. According to Radonic and Milos (*62*), the antiradical properties of essential oils were related to their chemical composition of free volatile compounds. Results showed that antiradical properties of **O** based films was significantly ($P \leq 0.05$) lower than **P** and **P/O** based films although oregano oil contained compounds with higher antiradical properties than those contained in pimento oil (*58*). However, **Table 6** showed no significant difference ($P > 0.05$) between the **O** and **P** based films during the whole storage time. In fact, the higher antiradical properties of pimento as compared to oregano oils could be explain by the higher concentration of phenolic compounds concentration present in them. According to Teisseidre and Waterhouse (*63*), antiradical activity of pimento oil was essentially due to the presence of eugenol. Bostsoglou et al. (*64*) showed that oregano oil increased the oxidative stability of breast and thigh turkey meat during frozen

storage. A high radical scavenging property has been demonstrated for the main components of oregano essential oil (cavacrol, γ-terpinene, thymol, *p*-cymene) (*65,66*). However, other compounds could contribute to the antiradical properties of oregano oil. Indeed, Lagouri and Boskou (*67*) showed that tocopherol, alpha-, beta, gamma- and delta-, were found to be present in all tested species of oregano. According to the same authors, total tocopherol content ranged from 288 ppm to 672 ppm and these compounds have powerful antioxidant properties. However, it is important to stress that a high antiradical activity protects not only the meat, but also the bacteria by reacting with the free radicals that would otherwise react with the bacteria. Chiasson (*68*) showed that the bactericidal action of carvacrol against *E. coli* ATCC 25922 in ground beef was eliminated or reduced with the addition of another compound with high antiradical properties, like ascorbic acid.

In summary, the incorporation of essential oils into milk protein-based edible film applied onto muscle meat help to reduce microbial load and increase antioxidative activity, during 7 days of storage. These results support also the hypothesis that the inhibition of *E. coli* O157:H7 and *Pseudomonas* sp. growth by essential oils depends on the nature of phenolic compounds. The phenolic compounds concentration could also play also an important role in the antimicrobial and antioxidant activities that they confer to essential oils used in this study. Oregano based films exhibited the most effective antimicrobial property, whereas pimento based films presented the highest antioxidant activity. Also, the films allow a progressive release of phenolic compounds during the storage. After 7 days the availability of the phenolic compounds in films being always significant, the films remain effective. The use of edible film containing essential oils as a preservation method of meat is promising. However, the formulation and the mechanical properties must be improved in order to control the moisture content of the film and the diffusion rate of essential oil from the film to the meat surface during storage. Other works are in hand in order to improve the physicochemical properties of films.

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Table 1. Effect of films composition on *Pseudomonas* sp. during storage at 4°C.

Samples[a]	Log CFU/cm[2b, c]				
	Day 1	**Day 4**	**Day 5**	**Day 6**	**Day 7**
M	$3.02 \pm 0.20^{a}a$	$3.03 \pm 0.30^{a}a$	$3.20 \pm 0.19^{a}ab$	$3.55 \pm 0.22^{a}c$	$3.33 \pm 0.11^{a}b$
C	^{a}a	$3.09 \pm 0.23^{a}a$	$3.57 \pm 0.27^{b}b$	$3.62 \pm 0.13^{a}b$	$3.67 \pm 0.10^{b}b$
O	^{a}a	$2.53 \pm 0.21^{bc}b$	$2.52 \pm 0.16^{c}b$	$2.44 \pm 0.11^{b}b$	$2.38 \pm 0.09^{c}b$
P	^{a}a	$2.77 \pm 0.28^{dc}b$	$2.96 \pm 0.34^{ad}ab$	$2.93 \pm 0.23^{c}ab$	$3.04 \pm 0.14^{d}a$
P/O	^{a}a	$2.45 \pm 0.12^{b}b$	$2.91 \pm 0.27^{d}ac$	$2.78 \pm 0.17^{c}c$	$2.87 \pm 0.12^{c}ac$

[a]**M**: meat without film; **C**: films without essential oil; **O**: films containing oregano essential oil; **P**: films containing pimento essential oil; **P/O**: films containing oregano and pimento essential oils.

[b]Means in the same column bearing the same superscript letters are not significantly different ($p > 0.05$).

[c]Means in the same row bearing the same lowercase letter are not significantly different ($p > 0.05$).

Table 2. Effect of Films composition on *E. coli* O157:H7 during storage at 4°C.

Samples[a]	Log CFU/cm^{2}[b, c]				
	Day 1	**Day 4**	**Day 5**	**Day 6**	**Day 7**
M	2.82 ± 0.26 ^{a}a	2.80 ± 0.18 ^{a}a	3.03 ± 0.33 ^{a}a	3.56 ± 0.20 ^{a}b	3.52 ± 0.30 ^{a}b
C	^{a}a	3.50 ± 0.24 ^{b}b	4.29 ± 0.26 ^{b}c	4.77 ± 0.32 ^{b}d	5.02 ± 0.40 ^{b}d
O	^{a}a	2.40 ± 0.02 ^{c}b	2.44 ± 0.11 ^{c}b	2.40 ± 0.10 ^{c}b	2.40 ± 0.13 ^{c}b
P	^{a}a	2.73 ± 0.21 ^{a}a	2.72 ± 0.22 ^{d}a	3.47 ± 0.36 ^{a}b	3.58 ± 0.21 ^{a}b
P/O	^{a}ad	2.42 ± 0.09 ^{c}b	2.52 ± 0.16 ^{c}bc	2.66 ± 0.37 ^{c}ac	2.92 ± 0.32 ^{d}d

[a]**M**: meat without film; **C**: films without essential oil; **O**: films containing oregano essential oil; **P**: films containing pimento essential oil; **P/O**: films containing oregano and pimento essential oils.

[b]Means in the same column bearing the same superscript letters are not significantly different ($p > 0.05$).

[c]Means in the same row bearing the same lowercase letter are not significantly different ($p > 0.05$).

Table 3. Concentration of TBARS in meat samples with films and without film during storage at 4°C.

Samples[a]	Concentration of TBARS (mg malonaldehyde/kg meat)[b, c]				
	Day 1	**Day 4**	**Day 5**	**Day 6**	**Day 7**
M	1.19 ± 0.16 ^{a}a	1.26 ± 0.37 ^{a}a	1.18 ± 0.10 ^{a}a	1.77 ± 0.05 ^{a}b	1.60 ± 0.24 ^{a}b
C	^{a}a	1.54 ± 0.19 ^{ab}ab	1.98 ± 0.15 ^{b}c	1.92 ± 0.12 ^{ab}c	2.02 ± 0.12 ^{a}c
O	^{a}a	1.94 ± 0.15 ^{b}b	1.93 ± 0.09 ^{b}b	2.04 ± 0.20 ^{ab}b	1.76 ± 0.18 ^{a}b
P	^{a}a	1.64 ± 0.21 ^{ab}ab	2.16 ± 0.16 ^{b}c	2.57 ± 0.21^{c}cd	2.81 ± 0.30 ^{b}d
P/O	^{a}a	1.65 ± 0.21 ^{ab}ab	2.18 ± 0.28 ^{b}bc	2.32 ± 0.30 ^{bc}c	2.67 ± 0.28 ^{b}c

[a]**M**: meat without film; **C**: films without essential oil; **O**: films containing oregano essential oil; **P**: films containing pimento essential oil; **P/O**: films containing oregano and pimento essential oils.

[b]Means in the same column bearing the same superscript letters are not significantly different ($p > 0.05$).

[c]Means in the same row bearing the same lowercase letter are not significantly different ($p > 0.05$).

Table 4. Availability of total phenolic compounds in films containing essential oils during storage at 4°C.

Films[a]	Total phenolic compounds (mg EAG/g film)[b, c]						
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
O	2.08 ± 0.05 [a]a	5.31 ± 0.37 [a]b	6.02 ± 0.91 [a]bc	7.02 ± 0.23 [a]c	6.39 ± 0.64 [a]bc	6.21 ± 0.52 [a]bc	6.60 ± 0.62 [a]c
P	4.46 ± 0.07 [b]a	10.02 ± 1.08 [b]bc	11.29 ± 1.01 [b]cd	11.90 ± 0.34 [b]d	10.39 ± 0.44 [b]bc	9.39 ± 0.77 [b]b	10.39 ± 0.28 [b]bc
P/O	2.92 ± 0.12 [c]a	6.42 ± 0.66 [a]b	8.32 ± 0.67 [c]c	10.22 ± 0.50 [c]d	9.66 ± 0.94 [b]d	7.27 ± 0.71 [a]bc	7.53 ± 0.71 [a]bc

[a]**O**: films containing oregano essential oil; **P**: films containing pimento essential oil; **P/O**: films containing oregano and pimento essential oils.

[b]Means in the same column bearing the same superscript letters are not significantly different ($p > 0.05$).

[c]Means in the same row bearing the same lowercase letter are not significantly different ($p > 0.05$).

Table 5. Antiradical properties of the different films during period storage at 4°C.

Films[a]	Antiradical properties (U.S.P./g film)[b, c]			
	Day 1	**Day 4**	**Day 5**	**Day 7**
C	58.78 ± 3.45 [a]a	80.22 ± 7.64 [a]b	80.20 ± 8.13 [a]b	74.98 ± 7.51 [a]b
O	70.60 ± 5.56 [a]a	89.33 ± 8.92 [ab]b	88.37 ± 8.78 [a]b	84.37 ± 8.59 [a]ab
P	132.46 ± 11.33 [b]a	125.28 ± 11.48 [c]a	128.11 ± 12.34 [b]a	109.37 ± 10.42 [b]b
P/O	111.22 ± 9.21 [c]a	114.81 ± 10.07 [bc]a	121.45 ± 11.95 [b]a	98.77 ± 9.01 [ab]b

[a]**C**: control films without essential oil; **O**: films containing oregano essential oil; **P**: films containing pimento essential oil; **P/O**: films containing oregano and pimento essential oils.

[b]Means in the same column bearing the same superscript letters are not significantly different ($p > 0.05$).

[c]Means in the same row bearing the same lowercase letter are not significantly different ($p > 0.05$).

Table 6. Antiradical properties related to the available phenolic compounds in films containing essential oils during storage at 4°C.

Films[a]	Antiradical properties (U.S.P./mg phenolic compounds)[b, c]			
	Day 1	Day 4	Day 5	Day 7
O	33.94 ± 2.67 ^{ab}a	12.73 ± 1.27 ^{a}b	13.83 ± 1.37 ^{a}b	12.78 ± 1.30 ^{ab}b
P	29.70 ± 2.54 ^{a}a	10.53 ± 0.96 ^{a}b	12.33 ± 1.19 ^{a}b	10.53 ± 1.00 ^{a}b
P/O	38.09 ± 3.15 ^{b}a	11.23 ± 0.99 ^{a}b	12.57 ± 1.24 ^{a}b	13.12 ± 1.20 ^{b}b

[a]**O**: films containing oregano essential oil; **P**: films containing pimento essential oil ;

P/O: films containing oregano and pimento essential oils.

[b]Means in the same column bearing the same superscript letters are not significantly different ($p > 0.05$).

[c]Means in the same row bearing the same lowercase letter are not significantl

Article 2: Soumis et accepté dans Meat Science

Running title: *Pseudomonas putida* inhibition by essential oils

Antimicrobial effects of selected plant essential oils on the growth of a *Pseudomonas putida* strain isolated from meat

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Résumé

L'effet inhibiteur de 60 huiles essentielles différentes a été évalué sur *Pseudomonas putida*, une bactérie liée à l'altération de la viande. Les huiles essentielles ont été testées aux concentrations de 0.003 à 0.8 % (wt/vol) pour déterminer les concentrations minimales inhibitrices et maximales tolérées (CMI et CMT, respectivement) sur un milieu gélosé. Des 60 huiles testées, l'huile essentielle de Capitatus de Corydothymus était la plus active en ayant une CMI de 0.025% (wt/vol) et une CMT de 0.06% (wt/vol). Sept huiles essentielles (Cinnamomum cassia, *Origanum compactum, Origanum heracleoticum*, *Satureja hortensis*, *Satureja montana*, *Thymus vulgaris carvacroliferum*, *Thymus vulgaris thymoliferum*) ont montré une importante activité antimicrobienne contre *P. putida* avec une CMI de 0.05% (wt/vol) et une CMT de 0.013 à 0.025% (wt/vol). Dix autres huiles (*Cinnamomum verum* (leaf and bark), *Eugenia caryophyllus*, *Cymbopogon martinii* var. *motia*, *Cymbopogon nardus*, *Melaleuca linariifolia, Origanum majorana, Pimenta dioica*, *Thymus satureoides, Thymus serpyllum*) ont aussi montré une activité antimicrobienne élevée avec une CMI de 0.1 à 0.4% (wt/vol), alors que le restant d'huiles testées étaient apparement moins actifs en ayant une CMI $\geq$ 0.8% (wt/vol).

Abstract

The inhibitory effect of 60 different essential oils was evaluated on a *Pseudomonas putida* strain of meat origin, associated with meat spoilage. Essential oils were tested at concentrations from 0.003 to 0.8 % (wt/vol) to determine minimum inhibitory and maximal tolerated concentrations (MIC and MTC, respectively) using an agar medium culture. Of the 60 samples tested, *Corydothymus capitatus* essential oil was the most active showing a MIC of 0.025% and a MTC of 0.06%. Seven essential oils (*Cinnamomum cassia, Origanum compactum, Origanum heracleoticum*, *Satureja hortensis*, *Satureja montana*, *Thymus vulgaris carvacroliferum*, *Thymus vulgaris thymoliferum*) have shown a strong antimicrobial activity against *P. putida* with a MIC of 0.05% and a MTC ranging from 0.013 to 0.025%. Ten other oils (*Cinnamomum verum* (leaf and bark), *Eugenia caryophyllus*, *Cymbopogon martinii* var. *motia*, *Cymbopogon nardus*, *Melaleuca linariifolia, Origanum majorana, Pimenta dioica*, *Thymus satureoides, Thymus serpyllum*) showed a high antimicrobial activity showing a MIC ranging from 0.1 to 0.4%, while the remaining were less active showing a MIC $\geq$ 0.8%.

Keywords: Antimicrobial activity; Essential oils; *Pseudomonas putida*; Minimum inhibitory concentration; Maximal tolerated concentration

Introduction

The assurance of inventory and the shelf life of meat represent an important challenge for meat's industries. Several attempts were made to control the spoilage and pathogen bacteria in order to assure innocuity, wholesome, and nutritious food supply and to reduce foodborne outbreaks. The spoilage of refrigerated meat is caused in part by *Pseudomonas* species bacteria which are responsible for the off-odours, off-flavours, discoloration, gas production and slime production in the meat. Antimicrobial agents, including food preservatives have been used to inhibit foodborne bacteria and extend the shelf life of processed food. Many naturally occuring extracts like essential oils from edible and medicinal plants, herbs and spices have been shown to possess antimicrobial functions and could serve as a source for antimicrobial agents against food spoilage and pathogens (Bagamboula, Uyttendaele & Debevere., 2003; Conner, 1993; Conner & Beuchat, 1984; Dorman & Deans, 2000; Zaika, 1988). 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; Sivropoulou, Papanikolaou, Nikolanou, Kokkini, Lanaras & Arsenakis, 1996).

Essentail oils are the odorous, volatile products of an aromatic plant's secondary metabolism, normally formed in special cells or groups of cells, found in many leaves and stems. They are commonly concentrated in one particular region 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 flavoring 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). Several references on the antimicrobial activity of essential oils are available in the literature (Araujo et al., 2003; Burt & Reinders, 2003; Conner & Beuchat, 1984; Cox et al., 2000; Delaquis, Stanich, Girard & Mazza, 2002; Helander et al., 1998; Panizzi, Flamini, Coni & Morelli, 1993). The antimicrobial activity of essential oils is assigned to a number of small terpenoid and phenolic compounds, which also in pure form have been shown to exhibit antibacterial or antifungal activity (Conner, 1993; Didry, Dubreuil & Pinkas, 1993; Karapinar & Aktung, 1987). The antibacterial properties of these compounds are in part associated with their lipophilic character, leading to accumulation in membranes and to

subsequent membrane-associated events such as energy depletion (Conner, 1993). Chemical analysis of these oils have shown that the principal active compounds of these oils are principally carvacrol, thymol, citral, eugenol, 1-8 cineole, limonene, pinene, linalool and their precursors (Demetzos & Perdetzoglou, 2001; Juliano, Mattana & Usai, 2000; Lataoui & Tantaoui-Elaraki, 1994). However, there are often large differences in the reported antibacterial activity of oils from the same essence. The reasons of this variability can be due to the geographical sources, the harvesting seasons, the genotype, the climate, the drying and the distilled part of the plant which are significant factors influencing the chemical composition and relative proportions of the individual constituents in the essential oils of the plant (Arrebola, Navarro, Jiménez & Ocána. 1994; Juliano et al., 2000; McGimpsey & Douglas, 1994; Rhyu, 1979; Salgueiro et al., 1997; Venskutonis, 1996). Also, a number of essential oils constituents exhibit significant antimicrobial properties when tested separately (Kim et al., 1995; Lambert, Skandamis, Coote & Nychas, 2001; Suresh, Ingle & Vijayalakshmi, 1992). 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 & Tantaoui-Elaraki, 1994). For this reason, the present study was carried out using whole essential oils. Also, since *Pseudomonas putida,* one of the most important spoilage bacteria in meat, was seldom included in previous antimicrobial studies of essential oils, this strain was to evaluate the antibacterial activity of sixty standardized and commercially essential oils with known concentration of each active components. The minimum inhibitory concentration (MIC) and maximal tolerated concentration (MTC) of tested essential oils were determined using the agar dilution method.

Materials and Methods

Preparation of Pseudomonas strain

Pseudomonas putida strain (CRDAV 372) was originally isolated from fresh beef and was provided by CRDA (Food Research and development Centre, St-Hyacinthe, QC, Canada). The bacterium was grown in brain heart infusion (BHI, Becton Dickinson-Difco, Sparks, MD, USA) at 35 ± 1°C from the stock cultures maintained at –80 ± 1°C in BHI containing 10%

glycerol. One mL of stock culture was standardized through two successive 24 h growth cycles at 35 ± 1°C in 9 mL of BHI broth. After 48 h, 100 µl of the suspension (10^9 CFU/ml) were then inoculated in fresh BHI broth and incubated at 25 ± 1°C for 2 h to obtain a working culture at exponential phase containing approximately $2x10^7$ CFU/ml.

Antibacterial agents

Sixty essential oils were used to determine their MICs and MTCs. All samples were provided by Robert et Fils (Montréal, QC, Canada) and stored at 4°C before use. The commercial and scientific names of essential oils tested are presented in Table 1.

Essential oil dispersion in the culture medium

First, 60 suspensions were prepared with plant essential oils. The oils were dispersed at room temperature for 2 min using a homogenizator Ultra-Turrax TP18/1059 (Janke & Funkel, Staufen, Germany) at 20000 rpm in sterile 10% (wt/vol) modified starch (Food starch-modified, Purity gum Be, National Starch & Chemical Co, Boucherville, QC, Canada) solution to obtain a colloidal suspension (10%, wt/vol) (Oussalah, Caillet, Salmiéri, Saucier & Lacroix, 2004). The presence of modified starch improved suspension stability and could improve the antimicrobial properties (Burt & Reinders, 2003). The starch-oil suspensions were added to sterile molten BHI agar (Becton Dickinson-Difco, Sparks, MD, USA) maintained at 45 ± 1°C and strongly mixed for 2 min using a vortex. For each oil, nine final concentrations ranging from 0.003 % to 0.8 % (wt/vol) were obtained by adding various volumes from the 10% suspension. The molten agar (15 ml) containing essential oils were poured into sterile petri plates and left to solidify.

Determination of minimal inhibitory concentration (MIC) and maximal tolerated concentration (MTC)

The MIC and MTC of tested essential oils were determined using the agar dilution method (Robert-Dernuet, 1995). Petri plates of BHI agar containing various concentrations of essential oils were inoculated with *P. putida*. The solution of working culture ($2x10^7$ CFU/ml)

was diluted to obtain 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} in peptone water (0.1% wt/vol) and 40 µL of the diluted culture was spread on the surface of the solidified agar plates. The positive control consisted of BHI agar without essential oil, inoculated with the diluted medium culture. Uninoculated plates containing essential oils, served as negative control. Test and control plates were then incubated at 35 ± 1°C. Plates were evaluated for the presence or the absence of colonies after 48h of incubation. For each treatment, the absence of colonies on all plates tested was considered as an inhibitory effect. The lowest concentration of essential oil required to inhibit completly the growth of *P. putida* was designated as the MIC. The MTC was the maximal concentration of essential oil in which tested essential oils did not affect the bacterial growth.

Statistical analysis of data

Analysis of variance and Duncan's multiple-range tests were employed to analyze data collected. Differences between means were considered significant when $P \leq 0.05$. Stat-Packets Statistical Analysis software (Walonick Associates, Inc. MN. USA) was used for the bacterial enumeration for the determination of the MTC. This experiment was conducted twice with four plates for each essential oil at each concentration.

Results and Discussion

These results of minimum inhibitory and tolerated maximal concentrations of each essential oil are presented in Table 1. Among all oils tested, thirty eight essential oils reduced significantly the microorganism level ($p \leq 0.05$) at concentrations < 0.8% and twenty eight essential oils destroyed the bacterium completely. Results showed that the antimicrobial effect varied between oils, and concentrations from 0.006% to 0.4% were required to prevent detectable growth of *P. putida* while concentrations from 0.025% to 0.8% were necessary to destroy the bacterial growth completly. Of the 60 samples tested, *Corydothymus capitatus* (spanish oregano) was the most active with a MIC of 0.025% and a MTC of 0.006% and seven essential oils (*Cinnamomum cassia, Origanum compactum, Origanum heracleoticum, Satureja hortensis, Satureja montana, Thymus vulgaris carvacroliferum, Thymus vulgaris thymoliferum*) have shown a strong antimicrobial activity against *P. putida* with a MIC of 0.05% and a MTC

value ranging between 0.013 and 0.025% as presented in Figure 1. Five oils (*Cinnamomum verum* (leaf and bark), *Eugenia caryophyllus*, *Pimenta dioica*, *Thymus serpyllum*) showed a high antimicrobial activity against bacterium showing a MIC of 0.1 % and a MTC value ranging between 0.025 to 0.05%. *Cymbopogon martinii* var. *motia, Melaleuca linariifolia* and *Thymus satureoides* showed a MIC and a MTC of 0.2% and 0.1% respectively. On the other hand, *Cymbopogon nardus* and *Origanum majorana* showed a MIC and a MTC of 0.4% and 0.1% respectively. Ten oils (*Coriandrum sativum*, *Cymbopogon citratus*, *Cymbopogon flexuosus*, *Inula graveolens*, *Lavandula hybrida reydovan*, *Melaleuca alternifolia*, *Salvia lavandulifolia*, *Thymus mastichina*, *Thymus vulgaris linaloliferum*, *Thymus vulgaris thymoliferum*) were less active and showed a MIC of 0.8%. The MTC values observed for these compounds were between 0.006 and 0.4%. Finally, ten oils (*Citrus limetta*, *Cuminum cyminum*, *Cymbopogon winterianus*, *Eucalyptus dives*, *Laurus nobilis*, *Lavandula latifolia spica cineolifera*, *Melaleuca cajeputii*, *Ocimum basilicum var. basilicum*, *Rosmarinus pyramidalis*, *Thymus vulgaris geranioliferum*) showed a weak antimicrobial activity against *P. putida* showing a MIC value above 0.8% and a MTC value ranging between 0.1 and 0.4%. The twenty two other oils (*Anethium graveolens*, *Apium graveolens*, *Artemisia dracunculus*, *Chamœmelum nobile*, *Cistus ladaniferus pineniferum*, *Citrus aurantium*, *Citrus bergamia*, *Citrus limon*, *Citrus reticulata*, *Citrus sinensis*, *Cupressus sempervirens*, *Curcuma longa*, *Melaleuca quinquenervia nerolidolifera*, *Myristica fragrans*, *Myrtus communis cineoliferum*, *Pinus sylvestris*, *Rosemarinus officinalis camphreiferum*, *Rosemarinus officinalis cineoliferum*, *Rosemarinus officinalis verbenoniferum*, *Salvia sclarea*, *Tsuga canadensis*, *Zingiber officinalis*) were inactive against *P. putida* showing a MTC value above 0.8%.

On the basis of these results, it is possible to conclude that the essential oils of oregano (*Corydothymus capitatus*, *Origanum compactum* and *O. heracleoticum*), savory (*Satureja hortensis* and *S. montana*), chinese cinnamon (*Cinnamomum cassia*), thymol thyme (*Thymus vulgaris thymoliferum*) and carvacrol thyme (*Thymus vulgaris carvacroliferum*) have the stronger antimicrobial activity between all essential oils tested. Zaika (1988) showed that the antimicrobial activity of essential oils can be classified as strong, medium, or weak. Conner (1993) showed that cinnamon, clove, pimento, thyme, oregano and rosemary had strong and consistent inhibitory effects against various pathogens and spoilage microorganisms. Hammer,

Carson and Riley (1999) showed that oregano, lemongrass and pimento inhibited the growth of *Pseudomonas aeruginosa* at a concentration 1.0% (vol/vol). Delaquis et al. (2002) estimated MIC against *Pseudomonas fragi* that ranged between 0.47% (vol/vol) for the cilantro essential oil, 0.3% for eucalyptus oil, 0.15% for coriander oil, and 0.3% for dill oil. Ouattara, Simard, Holley, Piette and Bégin (1997) found that cinnamon, clove, pimento and rosemary oils were very effective against *Pseudomonas fluorescens*.

Our results also indicate that the two types of ceylon cinnamon essential oils tested (*Cinnamomum verum*) from barks and leaves exhibited an antimicrobial effect against *P. putida*, without any significant difference ($P > 0.05$) between them. On the other hand, *P. putida* was two times more senstive to chinese cinammon (*Cinnamomum cassia*) with a MIC of 0.05% and MTC of 0.025% than ceylon cinnamom (*Cinnamomum verum*). In other context Lis-Balchin and Deans (1997) showed that chinese and ceylan cinnamom leaf had the same effectiveness against 20 strains of *Listeria monocytogenes*.

With regard to oregano, three oils tested (*i. e. Corydothymus capitatus*, *Origanum compactum* and *Origanum heracleoticum*) were strongly active despite their differences concerning the species and their geographic location, which is in agreement with previous studies. Finnish oregano (*Origanum vulgare*) oil showed broad antimicrobial activity, inhibiting many bacteria, including the Gram-negative organisms (Negi, Jayaprakasha, Jagan Mohan Rao & Sakariah, 1999). Bagamboula et al. (2003) showed that the MIC of oregano (*Origanum vulgare*) was 0.5% (wt/vol) for five Gram-negative bacterial strains (*Shigella sonnei* strain 6, *Shigella sonnei* CIP 82.49, *Shigella flexneri* strain1, *Shigella flexneri* CIP 82.48 and *Escherichia coli* LMG 8223). This is in agreement with the findings of Paster et al. (1990) who found that below 0.25% (wt/vol), two israeli oregano (*Origanum vulgare* and *Coridothymus capitatus*) essential oils were ineffective or very slightly effective against five bacteria tested (*Pseudomonas aeruginosa, Salmonella* Typhimurium, *Staphylococcus aureus*, *Campylobacter jejuni* and *Clostridium sporogenes*). Similarly, essential oils from other *Origanum* species have also been shown to possess high levels of antimicrobiol activity (Deans & Ritchie, 1987; Janssen, Scheffer & Baerheim Svendsen, 1987; Sivropoulou et al., 1996).

In our study, no obvious difference was observed between the MIC values of *Satureja hortensis* and *Satureja Montana*. However, the results showed that *P. putida* was twice more sensitive to *Satureja hortensis* with a MTC of 0.013%. According to several studies, the essential oil from *Satureja hortensis* possesses a wide antimicrobial spectrum, and it inhibits the growth of the human and plant pathogenic and food spoilage bacteria, fungi and yeast species (Gülluce et al., 2003; Nevas, Korhonen, Lindström, Turkki & Korkeala, 2004). Gülluce et al. (2003) showed that MIC values for three *Pseudomonas* strains, which were sensitive to the essential oil from *Satureja hortensis*, were respectively 0.03% (wt/vol) for *P. aeruginosa*-ATTC-9027, 0.06% for *P. fluorescens*-RK242 and 0.250% for *P. syringae* pv. Tomato-A35. On the other hand, *Satureja montana* did not show any activity against *Pseudomonas aeruginosa* (Panizzi et al., 1993).

Despite a common botanical origin, the compostion and antimicrobial activity of different essential oils: *thymus*, *cymbopogon* and *melaleuca* were very different (Table 1). *Melaleuca linariifolia* (narrow leal melaleuca) was very active against *Pseudomonas*, while *Melaleuca alternifolia* (tea-tree) and *Melaleuca cajeputii* (cajeput) were slightly active, and *Melaleuca quinquenervia* (niaouli) was ineffective. *Cymbopogon martinii* (indian palmorosa) and *Cymbopogon nardus* (ceylon citronella) presented a respective MIC of 0.4 and 0.2%, while *Cymbopogon citratus* (lemongrass) and *Cymbopogon flexuosus* (indian lemongrass) presented an identical MIC (0.8%). *Cymbopogon winterianus* (java citronella) was very slightly active showing a MIC > 0.8%. Among eight thyme oils tested, *Thymus vulgaris thymoliferum* and *Thymus vulgaris carvacroliferum* were most active showing a MIC of 0.05%, followed by *Thymus serpyllum* and *Thymus satureoides* showing a MIC of 0.1 and 0.2% respectively, while *Thymus vulgaris thuyanoliferum*, *Thymus vulgaris linaloliferum*, *Thymus vulgaris geranioliferum* and *Thymus mastichina* were slightly active showing a MIC ≥ 0.8%. A similar trend was observed by Lis-Balchin and Deans (1997) which showed that there was considerable variations between the antibacterial action of essential oils from several *Thymus vulgaris*, *Cymbopogon* (lemongrass, palmarosa) and *Melaleuca* (tea-tree, cajeput, niaouli, neroli) species. However, the comparison of the efficiency of oils between studies is difficult due to different uncontrollable and external parameters. The composition of plant oils is known to vary according to local climatic and environmental conditions (Janssen et al., 1987; Sivropoulou et al., 1996). Moreover, the antimicrobial properties can vary within the same plant species because the chemical

composition and relative proportions of the individual constituents in the essential oils of the plant are influenced by genotype (Arrebola et al., 1994). Furthermore, some oils with the same common name may be derived from different plant species (Windholz, Budavari, Blumetti & Otterbein, 1983). Similarly, the method used to assess antimicrobial activity, and the choice of tested organisms, varies between publications (Janssen et al., 1987). Agar disc diffusion technique is a method frequently used to screen plant oils for antimicrobial activity (Smith-Palmer, Stewart & Fyfe, 1998). Agar and broth dilution methods are also commonly used. The results obtained by each of these methods may differ as many factors vary between assays (Janssen et al., 1987). These include differences in microbial growth, time of exposition of microorganisms to plant oil, the solubility of oil, and the method used to solubilize or emulsifying them. These and other elements may account for the large differences in MICs obtained by the agar plates method in this study.

It is also worth mentioning to note that oils, such as sage (*Salvia* species), dill (*Anethium graveolens)*, lemon (*Citrus limon*), orange (*Citrus aurantium, C. sinensis*), bergamot (*Citrus bergamia*), tarragon (*Artemisia dracunculus*), niaouli (*Melaleuca quinquenervia*), tea-tree (*Melaleuca alternifolia*), turmeric (*Curcuma longa*), basil (*Ocimum basilicum*), celery (*Apium graveolens*) and rosemary (*Rosemarinus* species), with proven antimicrobial properties appeared to have little or no effect against the tested *P. putida* strain (Bagamboula et al., 2003; Cox et al., 2000; Jirovetz, Buchbauer, Stoyanova, Georgiev & Damianova. 2003; Kivanç & Akgül, 1986; Lis-Balchin and Deans, 1997; Negi et al., 1999; Shelef, Jyothi & Bulgarelli, 1984; Suppakul, Miltz, Sonneveld & Bigger, 2003; Tassou, Drosinos & Nychas, 1996). However, Hammer et al. (1999) showed that these oils were very active against some Gram-positive bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*) and *E. coli*, but presented a weak microbial activity against *Pseudomonas aeruginosa* with MICs above 2.0% (vol/vol). Gram-positive bacteria are generally more sensitive to the presence of essential oils from spices and herbs than Gram-negative bacteria, although Nychas and Tassou (2000) observed that a Gram-positive was as sensitive as Gram negative bacteria in presence of oregano oil.

The antimicrobial activities have been attributed to the presence of some volatile constituents in the oils. According to Farag, Daw, Hewedi and El-Baroty (1989), the antimicrobial activities of essential oils are predominantly related to their main components. Chemical analysis of essential oils provided by the manufacturer has shown that the major compounds identified in tested essential oils are phenolic monoterpenes, notably carvacrol, thymol or eugenol, monoterpenic hydrocarbons such as *p*-cimene, γ-terpinene, α-pinene or limonene, alcohol terpenoids such as borneol, linalool, 1,8-cineole or geraniol, aldehydes such as cinnamaldehyde, geranial or citronnellal and ketones such as piperitone or carvone. Bullerman, Lieu and Seier (1977) found that cinnamon and clove contained cinnamaldehyde and eugenol as major antimicrobial constituents which represented 65-75% and 93-95% of the total volatile oils, respectively. In oregano and thyme, the major antimicrobial constituents have been identified as carvacrol (62-79%), and thymol (42%) respectively (Farag et al., 1989; Sivropoulou et al., 1996). Gülluce et al. (2003) showed that *Satureja hortensis* essential oil has a high content of thymol (29.0%), carvacrol (26.5%), γ-terpinene (22.6%), and *p*-cymene (9.3%), while Ghannadi (2002) reported that carvacrol (59.7%), γ-terpinene (12.8%), and *p*-cymene (9.3%) were major constituents of the oil isolated from the seeds of *Satureja hortensis*. As reported by others (Tümen, Kirimer, Ermin & Baser, 1998), the genus *Satureja* may or may not contain phenols, and the phenol-containing species are divided into «carvacrol type» and «thymol type». In agreement with this point, the essential oils obtained from cultivated summer *Satureja hortensis* and *Satureja montana*, were characterized by the presence of carvacrol, while thymol was absent or detectable in small amounts or traces (Gora, Lis & Lewandowski, 1996; Milos, Radonic, Bezic & Dunki, 2001). In our case, *Satureja montana* essential oil had relatively high amount of thymol at concentration of 42.71% and *Stureja hortensis* had 40.89% of carvacrol. Nevas et al. (2004) showed a differences in the proportions of thymol, carvacrol, *p*-cymene and γ-terpinene in savory oil as compared to those reported in savory collected from Portugal (Esquivel, 1999).

The antimicrobial activity of essential oils would be related to the respective composition and structural configuration of the plant volatile oils, their functional groups and possible synergistic interactions between components (Dorman & Deans, 2000). Our study showed that the oils containing mainly components with phenolic structures, such as carvacrol, thymol and eugenol were highly active against *P. putida*. The antimicrobial potential of many essential oils

is related to their high phenolic contents (Sivropoulou et al., 1996). Phenolic compounds are known to be either bactericidal or bacteriostatic agents, depending upon the concentration used (Pelczar, Chan & Krieg, 1988). Among non-phenolic compounds of tested essential oils, cinnamaldhyde contained mainly in the *Cinnamomum cassia* and *Cinnamomum verum* (bark), presents also a powerful antimicrobial activity. On the other hand, aldehydes such as geranial, neral, citronnellal or cuminal mainly present in *Cymbopogon citratus*, *Cymbopogon winterianus* and *Cuminum cyminum*, displayed moderate activity against *P. putida*. A number of reports indicated that essential oils which contain cinnamaldehyde had high antibacterial properties by inhibiting amino acid decarboxylase activity (Didry et al., 1993; Wendakoon & Sakaguchi, 1995). A relationship was found between the inhibitory effect against *Pseudomonas fluorescens* and the presence of cinnamaldehyde in essential oils tested (Ouattara et al., 1997).

Conclusion

This study showed that many essential oils possess *in vitro* antibacterial activity against *P. putida*. Twenty eight oils appeared effective, and oregano, savory, chinese cinnamon, thymol thyme and carvacrol thyme essential oils showed the strongest antimicrobial activity. Also, this study provided comparable quantitative antimicrobial data for sixty essential oils. As food preservatives, volatile oils may have their greatest potential use. However, if essential oils were to be more widely applied as antibacterials in foods, the organoleptic impact would be important. Spices and herbs, which are used as integral ingredients in prepared foods or added as flavouring agents to food, are present in insufficient quantities for their antimicrobal properties to be significant. On the other hand, volatile oils, which often contain the principal aromatic and flavouring components of herbs and spices, even added into small quantity to foodstuffs, without affecting organoleptic properties, would retard bacterial contamination and therefore reduce the onset of spoilage. For practical addition of essential oils in food, it is important to know if oils at very low concentrations have efficient antimicrobial effects without affecting sensory qualities. The flavour of beef fillets treated with 0.8% (vol/wt) oregano oil was found to be acceptable after storage at 5°C and cooking (Tsigarida, Skandamis & Nychas, 2000). The flavour, odour and colour of minced beef containing 1% (vol/wt) oregano oil improved during storage under modified atmosphere packaging and vacuum at 5°C and was almost undetectable after cooking

(Skandamis & Nychas, 2001). However, if plant oils are be used for food preservation, issues of safety and toxicity will need to be addressed. Also, it should be undertaken in meat and processed meat to confirm the antimicrobial efficiency level of these essential oils. These essential oils could serve as potential antimicrobial agents to inhibit spoilage growth in food.

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Figure caption

Fig. 1. Survivor curves of *P. putida* in BHI agar containing the most active essential oils (E. O.)

Table 1. Minimum inhibitory concentration (MIC) and maximal tolerated concentration (MTC) of selected essential oils against *Pseudomonas putida*

Details of plant oils					Antimicrobial test	
Plant species	**Common name**	**origin**	**Distilled part**	**Main compounds (Area %)[a]**	**MIC (%wt/vol)**	**MTC(%wt/vol)**
Anethium graveolens	Dill weed	Hungary	Plant germinated	Carvone (39.72), α-phellandrene (24.97), limonene (21.95)	> 0.8	> 0.8
Apium graveolens	Celery	India	Fruit (seed)	Limonene (52.07), selinene (18.57)	> 0.8	> 0.8
Artemisia dracunculus	Tarragon	Iran	Flowering plant	Methylchavicol (75,23), trans- β-ocimene (10.05)	> 0.8	> 0.8
Chamæmelum nobile	Roman chamomille	France	Flower	Aliphatic esters (72.80)	> 0.8	> 0.8
Cinnamomum cassia	Chinese cinnamon	China	Leaf-branch	Cinnamaldehyde (64.59), methoxy-cinnamaldehyde (20.82)	0.05	0.025
Cinnamomum verum	Ceylon cinnamon	Skri Lanka	Bark	Cinnamaldehyde (86.63)	0.1	0.05
Cinnamomum verum	Ceylon cinnamon	Madagascar	Leaf	Eugenol (62.51), β-caryophyllene (5.19)	0.1	0.05
Cistus ladaniferus pineniferum	Pinene labdanum	Morocco	Branch	Bornyl acetate (14.00), camphene (13.48), borneol (5.28)	> 0.8	>0.8
Citrus aurantium	Bitter orange	Ivory Coast	Peel	Limonene (94.67)	> 0.8	> 0.8
Citrus bergamia	Bergamot	Ivory Coast	Peel	Linalyl acetate (36.61), Linalool (24.71), limonene (20.22)	> 0.8	> 0.8
Citrus limetta	Sweet lime	South america	Peel	Limonene (49.24), γ-terpinene (12.14)	> 0.8	0.2
Citrus limon	Lemon	Spain	Peel	Limonene (56.33), β-pinene (12.00), γ-terpinene (11.64)	> 0.8	> 0.8
Citrus reticulata	Mandarin	Argentine	Peel	Limonene (87.86)	> 0.8	> 0.8
Citrus sinensis	Sweet orange	Brazil	Peel	Limonene (94.66)	> 0.8	> 0.8
Coriandrum sativum	Coriander	Russia	Fruit	Linalool (70.29), α-pinene (5.93)	0.8	0.4
Corydothymus capitatus	Spanish oregano	Spain	Flowering plant	Carvacrol (75.53)	0.025	0.006
Cuminum cyminum	Cumin	Egypt	Fruit	Cuminal (47.88), γ-terpinene (16.06), β-pinene (12.17)	> 0.8	0.2
Cupressus sempervirens	Cypress	France	Branch	α-pinene (37.23), δ3-carene (22.77)	> 0.8	> 0.8

Curcuma longa	Turmeric	India	Root	Ar-turmerone (36.43), turmerone (26.19)	> 0.8	> 0.8
Cymbopogon citratus	Lemongrass	Guatemala	Herb grass	Geranial (45.28), neral (31.95), limonene (8.52)	0.8	0.4
Cymbopogon flexuosus	Indian lemongrass	India	Herb grass	Geranial (46.12), neral (31.45)	0.8	0.4
Cymbopogon martinii var. motia	Indian palmarosa	India	Herb grass	Geraniol (80.14), geranyl acetate (8.59)	0.2	0.1
Cymbopogon nardus	Ceylon citronella	Skri Lanka	Herb grass	Geraniol (19.11), limonene (9.92), camphene (9.02)	0.4	0.1
Cymbopogon winterianus	Java citronella	Vietnam	Herb grass	Citronnellal (34.11), geraniol (21.47), Citronnellol (11.48)	> 0.8	0.2
Eucalyptus dives	Broad-leal eucalyptus	Australia	Leaf	Piperitone (42.91), α-phellandrene (30.03)	> 0.8	0.2
Eugenia caryophyllus	Clove	Madagascar	Flower bud	Eugenol (78.00), eugenyl acetate (13.77)	0.1	0.05
Inula graveolens	Sweet inula	France	Flowering plant	Bornyl acetate (50.96), borneol (22.76), camphene (6.92)	0.8	0.2
Laurus nobilis	Sweet bay	Slovenia	Leaf	1,8-cineole (49.00), terpenyl acetate (9.92) sabinene (7.61)	> 0.8	0.4
Lavandula hybrida reydovan	Reydovan lavandin	France	Flowering plant	Linalool (50.58), linalyl acetate (19.38), camphor (8.04)	0.8	0.006
Lavandula latifolia spica cineolifera	Spike cineole lavender	France	Flowering plant	Linalool (33.65), 1,8-cineole (22.31), camphor (14.88)	> 0.8	0.4
Melaleuca alternifolia	Tea-tree	Australia	Leaf	Terpine ol4(40.22), γ-terpinene (21.04), α-terpinene (10.48)	0.8	0.4
Melaleuca cajeputii	Cajuput	Vietnam	Leaf	1,8-cineole (72.98), α-terpineol (9.55)	> 0.8	0.4
Melaleuca linariifolia	Narrow leaf melaleuca	Australia	Leaf	terpine ol4 (30.18), γ-terpinene (18.60), 1.8 cineole (14.19)	0.2	0.1
Melaleuca quinquenervia nerolidolifera	Nerolidol niaouli	Australia	Leaf	Nerolidol (90.23)	> 0.8	> 0.8
Myristica fragrans	Nutmeg	Indonesia	Fruit kernel	α-pinene (20.67), sabinene (19.92),), β-pinene (14.55)	> 0.8	> 0.8
Myrtus communis cineoliferum	Honey myrtle	France	Leaf	α-pinene (56.15), 1.8 cineole (22.76)	> 0.8	> 0.8
Ocimum basilicum var. basilicum	Sweet basil	Vietnam	Flowering plant	Methyl chavicol (86.72)	> 0.8	0.4
Origanum compactum	Oregano	Morocco	Flowering plant	Carvacrol (22.00), γ-terpinene (22.90), thymol (19.36)	0.05	0.025
Origanum heracleoticum	Greek oregano	France	Flowering plant	Carvacrol (54.38), paracymene (14.34), γ-terpinene (13.80)	0.05	0.025
Origanum majorana	Sweet marjoram	Egypt	Flowering plant	Terpinene-4-ol (26.22), γ-terpinene (12.16), thuyanol (9.94)	0.4	0.1
Pimenta dioica	Allspice	Antilles	Leaf	Eugenol (47.78), myrcene (26.76), geraniol (10.40)	0.1	0.05

Pinus sylvestris	Scotch pine	Slovenia	Needle	α-pinene (31.96), δ3-carene (13.60), β-myrcene (9.41)	> 0.8	> 0.8
Rosemarinus officinalis camphreiferum	Camphor rosemary	France	Flowering plant	1,8-cineole (23.59), camphor (18.20), α-pinene (12.55)	> 0.8	> 0.8
Rosemarinus officinalis cineoliferum	Cineole rosemary	Morocco	Flowering plant	1,8-cineole (42.15), camphor (13.33), α-pinene (12.14)	> 0.8	> 0.8
Rosemarinus officinalis verbenoniferum	Verbenone rosemary	France	Flowering plant	α-pinene (26.48), bornyl acetate (13.19), 1.8 cineole (10.71)	> 0.8	> 0.8
Rosmarinus pyramidalis	Rosemary pyramidalis	France	Flowering plant	α-pinene (31.26), 1.8 cineole (29.06), camphor (5.27)	> 0.8	0.4
Salvia lavandulifolia	Spanish sage	Spain	Flowering plant	1,8-cineole (26.56), camphor (22.50)	0.8	0.2
Salvia sclarea	Clary sage	France	Flowering plant	Linalyl acetate (57.27), linalool (18.56)	> 0.8	> 0.8
Satureja hortensis	Summer savory	France	Flowering plant	Carvacrol (40.89), γ-terpinene (32.60), *p*-cymene (5.63)	0.05	0.013
Satureja montana	Winter savory	Slovenia	Flowering plant	Thymol (42.71), *p*-cymene (11.68), γ-terpinene (8.63)	0.05	0.025
Thymus mastichina	Spanish thyme	Spain	Flowering plant	1.8 cineole (47.13), linalool (23.52), limonene (7.29)	0.8	0.4
Thymus satureoides	Borneol/carvacrol thyme	Morocco	Flowering plant	Borneol (26.00), camphene (9.05), carvacrol (6.77)	0.2	0.1
Thymus serpyllum	Mother of thyme	Albania	Flowering plant	Cavacrol (23.25), *p*-cymene (20.38), γ-terpinene (18.09)	0.1	0.025
Thymus vulgaris carvacroliferum	Common carvacrol thyme	France	Flowering plant	Cavacrol (32.90), *p*-cymene (24.02), thymol (11.85)	0.05	0.025
Thymus vulgaris geranioliferum	Common geraniol thyme	France	Flowering plant	Geranyl acetate (37.63), geraniol (26.04), thymol (5.16)	> 0.8	0.1
Thymus vulgaris linaloliferum	Commom linalool thyme	France	Flowering plant	Linalool (59.72), linalyl acetate (9.53)	0.8	0.4
Thymus vulgaris thuyanoliferum	Common thyjanol thyme	France	Flowering plant	Thujanol 4 (43.73), mycene 8-ol (13.00)	0.8	0.1
Thymus vulgaris thymoliferum	Common thymol thyme	France	Flowering plant	Thymol (38.12), *p*-cymene (19.44), γ-terpinene (17.39)	0.05	0.013
Tsuga canadensis	Hemlock	Canada	Needle	Bornyl acetate (34.89), α-pinene (15.57), camphene (11.90)	> 0.8	> 0.8
Zingiber officinalis	Ginger	Benin	Rootstock	α-zingiberene (23.64), β-sesquiphellandrene (10.99)	> 0.8	> 0.8

[a]According to the data of the gas chromatography analysis of essential oils provided by the manufacturer.

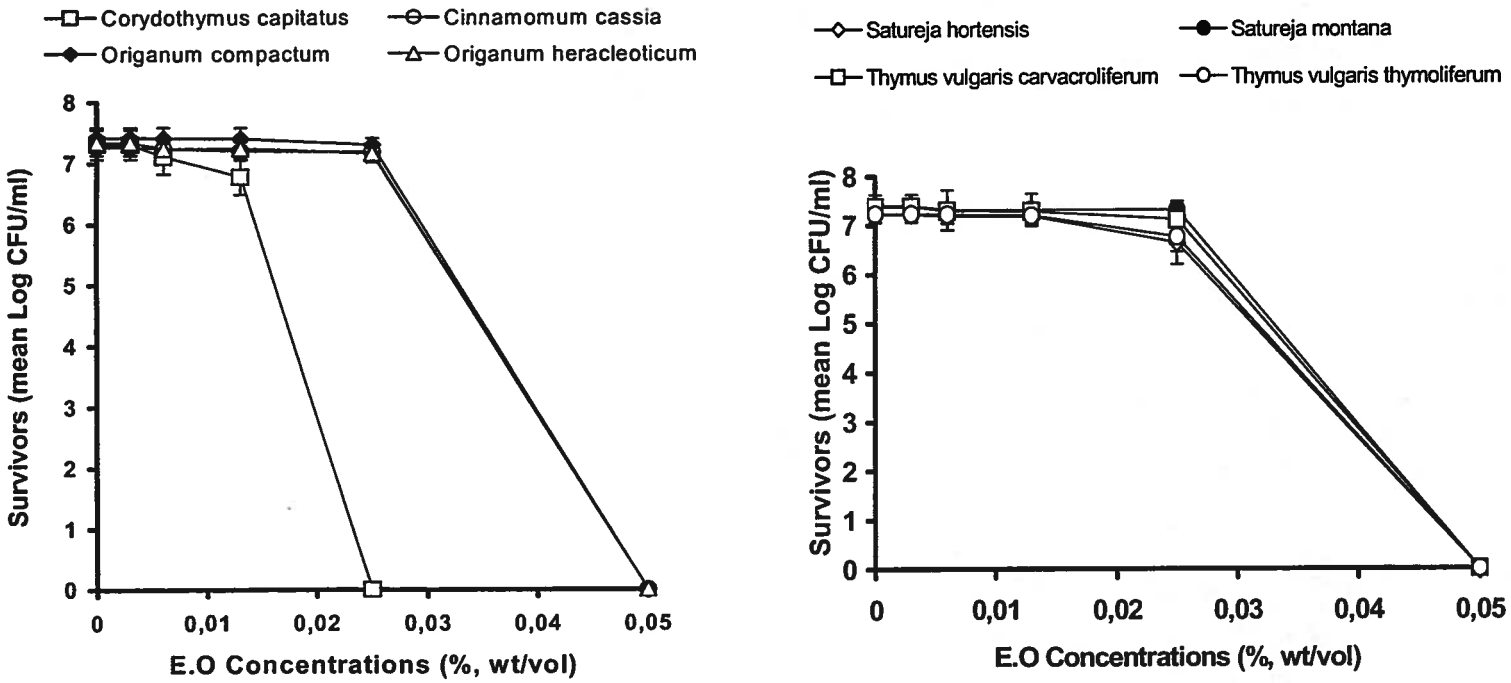
Corydothymus capitatus
Cinnamomum cassia
Origanum compactum
Origanum heracleoticum
Survivors (mean Log CFU/ml)
E.O Concentrations (%, wt/vol)
0
0,01
0,02
0,03
0,04
0,05
Satureja hortensis
Satureja montana
Thymus vulgaris carvacroliferum
Thymus vulgaris thymoliferum
Survivors (mean Log CFU/ml)
E.O Concentrations (%, wt/vol)

Fig. 1.

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Article 3: Soumis dans Food Control

Running title: Inhibition of foodborne pathogens by essential oils

Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157:H7, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*

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Résumé

Vingt huit huiles essentielles ont été évaluées pour leurs propriétés antibactériennes, contre quatre bactéries pathogènes *Escherichia coli* O157:H7, *Listeria monocytogenes* 2812 1/2a, *Salmonella* Typhimurium SL 1344 et *Staphylococcus aureus*. Les huiles essentielles ont été mélangées dans une gélose d'infusion de coeur et de cerveau (BHI) (15 ml) à une concentration de 0.003% à 0.8% (vol/vol) pour déterminer la concentration minimale inhibitrice (CMI) et la concentration maximale tolérée (CMT) contre chaque bactérie pathogène testée. Les résultats ont montré que les huiles essentielles les plus actives contre les bactéries testées étaient : *Corydothymus capitatus, Cinnamomum cassia, Origanum heracleoticum, Satureja montana*, et *Cinnamomum verum* (écorce). Ces huiles ont montré une CMI $\leq$ 0.05% (vol/vol) contre toutes les bactéries. La CMT des huiles essentielles de *Cinnamomum verum* et de *Cinnamomum cassia* était de 0.025% (vol/vol) contre *S.* Typhimurium et *L. monocytogenes*. Pour les trois autres meilleures huiles, la CMT était de 0.013% (vol/vol) avec toutes les bactéries testées. Trois huiles essentielles (*Satureja hortensis*, *Thymus vulgaris carvacroliferum, Origanum compactum*) ont montré une CMI $\leq$ 0.1% (vol/vol) contre toutes les bactéries testées. Sept huiles essentielles (*Thymus vulgaris thymoliferum, Thymus serpyllum, Thymus satureioides*, *Cymbopogon martinii*, *Pimenta dioica*, *Cinnamomum verum* (feuille), *Eugenia caryophyllus*) ont montré une faible activité antimicrobienne une CMI $\leq$ 0.4% (vol/vol) contre les quatre bactéries. En conclusion, treize huiles essentielles étaient apparement moins actives avec une CMI $\geq$ 0.8% (vol/vol) contre au moins une bactérie.

Abstract

Twenty eight essential oils were evaluated for their antibacterial properties, against four pathogenic bacteria (*Escherichia coli* O157:H7, *Listeria monocytogenes* 2812 1/2a, *Salmonella* Typhimurium SL 1344 and *Staphylococcus aureus*). Essential oils were introduced into Brain Heart Infusion agar (BHI) (15 ml) at a concentration of 0.003%, 0.006%, 0.013%, 0.025%, 0.05%, 0.1%, 0.2%, 0.4% and 0.8% (vol/vol) to determine the minimum inhibitory concentration (MIC) and the maximal tolerated concentration (MTC) for each pathogen evaluated. Results showed that the most active essential oils against bacteria tested were *Corydothymus capitatus, Cinnamomum cassia, Origanum heracleoticum, Satureja montana*, and *Cinnamomum verum* (bark). These showed a MIC ≤ 0.05% (vol/vol) for all bacteria tested. For the MTC, with the exception of *S.* Typhimurium and *L. monocytogenes* where a MTC of 0.025% (vol/vol) was observed in presence of *Cinnamomum verum* and *Cinnamomum cassia*, respectively, a MTC ≤ 0.013% (vol/vol) was observed for all other bacteria and the three other most active essential oils. Three oils (*Satureja hortensis*, *Thymus vulgaris carvacroliferum, Origanum compactum*) showed a MIC ≤ 0.1% (vol/vol) for all bacteria tested. Seven oils (*Thymus vulgaris thymoliferum, Thymus serpyllum, Thymus satureioides*, *Cymbopogon martinii*, *Pimenta dioica*, *Cinnamomum verum* (leaf), *Eugenia caryophyllus*) showed a lower antimicrobial activity showing a MIC ≤ 0.4% (vol/vol) against the four bacteria tested. Finally, thirteen essential oils were less active showing a MIC value ≥ 0.8% (vol/vol) against at least one bacterium.

Keywords: essential oils, foodborne pathogens, minimum inhibitory concentration

Introduction

Foodborne illness resulting from consumption of food contaminated with pathogenic bacteria has been of vital concern to public health. Among the reported outbreaks in the United States during 1993-1997 period for which the etiology was determined, bacterial pathogens caused the largest percentage of outbreaks (75%) and the largest percentage of cases (86%) (Olsen, MacKinnon, Goulding, Bean & Slutsker, 2000). *Salmonella* sp., *Listeria monocytogenes* and *E. coli* accounted for the largest number of outbreaks, cases, and deaths. In Canada, the cost to treat the foodborne disease due to meat and meat products contamination is estimated to $500 millions per year (Todd, 1989). To reduce health hazards and economic losses due to foodborne microorganisms, the use of natural products as antibacterial compounds (Conner, 1993; Dorman & Deans 2000) seem to be an interesting way to control the presence of pathogenic bacteria and to extend the shelf life of processed food. These compounds could be added during the food process. Among these products, essential oils from spices, medicinal plants and herbs have been shown to possess antimicrobial activities and could serve as a source of antimicrobial agents against food pathogens (Deans & Ritchie, 1987; Kim, Marshall & Vei, 1995). Several references on the antimicrobial and antifungal efficiency of essential oils are available in the literature (Burt & Reinders, 2003; Cox *et al.*, 2000; Delaquis, Stanich, Girard & Mazza, 2002; Mejholm & Dalgaard, 2002; Nielsen & Rios, 2000). More particularly, essential oils and their components are known to be active against a wide variety of microorganisms, including Gram-negative (Helander *et al.*, 1998; Sivropoulou, Papanikolaou, Nikolanou, Kokkini, Lanaras & Arsenakis, 1996) and Gram-positive bacteria (Kim *et al.*, 1995). Gram-negative bacteria were shown to be generally more resistant than Gram-positive ones to the antagonistic effects of essential oils because of the lipopolysaccharide present in the outer membrane (Russel, 1991) but this was not always true (Karapinar & Aktug, 1987).

Essential oils are the odorous, volatile products of an aromatic plant's secondary metabolism, normally formed in special cells or groups of cells, found in many leaves and stems. They are commonly concentrated in one particular region 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 flavoring 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 antimicrobial activity of essential oils is assigned to a number of small terpenoids and phenolic compounds (thymol, carvacrol, eugenol), which also in pure form demonstrate high antibacterial activity (Conner, 1993; Didry, Dubreuil & Pinkas, 1993; Karapinar & Aktug, 1987). The antimicrobial activities of oregano, savory, and thyme were first reported during 1950s and it was established that the inhibitory effect of oregano, savory, and thyme is likely due to their high content of thymol and carvacrol, which are among the most efficient herbal antibacterial agents known (Nevas, Korhonen, Lindstrom, Turkki & Korkela, 2004; Sivropoulou *et al.,* 1996). However, 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 this variability influences the chemical composition and the relative concentration of each constituent in the essential oils (McGimpsey & Douglas, 1994; Rhyu, 1977; Salgueiro *et al.*, 1997; Venskutonis, 1996). A number of essential oils constituents exhibit significant antimicrobial properties when tested separately (Kim *et al.*, 1995; Lambert, Skandamis, Coote & Nychas, 2001). 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 (Paster, Menasherov, Ravid & Juven, 1995).

Even if essential oils are considered as safe (GRAS) (Lambert *et al.*, 2001), their use is often limited by organoleptical criteria. For this reason, it will be necessary to determine the minimum concentration necessary to inhibit the growth of pathogenic bacteria without affecting the sensory quality of the food. Also, the majority of the preceding studies carried out with commercial essential oils seldom compared the antimicrobial activity of these oils on the basis of their chemical composition. The objective of the present study was to evaluate the minimum inhibitory concentration (MIC) and the maximal tolerated concentration (MTC) of whole essential oils. The antibacterial activity of twenty-eight oils was evaluated on four bacterial pathogens (Gram+ and Gram-).

Materials and Methods

Preparation of bacterial strains

Escherichia coli O157:H7 EDL 933 (INRS-Institut Armand Frappier, Laval, Qc, Canada), *Salmonella* Typhimurium SL1344 (INRS-IAF, Laval, Qc, Canada), *Listeria monocytogenes* 2812 1/2a (Health Canada, St-Hyacinthe, Qc, Canada) and *Staphylococcus aureus* ATCC 29213 were maintained at −80 ± 1°C in Brain Heart Infusion broth (BHI, Difco Laboratories, Detroit, MI, USA) containing 10% glycerol. Prior to the experiment, working cultures were prepared by subculturing 100 µl of each stock culture in 9 ml of BHI broth and incubated at 35 ± 1°C for 24 h. After incubation, 75 µl of *E. coli* O157:H7, 50 µl of *S.* Typhimurium SL1344, 150 µl of *S. aureus* and 100 µl of *L. monocytogenes* 2812 1/2a from each working culture were individually inoculated in fresh BHI broth and incubated at 35 ± 1°C for 1h 30 (*E. coli*) and 2 h (other strains), in order to obtain inocula containing cultures in the exponential growth phase of approximately 2×10^7 CFU/ml.

Antibacterial agents

Twenty eight essential oils were used in order to determine their MICs and MTCs. All samples were supplied by Robert et Fils (Montréal, QC, Canada) and stored at 4°C before use. The commercial and scientific names of essential oils tested are summarized in Table 1.

Essential oil dispersion in the culture medium

First, twenty-eight suspensions were prepared with plant essential oils. The oils were dispersed at room temperature for 2 min using a homogenizator Ultra-Turrax TP18/1059 (Janke & Funkel, Staufen, Germany) at 20,000 rpm in a sterile 10% (wt/vol) modified starch (Food starch-modified, Purity gum Be, National Starch & Chemical Co, Boucherville, QC, Canada) solution to obtain a colloidal suspension (10%, wt/vol) (Oussalah, Caillet, Salmiéri, Saucier & Lacroix, 2004). The presence of modified starch improved oil dispersion and improve the antimicrobial properties (Burt & Reinders, 2003). The starch-oil suspensions were added in

molten BHI agar (Difco Laboratories, Detroit, MI, USA) maintained at 62 ± 1°C and strongly mixed for 2 min using a vortex. Nine (9) final concentrations of oils (0.003%, 0.006%, 0.013%, 0.025%, 0.05%, 0.1%, 0.2%, 0.4% and 0.8% (vol/vol)) were obtained by adding various volumes from the 10% (wt/vol) suspension. The molten agar (15 ml) containing essential oils were poured into sterile petri plates and left to solidify.

Determination of the minimum inhibitory concentration (MIC) and the maximal tolerated concentration (MTC)

The MIC and MTC of the tested essential oils were determined using an agar dilution method (Robert-Dernuet, 1995). Briefly, Petri plates of BHI agar containing various concentrations of essential oils were inoculated with each bacterial strain evaluated. Each working culture ($2x10^7$ CFU/ml) was diluted to obtain 10^{-4} and 10^{-5} in peptone water (0.1% wt/vol) and 40 µL of each diluted culture was individually spread on the surface of the solidified agar plates. The positive control consisted of BHI agar without essential oil, inoculated with the diluted medium culture. Uninoculated plates containing essential oils served as negative control. Test and control plates were then incubated at 35 ± 1°C. Plates were evaluated for the presence or the absence of colonies after 48h of incubation. For each treatment, the absence of colonies on all plates tested was considered as an inhibitory effect. The lowest concentration of essential oil required to completely inhibit the growth of the tested microorganism was designated as the MIC. The MTC was the highest concentration of essential oil, which did not affect the bacterial growth.

Statistical analysis of data

Analysis of variance and Duncan's multiple-range was done using Stat-Packets Statistical Analysis software (Walonick Associates Inc., MN, USA) for the bacterial enumeration for the determination of the MTC. Differences between means of bacterial counts (Log CFU/ml) were considered significant when $P \leq 0.05$. These experiments were repeated twice with four plates for each essential oil at each concentration.

Results and Discussion

The MICs and MTCs of each essential oil are presented in Table 1. Of the 28 essential oils tested, *Corydothymus capitatus*, *Cinnamomum cassia, C. verum* (bark), *Satureja montana* and *Origanum heracleoticum* seem to be the most efficient essential oils against the four pathogenic bacteria tested. The MIC and MTC values obtained in the presence of *E. coli* O157:H7, *S.* Typhimurium, *S. aureus* and *L. monocytogenes* were ≤ 0.05% and ≤ 0.025% (vol/vol) for these five essential oils.

Even if all essential oils reduced the level of the four pathogenic bacteria, only fifteen of them destroyed the four bacteria completely. Eight essential oils (*Cinnamomum cassia, C. verum* (bark), *Corydothymus capitatus, Origanum compactum, O. heracleoticum, Satureja hortensis*, *S. montana,* and *Thymus vulgaris carvacroliferum*) showed a strong antimicrobial activity against the four bacteria tested showing a MIC ≤ 0.1% (vol/vol) and a MTC ≤ 0.025% (vol/vol). Seven oils (*Cinnamomum verum* (leaf), *Cymbopogon martinii*, *Eugenia caryophyllus*, *Pimenta dioica*, *Thymus satureoides*, *T. serpyllum*, *T. vulgaris thymoliferum*) showed a high antimicrobial activity against the four pathogenic bacteria tested showing a MIC ≤ 0.4% (vol/vol) and a MTC ≤ 0.1% (vol/vol). Finally, thirteen oils (*Coriandrum sativum*, *Cymbopogon citratus*, *C. flexuosus*, *C. nardus*, *C. winterianus*, *Inula gravolens*, *Lavandula hybrida reydovan*, *L. latifolia spica cineolifera*, *Melaleuca linariifolia*, *Origanum majorana*, *Thymus mastichina*, *T. vulgaris thuyanoliferum, T. vulgaris linaloliferum*) were less active against the four bacteria tested showing a MIC value ≥ 0.8% (vol/vol) in the case of at least one bacterium and a MTC value ≤ 0.8% (vol/vol). These results showed that all essential oils tested reduced the level of *E. coli* O157:H7, *S.* Typhimurium SL 1344 and *S. aureus* at concentrations ≤ 0.4% (vol/vol), whereas twenty seven reduced the level of *L. monocytogenes* at concentrations ≤ 0.4% (vol/vol). *E. coli, L. monocytogenes* and *S.* Tiphymurium were destroyed completely by seventeen, twenty and twenty six essential oils at concentrations ≤ 0.8% (vol/vol), whereas *S. aureus* was destroyed completely by twenty six essential oils at concentrations ≤ 0.4% (vol/vol). *S. aureus* was the most sensitive bacteria to essential oils. These results are in agreement with those obtained by Lambert *et al.* (2001) which observed a high sensitivity of *S. aureus* to oregano essential oil.

Our results also suggested a number of observations. *Corydothymus capitatus* (Spanish oregano) had a strong antibacterial activity. According the chemical analysis of essential oils provided by the manufacturer, *C. capitatus* is composed essentially of 76% of carvacrol and 5% of thymol (Table 1). *C. capitatus* was the most efficient essential oil against both Gram-positive and Gram-negative bacteria.

Our results also showed that for the seven genus of *Thymus* tested; all of them had different levels of sensitivity to the same bacteria tested. *T. vulgaris carvacroliferum* is composed essentially of carvacrol (33%) and *T. vulgaris thymoliferum* is composed essentially of thymol (38%) (Table 1). *T. vulgaris carvacroliferum* was twice as active against *S.* Typhimurium and *L. monocytogenes* as against *T. vulgaris thymoliferum,* whereas they had a same level of sensivity against *E. coli and S. aureus. T. vulgaris thymoliferum* and *T. serpyllum* with 23% of cavacrol had a same efficiency against *L. monocytogenes* and *S.* Typhimurium. However, *E. coli* O157:H7 *and S. aureus* were twice more sensitive to *T. vulgaris thymoliferum* as compared to *T. sepyllum.* Also, *S. aureus* was the most sensitive and *L. monocytogenes* was the most resistant to *T. satureoides* which is essentially composed of bornéol (26%). *T. mastichina* with 47% of 1,8-cineole had a significant ($p \leq 0.05$) lower antimicrobial activity than *T. vulgaris* linaloliferum with 60% of linalol and *T. vulgaris thujanoliferum* with 44% of *trans*-thujanol-4. *L. monocytogenes* was resistant to the presence of both oils. *S.* Typhimurium and *S. aureus* were more sensitive to the presence of oil rich in linalol than essential oil rich in *trans*-thujanol-4. On the contrary, *E. coli* was more sensitive to oils containing *trans*-thujanol-4 as compared to oils containing linalol. Pina-vaz *et al.* (2004) observed the antifungal activity of *T. vulgaris* containing 70% (vol/vol) of carvacrol and of *T. zygis* containing 40% (vol/vol) of thymol against *Candida.* The essential oil from *T. spinulosus* also showed an antibacterial activity against Gram-positive (*S. aureus*) and Gram-negative *(E. coli, S.* Typhimurium and *P. aeruginosa*) (De Feo, Bruno & Tahiri, 2003). This essential oil is mainly composed of monoterpene hydrocarbons, which are the biogenetics precursors of phenols as γ- terpinene and *p*-cymene. Carvacrol, thymol and *p*-cymene, are generally the most antimicrobial compounds found in *Thymus* species. Rasooli and Mirmostafa (2003) have observed that *T. kotschyanus* and *T. persicus*, essentially composed of thymol (between 7 and 27%), carvacrol (between 23 and 38%) and *p*-cymene (between 8 and 11%), were strongly bactericidal at low concentration against *E. coli*, *S. aureus* and *P.*

aeruginosa.

Three species of *Origanum* (*O. heracleoticum, O. compactum* and *O. majorana*) were also tested in our study. *O. majorana* was the least effective against the four pathogenic bacteria tested. A higher concentration of carvacrol could explain the efficiency of *O. heracleoticum* and *O. compactum*. The concentration of total phenolic compounds was 54% and 41% in *O. heracleoticum* and *O. compactum,* respectively, whereas *O. majorana* was rich in alcohol terpenoids (> 40%). These results are close to those obtained by others (Hammer, Carson & Riley, 1999) which showed that *O. majorana* had a MIC value of 0.5 and 0.25% (vol/vol) against *S.* Typhimurium ATTC 13311 and *S. aureus* NCTC 6571, respectively. On the other hand, the strain of *E. coli* NCTC 10418 tested by these authors seems more sensitive to *O. majorana* with a value of MIC of 0.5% (vol/vol) than *E. coli* O157:H7. Other species of *Origanum* were previously tested on various pathogenic bacteria (Aligiannis, Kalpoutzakis, Mitaku & Chinou, 2001). Their studies showed that *O. scabrun* which contains 75% of carvacrol had a very high antibacterial effect against *S. aureus* and *E. coli*. On the other hand *O. microphyllum,* which is poor in carvacrol as compared to the concentration present in *O. scabrum,* had a weaker antibacterial activity against *S. aureus* and *E. coli.*

Two strains of *Satureja* were analysed in our study, *S. hortensis* and *S. montana* with a concentration of carvacrol of 41% and 43%, respectively. Both strains had a strong antibacterial activity against all pathogenic bacteria tested. However, *S. aureus* was four times more sensitive than *E. coli* O157:H7 and *S.* Typhimurium to these oils. *L. monocytogenes* was twice as sensitive to *S. montana* as compared to *S. hortensis*. These results are in agreement with those obtained by Gülluce *et al.* (2003) who showed that the active compounds present in S. *hortensis* had a stronger and a broader spectrum of antimicrobial activity. The study also showed a strong correlation between their antimicrobial efficiency and the concentration of phenolic compounds. At a concentration of 300 µg/ml, *S. hortensis* had both antibacterial and anticandidal effects. Indeed, it showed this effect against bacterial strains composed essentially of Gram+ and Gram– bacteria which were human plant pathogenic and food spoilage strains (Sahin *et al.*, 2003). Skočibušić, Bezić, and Dunkić (2005) showed that the essential oil of *S. subspicata* possesses antimicrobial properties against 13 bacteria. Their study showed that *S. aureus* ATCC 25923 is

more sensitive to *S. subspicata* than *E. coli* O157:H7 ATCC 43895, *L. monocytogenes* ATCC 15313 and *S.* Typhimurium ATCC 19430.

The oils of *Cinnamomum verum* from bark and *C. cassia* were very active against the four pathogenic bacteria tested. On the other hand, *C. verum* from leaves was less effective against the four pathogens. *C. verum* from leaves is composed essentially of eugenol (63%) whereas cinnamaldehyde is the main compound in *C. verum* from bark (87%) and *C. cassia* (65%). Kwon, Yu and Park (2003) have evaluated the antimicrobial activity of cinnamic aldehyde on *B. cereus* and demonstrated its bactericidal effect at 0.03% (vol/vol). *C. cassia* and *C. verum* from bark were also found effective against a wide range of bacteria, such *L. monocytogenes*, *E. coli and B. subtilis* (Mau, Chen & Hsieh, 2001). Another report found that the MIC of *C. osmophloeum* on *E. coli*, *P. aeruginosa, K. pneumoniae* and *Salmonella* sp. was less than with cinnamaldehyde alone (Chang, Chen & Chang, 2001). The higher effectiveness of oils as compared to the individual active ingredients can come from the synergetic effect of the constituents presents in oils.

The essential oil of *Eugenia caryophillus* (clove) containing 78% of eugenol was effective against the four pathogenic bacteria. However, *L. monocytogenes* was the most resistant to this oil. According to Leuschner and Lelsch (2003), clove oil has a bactericidal effect at a concentration of 1% (vol/vol) and reduced the initial count of *L. monocytogenes* from 6.77 log to 1 CFU/ml when incubated at 37°C and 3 log when incubated at 4°C. *Cymbopognon martinii* also showed an antibacterial effect against the four pathogenic bacteria tested. This essential oil had a similar MIC value on *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* whereas *S. aureus* was the most sensitive. This oil was especially known to have a fungistatic effect (Delespaul, De Billerbeck, Roques & Michel, 2000). Our results also showed antibacterial properties of *Coriandrum sativum* against *E. coli* O157:H7, *S.* Typhimurium and *S. aureus* whereas *L. monocytogenes* was more resistant. *C. sativum* was found to be effective against *S. choleraesuis* with a maximal bactericidal concentration (MBC) value of 6.25 µg/ml (Kubo, Fujita, Kubo, Nihel & Ogura, 2004). These observations suggest that the *C. sativum* is more efficient against the Gram-negative than the Gram-positive bacteria.

Two recent studies (Freidman, Henika & Mandrell, 2002; Nevas *et al.*, 2004) showed that oregano, savory and thyme had an important antibacterial activity on *E .coli, L. monocytogenes, S.* Typhimurium, *S. aureus, C. perfringens* and *C. botulinum* and that oregano, cinnamon, thyme and clove bud have a strong bactericidal activity against *Campylobacter jejuni, E. coli* O157:H7, *L. monocytogenes* and *S. enterica*. Smith-Palmer, Stewart and Fyfe (1998) showed that cinnamon and thyme oils are active against *S. enterica, E. coli, S. aureus and L. monocytogenes*. Their effective bacteriostatic and bactericidal concentrations were respectively 0.23% and 1% (vol/vol). It appears that the difference in antibacterial activities may be related to the concentration and nature of contents, to the respective composition, the functional groups, the structural configuration of the components and their possible synergistic interaction. These differences found between essential oils are due to ecological and plant growth factors (Chang *et al.*, 2001). As showed in Table 1, the major chemical constituents of these oils are essentially monoterpene phenolics and monoterpene hydrocarbons. Eugenol, carvacrol and thymol are phenolic compounds that appeared to be highly active against the four pathogenic bacteria. Kim *et al.* (1995) observed that carvacrol was the most active constituent of eleven essential oils tested and this constituent was present at high concentration in *C. capitatus, T. vulgaris, T. serpyllum, S. hortensis* and *S. montana*. Among the non-phenolic compounds, cinnamaldehyde presents a powerful antimicrobial activity. However, the other aldehydes such as geranial, neral, citronnellal or cuminal displayed moderate activity against the four pathogenic bacteria tested.

Deans and Ritchie (1987) and Deans, Noble, Hiltunen, Wuryani and Penzes (1995) had observed that the susceptibility of Gram-positive and Gram-negative bacteria to plant volatile oils has a little influence on growth inhibition. However, some oils appeared more active with respect to Gram reaction, exerting a greater inhibitory activity against Gram-positive bacteria. It was often reported that Gram-negative bacteria were more resistant to the essential oils present in plants (Smith-Palmer *et al.*, 1998). The cell wall structure of Gram-negative bacteria is constituted essentially with LPS. This constituent avoids the accumulation of the oils on the cell membrane (Bezić, Skočibušić, Dunkić & Radonić, 2003). The results obtained in our study showed that *E. coli* O157:H7 and *S.* Typhimurium (Gram-negative) was less sensitive than *S. aureus* (Gram-positive). However, *L. monocytogenes,* a Gram-positive bacterium is more resistant than other bacteria tested which is in agreement with the work of Kim *et al.* (1995). *S.*

aureus which have a single layer wall was the most sensitive to over eighteen oils tested as compared to *E. coli* O157:H7 and *S.* Typhimurium which have a multi-layered structure (Essawi & Srour, 2000).

Conclusions

Food contamination is still an enormous public health problem, but may be better controlled by the use of natural preservatives. This study showed that many essential oils possess an antibacterial activity against the four bacteria tested. All of them influence bacterial growth at different concentrations. Among the essential oils tested, *Corydothymus capitatus*, *Cinnamomum cassia, C. verum* from bark, *Satureja montana* and *Origanum heracleoticum* were the most active with a smaller MIC on the bacteria tested. Between them, *Corydothymus capitatus* was the most active with a MIC value ≤ 0.025% on *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes* and *S. aureus*. Also, this study created directly comparable, quantitative, antimicrobial data for oils. As food preservatives, volatile oils may have their greatest potential use. Spices and herbs, which are used as integral ingredients in prepared foods or added as flavouring agents to food, are present in insufficient quantities for their antimicrobial properties to be significant. On the other hand, volatile oils, which often contain the principal aromatic and flavouring components of herbs and spices, even added in small quantity to foodstuffs, without affecting organoleptic properties, would reduce bacterial contamination. However, a studies should be undertaken in food to confirm the antimicrobial efficiency level of these essential oils, and their organoleptic impact.

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Table 1- Minimum inhibitory concentrations (MICs) and maximal tolerated concentration (MTCs) of selected essential oils against four pathogenic bacteria.

Details of plant oils					Test organism (MIC/MTC (vol/vol))			
Plant species	**Common name**	**Origin**	**Distilled part**	**Main compounds (Area %)[a]**	***E. coli O157:H7***	***S.*** Typhimurium	***S. aureus***	***L. mono-cytogenes***
Cinnamomum cassia	Chinese cinnamon	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	Ceylon cinnamon	Skri Lanka	Bark	Cinnamaldehyde (87)	0.025/0.006	0.05/0.025	0.025/0.013	0.05/0.013
Cinnamomum verum	Ceylon cinnamon	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	Coriander	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	Spanish oregano	Spain	Flowering plant	Carvacrol (76), thymol (5%)	0.025/0.003	0.025/0.006	0.013/0.006	0.025/0.013
Cymbopogon citratus	Lemongrass	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	Indian lemongrass	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	Indian palmarosa	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	Ceylon citronella	Skri 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	Java citronella	Vietnam	Herb grass	Citronnellal (34), geraniol (21), Citronnellol (11)	>0.8/0.05	0.4/0.1	0.05/0.025	0.4/0.2
Eugenia caryophyllus	Clove	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	Sweet inula	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	Reydovan lavandin	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	Spike cineole lavander	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	Narrow leaf melaleuca	Australia	Leaf	Terpine-ol-4 (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	Oregano	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	Greek oregano	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	Sweet marjoram	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	Allspice	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	Summer savory	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	Winter savory	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	Spanish thyme	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	Borneol/carvacrol thyme	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	Mother of thyme	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	Common carvacrol thyme	France	Flowering plant	Cavacrol (33), *p*-cymene (24), thymol (12)	0.05/0.003	0.05/0.006	0.025/0.013	0.1/0.013
Thymus vulgaris linaloliferum	Common linalol thyme	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	Common thyjanol thyme	France	Flowering plant	*Trans*-thujanol-4 (44), mycene 8-ol (13)	0.8/0.05	0.4/0.1	0.4/0.2	>0.8/0.4
Thymus vulgaris thymoliferum	Common thymol thyme	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

[a] According to the data of the gas chromatography analysis of essential oils provided by the manufacturer

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Running title: Antimicrobial effects of alginate based film containing essential oils for preservation of meat.

Antimicrobial Effects of Alginate Based Film Containing Essential oils for the preservation of Whole Beef Muscle

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Résumé

Des films biodégradables à base d'alginate, contenant 1% (wt/v) d'huile essentielle d'origan d'Espagne (o), 1% (wt/v) d'huile essentielle de cannelle de Chine (c) et 1% (wt/v) d'huile essentielle de sarriette des montagnes ont été traités dans 2% (wt/v) ou 20% (wt/v) de solution de $CaCl_2$ et appliqués sur des tranches de muscle de boeuf pour contrôler la croissance d'*Escherichia coli* O157:H7 et de *Salmonella* Typhimurium. Des surfaces entières de muscle de boeuf ont été inoculées avec 10^3 UFC/cm^2 de ces bactéries. Des échantillons de viande ont été quotidienement pris pendant 5 jours de stockage pour l'analyse microbiologique. La disponibilité des composés actifs des huiles essentielles présents dans les films a été évaluée par la détermination des composés phénoliques totaux pour les films à base d'origan d'Espagne et de sarriette des montagnes et des aldéhydes totaux pour les films à base de cannelle de Chine pendant le stockage. Après 5 jours d'entreposage, l'application des films bioactifs sur des surfaces de viande a montré que les films à base d'origan d'Espagne ou de cannelle de Chine étaient les plus efficaces contre *S.* Typhimurium indépendement du type du prétraitement utilisé (2% ou 20% de $CaCl_2$). Pendant la même période, la viande inoculée avec *E. coli* O157:H7 et enduite avec des films à base d'origan d'Espagne traités dans 2% de $CaCl_2$ a montré un niveau bactérien significativement inférieur ($P \leq 0.05$) par rapport au niveau bactérien trouvé lorsque les films à base de cannelle de Chine ou de sarriette des montagnes ont été appliqués (1.33, 1.83 et 1.98 UFC/cm^2 respectivement). Les comptes cellulaires pour *E. coli* O157:H7 étaitent plus élevées quand les films ont été traités préalablement avec 20% de $CaCl_2$, des valeurs respectives de 2.29, 2.36 et de 3.04 UFC/cm^2 ont été retrouvées dans les échantillons enduits de films à base d'origan, de cannelle et de sarriette à la fin de la période de stockage. L'évaluation de la disponibilité des composés actifs dans les films a prouvé que la disponibilité des composés phénoliques dans les films à base d'origan d'Espagne et de sarriette des montagnes était sensiblement plus d'importante ($P \leq 0.05$) que celle des aldéhydes dans les films à base de cannelle de Chine indépendement du prétraitement utilisé (2% ou 20% de $CaCl_2$). En effet, à la fin du stockage, un taux de libération de 40%, de 60% et de 77% a été noté dans les films à base d'origan, de sarriette et de cannelle traités dans 2% de $CaCl_2$ et un taux de 65%, de 62% et de 90% respectivement a été noté dans les mêmes films traités dans 20% de $CaCl_2$.

Abstract

Alginate based edible films, containing 1% (wt/v) of Spanish oregano (O), 1% (wt/v) of Chinese cinnamon (C) or 1% (wt/v) of savory (S) essential oils were treated in a 2% (wt/v) or a 20% (wt/v) $CaCl_2$ solution which was applied on beef muscle slices to control the growth of *E. coli* O157:H7 and *S.* Typhimurium. Whole beef muscle surfaces were inoculated with 10^3 CFU/cm^2 with one of these strains. Meat samples were periodically sampled during the 5-day storage for microbiological analysis. The availability of active compounds from essential oils present in films was evaluated by the determination of total phenolic compounds for Spanish oregano and savory-based films and of total aldehydes for Chinese cinnamon-based films during storage. After 5 days of storage, the application of bioactive films on meat surfaces showed that films containing Spanish oregano or Chinese cinnamon were the most effective against *S.* Typhimurium nevertheless the type of the pretreatment used (2% or 20% of $CaCl_2$). During the same period, meat inoculated with *E. coli* O157:H7 and coated with films treated with 2% of $CaCl_2$ showed a significantly lower ($P \leq 0.05$) content of bacteria when Spanish oregano-based films was used as compared to films containing Chinese cinnamon or savory (1.33, 1.83 and 1.98 log CFU/cm^2, respectively). Whereas the content of *E. coli* O157:H7 was higher when films were pretreated with 20% of $CaCl_2$, with respective values of 2.29, 2.36 and 3.04 log CFU/cm^2 in O, C and S-based films at the end of storage period. The evaluation of the active compounds availability in films showed that this availability in Spanish oregano and savory based films was significantly more important ($P \leq 0.05$) than the one in Chinese cinnamon based films independently of the type of the pretreatment used (2% or 20% of $CaCl_2$). Indeed, at the end of the storage, a release rate of active compounds of 40%, 60% and 77% was noted in O, S and C-based films treated in 2% of $CaCl_2$ and a release rate of 65%, 62% and 90% was noted in the same films but treated in 20% of $CaCl_2$.

Keywords: Whole muscle meat, antimicrobial edible film, essential oils, phenolic compounds, aldehydes, *E. coli* O157:H7, *S.* Typhimurium.

Introduction

A variety of foods have been associated with *Escherichia coli* O157:H7 outbreaks, mainly raw or under cooked foods of bovine origin *(1)* and also other foods are contaminated with animal feces *(2, 3)*. The contamination by *E. coli* O157:H7 has been reported in cattle carcasses at slaughter *(4, 5)* and in minced beef *(5, 6)*. The serotype O157:H7 of *E. coli* strain is more frequently associated with haemorrhagic colitis and haemolytic uraemic syndrome *(7, 5)*. Beef-processing equipment is potentially a source of contamination by pathogenic bacteria such as *E. coli* O157:H7 and *Salmonella* sp. *(8, 9)*. The emergence of *Salmonella* Typhimurium DT104, an antibiotic-resistant organism has been reported in many countries, including the United states and Canada *(10)*. Indeed, resistance to antibiotcs of *Salmonella* sp. and *E. coli* are reported to be high and are rising in certain countries *(11-15)*.

Nowadays, it remains an important aim for the meat industry to control the prevalence of these pathogenic bacteria. The use of natural compounds to control their growth has become preferred by customers and hence is of great interest *(16)*. Essential oils extracted from plants or spices are rich sources of biological active compounds *(17, 18)* such as flavonoids and phenolic acids, which exibit a wide range of antimicrobial properties *(19, 20)*. According to Conner et al. *(21)* the active compounds present in essential oils would be responsable of the inactivation of a variety of enzyme systems in the microbial cells and making them more sensitive to antimicrobial preservatives. Several studies showed the antimicrobial activity of natural extracts. Smith-Palmer et al. *(22)* showed that cinnamon and thyme oils are active against *Campylobacter jejuni, Salmonella enterica, E. coli, Staphylococcus aureus and Listeria monocytogenes.* According to Friedman et al. *(23),* oregano, cinnamon, thyme and clove bud essential oils had a bactericidal activity against *Campylobacter jejuni, E. coli* O157:H7, *L. monocytogenes* and *S. enterica. Satureja* essential oils was found to possess a high antibacterial potential against a wide range of microorganisms *(24)*. The addition of oregano , mint and finely ground rosemary essential oils in liver sausages, aubergine salad, fish, pâté, taramasalad, tzatziki and sterile beef extracts has been found to inhibit *L. monocytogenes, S. aureus, E. coli* and *Salmonella* sp. *(25- 31)*.

The incorporation of natural antimicrobial compounds into edible packaging films or coatings is a new method to control pathogenic growth and so, food contamination and quality loss must be acessed. Antimicrobial packaging is part of the broader area of active packaging who became in the last decade, one of the major areas of research in food packaging *(32, 33)*, since their biodegradability and environmental compatibility are assured *(34, 35)*. It can play an important role in reducing the risk of pathogen development, as well as extending the shelf life of foodstuffs *(36)*. The coating can especially be useful as a carrier of antimicrobial compounds in order to maintain high concentrations of these compounds on the food surface. The incorporation of organic acids and essential oils in edible films packaging or coatings contributed greatly to control bacterial growth during the storage of meat and oranges *(37-41)*. Active milk protein based film could allow a progressive release of the antimicrobial compounds *(38)*. However, in order to improve the water resistance and the mechanical properties of the films, films based on a mixture of alginic acid and polycaprolactone has been developped. Poly (ε-caprolactone) (PCL) is a semicrystalline biodegradable polyester, derived from chemical synthesis. It has good water resistance, a low melting point, a low glass transition temperature and a good processability *(42, 43)*. It has been shown that PCL exhibited desirable characteristics as a diffusion-controlled delivery system, including biodegradability, biocompatibility, commercial availability and affordability *(44)*. In this study, PCL was used as both an hydrophobic and emulsifying agent to stabilize essential oils in the alginate matrix. Alginate, a natural polysaccharide extracted from seaweed, is a copolymer of alterning sequences of β-D-mannuronic and α-L-guluronic acid residues linked by 1→ 4 glycosidic bonds *(45)*. Several studies suggested alginate as an interesting film matrix, on account of its biocompatibility, low toxicity *(46)* and insoluble film formation by ionotropic gelation *(47)*. It is recognized that alginate films possess good oxygen barrier properties *(48)*, prevent lipid oxidation *(49)* and improve food flavor and texture *(34)*.

The aim of this study was (i) to evaluate the type of the pretreatment used (2% or 20% of $CaCl_2$) and the antimicrobial potential of Chinese cinnamon, Spanish oregano and savory-based films against the growth of two pathogenic bacteria *E. coli* O157:H7 and *S.* Typhimurium on beef muscle; (ii) to evaluate the controlled release of active compounds present in films toward meat during storage.

Material and Methods

Films preparation.

Alginate-based films were prepared according to a method developed in our laboratory (50). Sodium alginate and polycaprolactone diol (m_n 1250) were purchased from Sigma-Aldrich Ltd. (Oakville, Ont, Canada). The polycaprolactone (1% wt/v) was slowly solubilized in distilled water at 45 °C for 15 min. Meanwhile, alginate was solubilized at a concentration of 3 % (wt/v) in distilled water at room temperature under agitation during two hours. The Polycaprolactone solution was then mixed with the alginate solution. Glycerol was added to this mixture at a final concentration of 2% (wt/v) *(51)*. The alginate-polycaprolactone solution was then heat-sterilized at 121°C during 30 min. The mixture was maintained at 40-50°C, under strong agitation in order to maintain the miscibility of the solution. Spanish oregano (*Corydothymus capitatus*), Chinese cinnamon (*Cinnamomum cassia*) and savory (*Satureja montana*) essential oils (Robert et fils, Qc, Canada) was added at a final concentration of 1% (wt/v). The film solution was homogenized at room temperature at 25.000 rpm during 1 min using an homogenizer Ultra-Turrax TP18/1059 (Janke & Kunkel, Staufen, Allemagne). For each film solution, a volume of 5 ml was spread in Petri dishes (50X11 mm) and dried at ambient temperature under 40-50 % of relative humidity (R.H) for 24h, to form a homogeneous and transparent film. After drying, the water-soluble films we immersed in a $CaCl_2$ solution (2% for 1 min or 20% for 20 min), in order to compare the effect of this treatment on the release of active compounds towards beef muscle. The immersion treatment allowed the formation of an insoluble gel by the formation of ionic connections in the presence of multivalents cations of the calcium.

Preparation of bacterial strains.

E. coli O157:H7 EDL 933 (INRS-Institut Armand Frappier, Laval, Qc, Canada) and *S.* Typhimurium SL1344 (INRS-IAF, Laval, Qc, Canada) stock cultures were maintened at –80 °C in BHI containing 10% glycerol. Prior to the experiment, strains were individually subcultured twice [1.0% (v/v)] in Brain-Heart Infusion (BHI, Difco Laboratoires, Detroit, MI) and incubated at 35 ± 1°C.

Sample prepatation.

Beef from the semimembranous (inside roud) (15 kg), was purchased at a local grocery stire (IGA, Laval, Qc, Canada) and transported to the INRS-Institut Armand Frappier and the Canadian Irradiation Center (CIC) under refrigerated conditions in an ice-filled cooler. Beef muscle, was cut into 225 pieces (150 pieces for microbiological analysis and 75 pieces for biochemical analysis) of equal thickness (7.5 mm) and diameter (47 mm) using a pastry cutter. The 225 beef muscle pieces (10 pieces/package) were placed in a sterile bags (0.5 mm mettalized polyester/2 mm EVA copolymer; 305 mm X 210 mm, Winpak, St-leonard, Qc, Canada) and sealed under vacuum. The packages were stored overnight at –80 ± 1°C until sterilization treatment by irradiation. The irradiation treatment was done at a dose of 25 kGy and at a dose rate of 22.928 kGy/h, using a ^{60}Co source UC-15A (MDS-Nordion International Inc, Kanata, On, Canada). After irradiation, 150 pieces of beef muscle was inoculated with *E. coli* O157:H7 and *S.* Typhimurium to obtain a final concentration of 10^3 colony-forming units (CFU)/cm^2. Each sample of beef muscle was inoculated with a volume of 50 µl, which were spread over the entire upper surface of samples. For the biochemical analysis, the 75 pieces of beef muscle were not inoculated. Meat samples were divided into five separate groups: (i) the control group whithout films (M); (ii) alginate-based films coating whithout essential oil (w); (iii) alginate-based films coating containing Spanish oregano oil (O); (iv) alginate-based film coating containing Chinese cinnamon oil (C); and (v) alginate-based film coating containing savory oil (S). Beef muscle samples of each group were placed individually into sterile Petri dishes (50 X 11 mm) and, except for the control, all samples were covered on either side with one of the corresponding films. Petri dishes were sealed hermetically and stored at 4 ± 1°C; samples were tested periodically (days 1, 2, 3, 4, and 5) for microbiological and biochemical analysis. Day 1 corresponded to the day of samples preparation.

Bacterial Enumeration

Each beef muscle sample was homogenized for 2 min in 153 ml of sterile peptone water [0.1% (wt/v), Difco Laboratories] using a Lab-blender 400 stomacher (Seward Medical, London,

U.K.). From this homogenate, serial dilutions were prepared, and appropriate dilutions were spread plated on sterile Petri plates containing TSA (Tryptic Soya Agar, Difco laboratories). Then, TSA plates were incubated for 24 h at 35 ± 1°C, and results were expressed as log CFU/cm^2 of bacteria in meat.

Active Compound Availability.

To evaluate the availability of the active compounds in the packaging films, 1 g of each film samples were cut using a bistoury. The obtained thin strips of films were placed in 50 ml of ethanol solution and kept under agitation with a magnetic stir bar at room temperature for 30 min. Then, the extract was filtered through a Whatman 0.45 μm filter paper (Whatman International Ltd.). Total phenolic compounds content in each film extract was determined according to the procedure of Folin-Ciocalteu *(52)*. The availability of total aldehydes in films containing cinnamon oil was determined following the Kleber method using phenylhydrazine reagent *(53)*.

Statistical analysis of data

Experiments for microbiological analysis were done using a 1 X 2 X 5 X 2 X 2 X 5 factorial design [1 kind of meat (beef muscle), 2 replicates, 5 treatments (control, film whithout essential oils, film with Spanish oregano, Chinese cinnamon or savory essential oils), 2 bacteria (*E. coli* O157:H7 and *S.* Typhimurium), 2 kind of films (insolubilized in 2% of $CaCl_2$ or 20% of $CaCl_2$ solution), and 5 storage period (1, 2, 3, 4, and 5 days)]. Analysis of variance and Duncan's multiple-range tests were performed on all results. Differences between means were considered to be significant when $p \leq 0.05$. Stat-Packets Statistical Analysis sofware (Walonick Associates, Inc., Minneapolis, MN) was used for the analysis. The experiment was done twice and for each measurement, three samples in each replication were tested.

Results and Discussion

Microbiology.

Effects of edible films on *E. coli* O157:H7 and *S.* Typhimurium counts during storage are presented in Tables 1 and 2, respectively. Results showed that the *E. coli* O157:H7 content was stable in M samples during the whole storage period (Table 1). The use of film containing essential oils also reduced significantly ($P \leq 0.05$) the *E. coli* level on the meat surface as compared to meat samples coated with film without essential oil during storage. Films immersed in $CaCl_2$ solution (2%) had higher antimicrobial properties than films immersed in $CaCl_2$ solution (20%). A same effect was observed with W-based film pre-treated in $CaCl_2$ solution (2%), whereas a significant increase of microorganism level was noted at day 2 in meat coated with W-based films treated with $CaCl_2$ solution (20%), showing a count of 3.93 log CFU/cm^2 as compared to 3.27 log CFU/cm^2 at day 1. This level was maintened during 4 days of storage, then a significant decrease ($P \leq 0.05$) was observed at day 5, to reach 3.29 log CFU/cm^2. When meat is coated with O-based films, a significant decrease ($P \leq 0.05$) of the microbial count was observed only at day 5 with a value of 1.33 log CFU/cm^2, whereas meat coated with C and S-based films showed a significant decrease ($P \leq 0.05$) of microbial count at day 2, with respective values of 2.15 and 2.59 log CFU/cm^2. Indeed, after two days of storage, a 1.15 log and 0.7 log reduction of *E. coli* O157:H7 was observed respectively in meat covered with C and S-based films. After five days of storage, a 1.47 and 1.32 log reduction was observed respectively for the same treatment. Until day 5, C and S-based films were significantly ($P \leq 0.05$) more efficient than O-based films. In O-based films, the *E. coli* content at day 5 was significantly lower ($P \leq 0.05$) than the content observed in C and S-based films, showing a respective values of 1.33, 1.83, and 1.98 log CFU/cm^2.

The results obtained on meat coated with O based films and treated in $CaCl_2$ solution (20%) showed a significant decrease of *E. coli* O157:H7 contents only after 4 days of storage with a level of 2.45 log CFU/cm^2. This value was similar to the one observed at day 4 in meat coated with C-based films (2.42 log CFU/cm^2). At day 5, a 1 log reduction ($P \leq 0.05$) was observed in O and C-based films, showing a respective values of 2.29 and 2.36 log CFU/cm^2,

whereas a significant difference ($P > 0.05$) was observed in meat coated with S-based films between the days of storage, showing a microbial stability during the whole storage period.

Results on *S.* Typhimurium are presented in Table 2. Results showed that at day 2 of storage, a significant increase ($P \leq 0.05$) of 1 log was observed in M samples. A minor increase of microbial growth was noted in meat coated with W-based films independently of the pre-treatment used (immersion in 2% vs 20% of $Cacl_2$), similar content of *S.* Typhimurium (3.92 and 3.44 log CFU/cm^2 respectively) after 5 days of storage. A significant decrease ($P \leq 0.05$) of 0.76 log was observed in meat coated with O-based films treated in $CaCl_2$ (2%) at day 3 of storage, with a value of 2.32 log CFU/cm^2, whereas in meat coated with C and S-based films, a significant reduction ($P \leq 0.05$) occured at day 2, with a microbial count of 2.49 and 2.87 log CFU/cm^2, respectively. At the end of storage, the content of *S.* Typhimurium in meat coated with O, C and S-based films were, respectively, 2.10, 2.12 and 2.47 log CFU/cm^2. These results showed that O, C and S-based films were able to reduce by 0.98, 0.96 and 0.61 log the content of *S.* Typhimurium after five days of storage. Also, O and C-based films were significantly ($P \leq 0.05$) more efficient than S-based films.

Results obtained in films immersed in $CaCl_2$ solution (20%) showed that O, C and S-based films were efficient to control the growth of *S.* Typhimurium. At day 2, a 0.98, 0.81 and 0.86 log reduction ($P > 0.05$) was observed in meat coated with O, C and S-based films, respectively. Also, any significant difference ($P \geq 0.05$) was observed between these samples at day 2 of storage. At the end of the storage, the content of *S.* Typhimurium in O, C and S-based film was respectively, 2.21, 2.24 and 2.49 log CFU/cm^2. These results showed that O and C-based films were most efficient against the growth of *S.* Typhimurium, by allowing a significant reduction ($P \leq 0.05$) of 0.91 and 0.88 log, respectively.

Our study showed that the incorporation of essential oils at 1% (w/v) into alginate coating film formulation allowed a significant decrease ($P \leq 0.05$) of *S.* Typhimurium and *E. coli* O157:H7 content on beef muscle samples. Independently of the pre-treatment used (immersion in 2% vs 20% of $CaCl_2$), the level of microorganism tested in meat coated with O, C and S-based films was significantly ($P \leq 0.05$) lower than the one in W-based films and M samples. Our data

also suggest that O, C and S-based-coating films treated by immersion in $CaCl_2$ solution (2%) were a little more efficient against *E. coli* O157:H7 than the ones insolubilized in $CaCl_2$ solution (20%). The calcium seems to have a protective effect on *E. coli* O157:H7 growth. This phenomena can be explained by the fact that calcium play a role for the development of bacterial competence, an inducible property of many bacterial species *(54)*. In presence of $CaCl_2$, the LPS of certain *E. coli* is modified by induction of a phosphoethanolamine transferase, which might ensure resistance to environmental stresses, such as exposure to certain detergeants and antibiotics *(55)*. Also, Hauben et al. *(56)* showed that the presence of divalents cations such as 0.5 m mol/l of $CaCl_2$, provided a protection effect on *E. coli,* by reducing high pressure hydrostatic inactivation. The reduction level observed with *S.* Typhimurium on beef muscle coated with either essential oils-based films insolubilized with 2% or 20% of $CaCl_2$ solution was similar and no difference was observed between them ($P > 0.05$).

In absence of essential oil in films, both bacteria seem to use the content of the film, like alginate as a nutrient to maintain their growth. The essential oils evaluated in this study were already found as being effective *in vitro* against a wide spectrum on microorganism. Yuste and Fung *(57)*, founded that cinnamon added to the apple juice with nisin contributed greatly to the inactivation of *S.* Typhimurium and *E. coli* O157:H7 after 3 and 7 days of storage, respectively. Chang et al. *(58)* founded that cinnamon essential oils had a minimal inhibitory concentration (MIC) of 500 µg/ml and 250 µg/ml against *Salmonella* sp. and *E. coli* O157:H7, respectively. Cinnamon extract mixed with two other extracts such as Chinese chive and corni fructus led to the expected antimicrobial effect in orange juice, pork and milk on *E. coli* and *L. monocytogenes (59)*. As observed by Dadalioglu and Evrendilek *(60)*, oregano essential oils from Turkia exhibited a very strong antibacterial activity against *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium, and *S. aureus*. At 0.8%, oregano essential oil was able to reduce by 2 to 3 log the lactic acid bacteria and *L. monocytogenes* on beef meat fillets *(29)*. These results are in according with those obtained by Skandamis et al. *(61),* who showed that 0.8% of oregano essential oils reduced the majority of the microbial population of lactic bacteria and *S.* Typhimurium on meat. Also, Chorianopoulos et al. *(62)* showed that savory species from Greece possess remarkable bactericidal properties against *Salmonella* sp. *E. coli* O157:H7, *S. aureus, Bacillus cereus* and *L. monocytogenes*. Our results also showed that the antimicrobial properties

of essential oils tested were not affected by their incorporation in alginate based films. Indeed, the incorporation of antimicrobial agents in films was effective to prolong their antimicrobial effect. Cutter and Siragusa. *(63)*, have shown that *Brochothrix thermosphacta* contents was reduced by 2 log after 7 days of storage, by immobilization of nisin in a calcium alginate gel; it may be a more effective delivery system of the bacteriocin to the carcass beef surfaces than direct application. Mauriello et al. *(36)*, observed a decrease of about 1 log of *L. monocytogenes* contents in pork steak and ground beef, by incorporation of bacteriocin in an active polythene films, an application which allowed a highest antimicrobial activity. Application of milk protein-based edible films containing 1% (wt/v) of oregano essential oils allowed a significant ($P \leq 0.05$) reduction of 0.95 and 1.12 log of *Pseudomonas* sp. and *E. coli* O157:H7 level, respectively after 7 days of storage *(55)*. Pronato et al. *(64)* also showed that the edible alginate-based films containing garlic oil exhibited an antimicrobial activity on *S. aureus* and *B. cereus*, tested by agar diffusion assay.

Chemical analysis of essential oils provided by the manufacturer showed that Spanish oregano essential oils was composed essentially of carvacrol (76%) and thymol (4.47%), two phenolic monoterpenes. Tested Chinese cinnamon essential oils was rich in aldehydes: cinnamaldehyde (65%), and methoxy-cinnamaldehyde (21%). Savory essential oils were composed of phenolic monoterpenes: carvacrol (5.70%) and thymol (42.71%), and nonoterpenic hydrocarbons: γ-terpinene (8.63%) and *p*-cymene (11.68%). The hydroxyl group present in the structure of phenolic compounds confers its antimicrobial activity, and the relative position of the hydroxyl group has a great importance on the effectiveness of these components *(65)*. This fact can explain the difference obtained for the antimicrobial activity of carvacrol and thymol. This hypothesis is consistant with our results, showing that Spanish oregano essential oils had better antimicrobial activity than savory essential oils. It is known that the antimicrobial activities of essential oils are predominately related to their main components *(66)*. The antimicrobial potential of Spanish oregano and savory essential oils is related to their high concentration in phenolic content *i.e.* carvacrol and thymol *(67, 68)*. Cinnamaldehyde is one of the most active compound present in Chinese cinnamon essential oils. Ultee et al. *(69)* founded that 0.5 mM of carvacrol and 0.25 mM of cymene reduced the viable count of *B. cereus* in rice to 50% within 48 min. Indeed, it seems that carvacrol is very effective against foodborne pathogens. Kim et al. *(70)*

have shown a strong bactericidal activity on *E. coli* O157:H, *L. monocytogenes*, *S.* Typhimurium and *Vibrio vulnificus*. Lambert et al. *(68)* have observed a high sensitivity of *S. aureus* to a mixture of thymol and carvacrol. Karapinar et al. *(67)* showed that thymol was effective against the growth of *S. aureus* and *V. parahaemolyticus* showing a MIC of 75 µg/ml for both bacteria. Burt *(71)* reviewed that carvacrol, thymol, eugenol, perillaldehyde, cinnamaldehyde and cinnamic acid have been identified as antibacterial agents on *L. monocytogenes, S.* Typhimurium, *E. coli* O157:H7, *Shigella dysenteria, B. cereus* and *S. aureus*, and exhibit a minimum inhibitory concentrations (MICs) of 0.05-5 µl/ml *in vitro*, whereas a higher concentration, around 0.5-20 µl/g is needed to achieve the same effect in foods. Kwon et al. *(72)* have evaluated the antimicrobial activity of cinnamic aldehyde on *B. cereus* and have estimated its bacteriocidal effect at 0.3 ml/l. Chang et al. *(58)* founded that cinnamaldehyde was effective on *E. coli*, *P. aeruginosa, K. pneumoniae* and *Salmonella* sp. Cinnamaldehyde has been well known to possess strong inhibitory effects against various micro-organisms as well as against toxin production *(72-74)*. According to Ultee et al. *(69)*, carvacrol attacks the membrane, while making it permeable to the potassium ion and to protons, thus acidifying the cytoplasm of the bacteria, making impossible the synthesis of ATP and so decreasing of the quantity of intracellular ATP. Also, carvacrol has been reported to desintegrate the outer membrane and increased the permeability of ATP through cytoplasmic membrane *(72)*. Indeed, Helander et al. *(75)*, founded that thymol and carvacrol have the ability to desintegrate the membrane of *E. coli* and *S.* Typhimurium at a concentration close to the MIC. Whereas, cinnamaldehyde being electronegative compound *(76)* act in its turn by the interferance on the level of the transfer of electron and while reacting by substitution with the nucleic acids and the proteins *(65)* or by inhibiting amino acid decarboxylase activity *(77, 78)*. Contrary to several antibiotics, the hydrophobic compounds presents in essential oils can reach the periplasmic space of Gram- negative bacteria through the proteins porins *(75, 68)*, inhibiting the direct transport of cellular proteins and also the active transport of the nutrients and interfering with the elements which play a role in FMP (Force proton motrice) *(79)*. The hydrophobicity of essential oils enables them to partition in the lipids of the cell membrane and mitochondria, rendering them permeable and leading to leakage of cell contents *(71)*.

Active compounds availability in films applied on meat

The availability of active compounds in Spanish oregano, Chinese cinnamon and savory-based films was determined during the whole storage period at 4°C (Table 3). The active compounds are respectively phenolic compounds (carvacrol and thymol) in O and S-based films and aldehydes (trans and methoxy-cinnamaldehyde) in C-based films. Initial percentage of active compounds was 100% in O, C and S-films nevertheless the type of the pre-treatment used (immersion in 2% or 20% of $Cacl_2$ solution). During all the storage period, a progressive release of the active compounds was noted for the two types of films. Results obtained with films treated with 20% of $CaCl_2$ solution showed a significant decrease ($P \leq 0.05$) of active compounds during time independently of the film used. At day 2, the concentration of active compounds present in films was: 50.16, 42.74 and 63.60 % in O, C, and S-based films, respectively. Our results showed that during the whole storage period, the availability of active compounds in C-based films was significantly lower ($P \leq 0.05$) than the active compounds availability observed in O and S-based films. Moreover, the results showed that during storage, the availability of phenolic compounds in S-based films was significantly higher ($P \leq 0.05$) than those in O-based films. However, any significant difference ($P > 0.05$) was noted between the concentration of phenolic compounds present in O and S-based films at day 4. These data suggest that the active compounds migration in films containing Chinese cinnamon to beef muscle is more important than the one occurred with Spanish oregano and savory based films. Indeed, a release level of active compounds of 65%, 62% and 90% was recorded from O, S and C-based films, respectively, after 5 days of storage. The active compounds availability present in films treated with $CaCl_2$ solution (2%) was also determined and the results are presented in Table 3. Between days 1 and 5, a significant decrease ($P \leq 0.05$) of the active compounds availability was observed in all films evaluated. At day 2, the availability of active compounds in films was 71.91, 60.92 and 53.75% in O, C and S-based films respectively. This level remained stable between days 2 and 3 in O and S-based films, whereas, a progressive loss of active compounds was observed in C-based films at the third day of storage, showing a concentration of 30.04%. A significant ($P \leq 0.05$) decrease of active compounds availability occured at the fourth day of storage for O and S-based films, showing respective levels of 61.21 and 39.75%. However, the level of active compounds was then stable until the last day of storage. The results also showed a significant decrease ($P \leq 0.05$) of active

compounds availability at day 4 in C-based films, showing a level of 23.59%. As for the two other types of films containing essential oils, the active compounds release doesn't change during the two last days of storage as compared to the results obtained at day 4. According to these results, it seems that even for the films treated with $CaCl_2$ solution (2%), active compounds present in Chinese cinnamon were more released towards the beef muscle than those of Spanish oregano and savory essential oils during all period of storage. Also, savory based films salted out more than the oregano based films during the same period of time. Indeed, a release of 60%, 40% and 77% of the active compounds was noted for the S, O and C-based films, respectively, at the end of storage.

The comparison between the films immersed in $CaCl_2$ solution (2% and 20%) showed a similar behavior between the releases of the active compounds during the storage period. Between days 2 and 4, a mean release of 39% of the active compounds was observed in the C-based films treated in 2% and 20% of $CaCl_2$. The phenolic compounds release from S-based films treated with $CaCl_2$ solution (20%) was similar to the release observed in films treated with $CaCl_2$ solution (2%). Between day 2 and 4, a respective release of 62 and 74 % was observed. In O-based films, between day 2 and 4, a more important release was noted in films treated with $CaCl_2$ (2%) as compared to films treated with $CaCl_2$ (20%).

Many interactions may occur during oils diffusion from polymer to beef muscle. In particular, water uptake by the film from the meat may cause the polymer to swell *(80)* and migration rates change continuously because of a time-dependent relaxation process resulting from swelling stress *(81)*. The oil diffusion would also be expected to vary with respect to their respective composition, their polarity due to the structural configuration of the constituent compounds and their functionnal groups. Indeed, the hydrophobicity of these compounds, which is related to their structure, could have an influence on the migration towards the beef musle *(82, 83)*. The high release rate of Chinese cinnamon oil can be explained by the polar character of trans-cinnamaldehyde and 2-methoxycinnamaldehyde, which are the main components of Chinese cinnamon oil, implying a better affinity with water present in beef muscle, as compared to carvacrol and thymol, the main components of Spanish oregano and savory oils. Indeed, Helander *et al. (75)* showed that the solubility of cinnamaldehdye in water at 20°C was higher

than the solubility of carvacrol and thymol. Moreover, it seems that a better insolubilization treatment of films (20% *vs* 2 % $CaCl_2$) provides a similar level of availability of active compounds in O and S-based films the end of storage. The release of active compounds in C-based films was faster than with O- and S-based films during the first three days of storage. It is also interesting to note that there was any significant difference ($p > 0.05$) between mechanical properties of films treated with 2% $CaCl_2$. Nevertheless, for films treated with 20% $CaCl_2$, puncture resistance of savory films (68.11 Newton/mm) was significantly higher ($p \leq 0.05$) than for film containing Spanish oregano and Chinese cinnamon (57.65 and 49.29 Newton/mm, respectively) (data not shown). These results show that the release profile of active compounds is a complexe phenomena.

In conclusion, the incorporation of essential oils into alginate-based edible films helped to reduce *E. coli* O157:H7 and *S.* Typhimurium growth onto beef muscle during 5 days of storage. A higher concentration of the active compounds was maintained with the films treated with 2 % of $CaCl_2$. This means that films treated with 20% of $CaCl_2$ would be used with a shorter shelf life foodstuff than films treated in 2% of $CaCl_2$. Also, the treatment of the films in 2% and 20% of $CaCl_2$ may make to its structure a perfect obstruction to the active compounds release towards beef muscle during storage. The use of edible films containing Spanish oregano, Chinese cinnamon and savory essential oils, as a preservation method of beef muscle, is promising.

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Table 1. Growth of *E. coli* O157:H7 during storage at 4°C.

		Log CFU/cm$^{2\ a,\ b}$				
Treatment	**Sample[c]**	**day 1**	**day 2**	**day 3**	**day 4**	**day 5**
2% $CaCl_2$	**M**	3.30 ± 0.20 a A	3.20 ± 0.09 c A	3.16 ± 0.38 b A	3.11 ± 0.10 b A	3.12 ± 0.41 c A
	W	3.30 ± 0.20 a A	3.27 ± 0.48 c A	3.08 ± 0.42 b A	3.07 ± 0.44 b A	3.25 ± 0.39 c A
	O	3.30 ± 0.20 a B	3.08 ± 0.31 c B	3.04 ± 0.51 b B	3.07 ± 0.49 b B	1.33 ± 0.29 a A
	C	3.30 ± 0.20 a C	2.15 ± 0.14 a B	2.00 ± 0.35 a A B	1.97 ± 0.22 a A B	1.83 ± 0.23 b A
	S	3.30 ± 0.20 a C	2.59 ± 0.30 b B	1.99 ± 0.22 a A	1.99 ± 0.22 a A	1.98 ± 0.15 b A
20% $CaCl_2$	**M**	3.27 ± 0.27 a A	3.20 ± 0.43 a A	3.14 ± 0.32 a A	3.06 ± 0.16 b A	3.04 ± 0.44 b A
	W	3.27 ± 0.27 a A	3.93 ± 0.36 b B	3.74 ± 0.31 b B	3.76 ± 0.34 c B	3.29 ± 0.39 b A
	O	3.27 ± 0.27 a B	3.27 ± 0.39 a B	3.19 ± 0.19 a B	2.45 ± 0.28 a A	2.29 ± 0.37 a A
	C	3.27 ± 0.27 a C	2.95 ± 0.32 a B	2.96 ± 0.34 a B	2.42 ± 0.24 a A	2.36 ± 0.20 a A
	S	3.27 ± 0.27 a A	2.98 ± 0.19 a A	2.98 ± 0.40 a A	3.03 ± 0.44 b A	3.04 ± 0.39 b A

[a] Means for each treatment and in the same column bearing the same lower case letters are not significantly different ($p > 0.05$). [b] Means in the same row bearing the same upper case letters are not significantly different ($p > 0.05$); [c]M, meat whithout film; W, films whitout essential oil; O, films containing Spanish oregano essential oil; C, films containing Chinese cinnamon essential oil; S, films containing winter savory essential oil.

Table 2. Growth of *S.* Typhimurium during storage at 4°C.

		Log CFU/cm$^{2\ a,\ b}$				
Treatment	**Sample[c]**	**day 1**	**day 2**	**day 3**	**day 4**	**day 5**
2% $CaCl_2$	**M**	3.08 ± 0.22 a A	4.03 ± 0.65 c B	4.44 ± 0.23 d C	4.25 ± 0.59 c B C	3.11 ± 0.55 c A
	W	3.08 ± 0.22 a A	3.79 ± 0.07 b c B	3.91 ± 0.38 c B C	4.12 ± 0.33 c C	3.92 ± 0.18 d B C
	O	3.08 ± 0.22 a C	3.46 ± 0.21 b D	2.32 ± 0.34 a B	2.09 ± 0.29 a A	2.10 ± 0.39 a A
	C	3.08 ± 0.22 a C	2.49 ± 0.35 a B	2.32 ± 0.24 a A B	2.15 ± 0.33 a A	2.12 ± 0.33 a A
	S	3.08 ± 0.22 a C	2.87 ± 0.29 a B	2.60 ± 0.24 b A	2.53 ± 0.19 b A	2.47 ± 0.27 b A
20% $CaCl_2$	**M**	3.12 ± 0.15 a A	4.04 ± 0.63 c B	4.36 ± 0.29 d B	4.39 ± 0.06 d B	3.06 ± 0.57 c A
	W	3.12 ± 0.15 a A	3.44 ± 0.10 b B	3.47 ± 0.33 c B	3.37 ± 0.41 c B	3.44 ± 0.26 d B
	O	3.12 ± 0.15 a B	2.14 ± 0.22 a A	2.19 ± 0.28 a A	2.19 ± 0.33 a A	2.21 ± 0.29 a A
	C	3.12 ± 0.15 a C	2.31 ± 0.26 a A B	2.51 ± 0.09 b B	2.39 ± 0.27 a b A	2.24 ± 0.30 a A
	S	3.12 ± 0.15 a C	2.26 ± 0.16 a A	2.66 ± 0.31 b B	2.50 ± 0.22 b B	2.49 ± 0.22 b B

[a] Means for each treatment and in the same column bearing the same lower case letters are not significantly different ($p > 0.05$). [b] Means in the same row bearing the same upper case letters are not significantly different ($p > 0.05$); [c]M, meat whithout film; W, films whitout essential oil; O, films containing Spanish oregano essential oil; C, films containing Chinese cinnamon essential oil; S, films containing winter savory essential oil.

Table 3 - Active compounds availability in films during storage at 4°C.

		Percentage of active compounds availability[a, b] (%)				
Treatment	**Film[c]**	**day 1**	**day 2**	**day 3**	**day 4**	**day 5**
2% $CaCl_2$	**O**	100.00 a C	71.91 ± 2.13 b B	70.85 ± 1.82 c B	61.21 ± 4.64 c A	60.62 ± 4.65 c A
	S	100.00 a C	53.75 ± 0.28 a B	53.34 ± 2.18 b B	39.75 ± 2.39 b A	39.13 ± 4.44 b A
	C	100.00 a D	60.92 ± 6.74 a C	30.04 ± 1.19 a B	23.59 ± 3.37 a A	22.78 ± 0.28 a A
20% $CaCl_2$	**O**	100.00 a C	50.16 ± 4.40 b B	47.07 ± 1.74 b B	37.39 ± 2.71 b A	34.86 ± 1.89 b A
	S	100.00 a C	63.60 ± 1.20 c B	59.41 ± 2.48 c B	39.51 ± 2.38 b A	37.84 ± 1.93 c A
	C	100.00 a E	42.74 ± 3.57 a D	24.11 ± 2.06 a C	16.86 ± 0.85 a B	9.94 ± 0.35 a A

[a] Means for each treatment and in the same column bearing the same lower case letters are not significantly different ($p > 0.05$). [b] Means in the same row bearing the same upper case letters are not significantly different ($p > 0.05$); [c]O, films containing Spanish oregano essential oil; S, films containing winter savory essential oil; C, films containing Chinese cinnamon essential oil.

Article 5: Soumis dans Journal of Agriculture and Food Chemistry

Running title: Antimicrobial properties of alginate based films containing essential oils.

Antimicrobial Effects of Alginate Based Films Containing Essential oils for the preservation of Bologna and Ham

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Résumé

Un nombre de 150 tranches de bologne et de jambon ont été inoculées avec *Listeria monocytogenes* et *Salmonella* Typhimurium à une concentration de 10^3 UFC/cm^2. Les films à base d'alginate et d'huiles essentielles d'origan d'Espagne 1% (w/v) (o), de cannelle de Chine 1% (w/v) (c), ou de sarriette des montagnes 1% (w/v) préalablement immergés dans 2% (w/v) ou 20% (w/v) de $CaCl_2$ ont été appliqués sur des tranches de bologne ou de jambon pour contrôler la croissance des bactéries pathogènes. Les résultats ont montré que sur la bologne, les films à base de cannelle traités dans du $CaCl_2$ (20%) étaient les plus efficaces contre la croissance de *L. monocytogenes* et de *S.* Typhimurium. *L. monocytogenes* a été la plus sensible des deux en présence des films à base d'origan d'Espagne, de cannelle de Chine et de sarriette des montagnes. Les comptes étaient sous le seuil de détection après cinq jours d'entreposage avec les échantillons de bologne enduits de films à base d'origan, de cannelle ou de sarriette traités dans du $CaCl_2$ (20%). Les résultats obtenus sur le jambon ont montré une réduction significative ($P \leq 0.05$) de 1.85 log UFC/cm^2 de *S.* Typhimurium avec les échantillons enduits de films à base de cannelle indépendement du prétraitement reçu ou avec les films à base d'origan traités dans 20% de $CaCl_2$ après 5 jours de conservation. Les résultats obtenus ont aussi prouvé que *L. monocytogenes* a été fortement résistante avec les échantillons de jambon enduits de films à base d'origan, de cannelle et de sarriette. Cependant, les films à base de cannelle de Chine traités avec du $CaCl_2$ (20%) étaient les plus efficaces contre la croissance de *L. monocytogenes*. L'évaluation de la disponibilité des composés actifs dans les films a montré une libération significativement ($P \leq 0.05$) importante avec les films à base de cannelle indépendement du prétraitement et des substrats alimentaires utilisés (bologne ou jambon). La disponibilité des composés actifs a été plus élevé dans les films à base d'origan que celle dans les autres films. Le taux de libération des composés actifs des films à base d'origan et de sarriette traités avec du $CaCl_2$ (20%) était plus rapide que celui avec les films traités avec du $CaCl_2$ (2%) indépendement du substrat utilisé (bologne ou jambon).

Abstract

A number of 150 slices of bologna and ham were inoculated with 10^3 CFU/cm^2 of *Salmonella* Typhimurium or *Listeria monocytogenes*. Alginate based edible films prealably immersed in 2% (w/v) or 20% (w/v) $CaCl_2$ solution and containing 1% (w/v) Spanish oregano (O), 1% (w/v) Chinese cinnamon (C), or 1% (w/v) savory (S) essential oils were then applied on bologna or on ham slices to control the growth of the pathogenic bacteria evaluated. Results showed that on bologna, C-based films treated with $CaCl_2$ (20%) were the most effective against the growth of *S.* Typhimurium and *L. monocytogenes*. Moreover, *L. monocytogenes* was the most sensitive bacterium in presence of O-, C- and S-based films. Counts were below detection level

(< 10 UFC/ml) after five days of storage on bologna coated with O-, C- or S-based films treated with $CaCl_2$ (20%). Results obtained on ham showed a 1.85 log CFU/cm^2 reduction of *S.* Typhimurium ($P \leq 0.05$) after 5 days of storage when coated with C- based films independently the type of the pre-treatment used (2% or 20% $CaCl_2$) or when coated with O-based films treated with $CaCl_2$ (20%). Results on *L. monocytogenes* counts showed that this bacterium was highly resistant in ham even in presence of O-, C- or S-based films. However, C-based films treated with $CaCl_2$ (20%) were the most effective against the growth of *L. monocytogenes*. The evaluation of the active compounds availability in films showed a significant higher release level of active compounds in C-based films ($P \leq 0.05$) independently the pre-treatment used or the media of application (bologna or ham). O-based films showed the lower release level of active compounds. The release rate of active compounds from O- and S-based films treated with $CaCl_2$ (20%) was faster than when the same respective films were treated with $CaCl_2$ (2%) independently of the meat matrix used (bologna *vs.* ham).

Keywords: Bologna, ham, antimicrobial edible film, essential oils, phenolic compounds, aldehydes, *L. monocytogenes*, *S.* **Typhimurium**.

Introduction

Contamination of ready-to-eat (RTE) meats with *Listeria monocytogenes* and *Salmonella* sp. occurs mainly during post processing *(1)*. Indeed, the contamination of raw and processed foods is mainly related to *L. monocytogenes*, causing listeriosis, a cause to human deseases *(2)* sucg as meningitis, meningoencephalitis, septicaemia and severe gastroenteritis *(3, 4)* with an overall mortality rate of more than 20% *(4,5)*. *L. monocytogenes* is able to grow under aerobic or anaerobic condition, between -4°C and 50°C, and can survive for long periods of time *(6)*. The incidence of *L. monocytogenes* reaches 1% to 2% on cooked meat products directly after packaging, which can become a significant health hazard when growth continue during storage *(7)*. *Salmonellae* are widely distributed in nature, with humans and animals, being their primary reservoirs *(1)*. The principal origin of salmonellose in humans comes mainly from contaminated eggs, poultry, meat and meat products *(8, 9, 10, 1)*. Several factors have to be taken in consideration with respect to growth control of this microorganism. These parameters are principally: the pH, the a_w, the composition of the food product, the presence of natural antimicrobial components, the biological structure of the food product, the temperature and the storage atmosphere conditions *(11)*. A novel way to reduce the proliferation of microrganisms is the use essential oils extracted from spices. According to Gill et al. (*12*), clove, cinnamon, nutmeg and allspice extracts are able to inhibit the enzymatic activities of histidine, lysine and ornithine decarboxylases of *Enterobacter aerogenes* which cause food putrefaction. The incorporation of these natural preservatives into edible packaging is one of the latest methods to control pathogenic growth and consequently, food contamination and quality loss. Antimicrobial packaging is part of a broader area of active packaging who has became in the past decade, one of the major areas of research in food packaging *(13, 14)*. Since their biodegradability and environmental compatibility are assured *(15, 16)*. It can play an important role in reducing the risk of pathogen development, as well as extending the shelf life of foodstuffs *(17)*. Coating application can especially be useful as a carrier of antimicrobial agents in order to maintain high concentrations of preservatives on the food surface. Moreover, the incorporation of organic acids and essential oils in edible films and coatings contributed greatly to control bacterial growth during the storage of meat *(18, 19)*. Also, application of essential oils emulsion or volatile compounds based coating on oranges allowed a longer shelf life than uncoated ones *(14, 20)*.

Oussalah et al. *(21)* have studied the effect of milk protein-based edible films containing essential oils. These films allowed the control of *Pseudomonas* sp. and *E. coli* O157:H7 growth on whole beef muscle. However, the formulation and the mechanical properties of the films had to be improved and a better control of the essential oils diffusion from the film to the meat surface during storage should be obtained.

Poly (ε-caprolactone; PCL) is semicrystalline biodegradable polyester, derived from a chemical synthesis. It has good water resistance, a low melting point, a low glass transition temperature and a good processability *(22, 23)*. It has been shown that PCL exhibited desirable characteristics as a diffusion-controlled delivery system, including biodegradability, biocompatibility, commercial availability and affordability *(24)*. In this study, PCL was used as both a hydrophobic and an emulsifying agent to stabilize essential oils in the alginate matrix. Alginate, a natural polysaccharide extracted from seaweed, is a copolymer of alterning sequences of β-D-mannuronic and α-L-guluronic acid residues linked by $1\rightarrow 4$ glycosidic bonds *(25)*. Several studies suggested alginate as an interesting film matrix, on account of its biocompatibility, low toxicity *(26)* and insoluble film formation by ionotropic gelation *(27)*. Alginate films generally possess good oxygen barrier properties *(28)*, prevent lipid oxidation *(29)* and improve the food flavour and texture *(15)*.

Film based on alginate and polycaprolactone have showed good physico-chemical properties through the storage when applied on meat. In this study, alginate film efficiency was studied to: 1) control the growth of *S.* Typhimurium and *L. monocytogenes* on bologna and ham and to: 2) control the release of active compounds from the film to the meat during storage. Spanish oregano, Chinese cinnamon and savory essential oils was immobilized in films and then, the films were treated in Ca Cl_2 solution (2 or 20% w/v).

Material and Methods

Films Preparation

Sodium alginate and polycaprolactone diol (m_n 1250) were purchased from Sigma-Aldrich Ltd. (Oakville, Ont, Canada). The polycaprolactone was slowly solubilized in distilled water at 45°C for 15 min, at a concentration of 1% (w/v). Meanwhile, alginate 3% (wt/v) was kept under agitation in distilled water at room temperature during two hours to obtain a complete solubilization. Polycaprolactone solution was then, mixed with the alginate solution at room temperature. Glycerol was added as a plasticizer at a total concentration of 2% (wt/v) *(30)*. The alginate-polycaprolactone solution was heat-sterilized at 121°C during 30 min. In order to keep the polymers miscible, the mixture was maintained at 40-50°C, under strong agitation. Spanish oregano (*Corydothymus capitatus*); Chinese cinnamon (*Cinnamomum cassia*); and savory (*Satureja montana*); essential oils (Robert et fils, Montréal, Qc, Canada) were added singly at a final concentration of 1% (wt/v). The film forming solutions was homogenized at room temperature during 1 min with a homogenizer Ultra-Turrax TP18/1059 (Janke & Kunkel, Staufen, Germany) at 25.000 tr/min. For each film solution, a volume of 5 ml was deposited in Petri dishes (50X11 mm) and dried at ambient temperature under 40-50% of relative humidity (R.H.) for 24h, to form a homogeneous and transparent film. After drying, the water-soluble films underwent to an insolubilisation treatment by immersing them in a $CaCl_2$ solution. Two concentrations of $CaCl_2$ were evaluated in order to compare their effect on the active compounds release towards ham and bologna slices. This treatment allowed a gel formation by ionic bonds formation with calcium. The treatments were done for 1 and 20 min respectively for 2 and 20% (wt/v) of $CaCl_2$ solution.

Bacterial Strains Preparation

L. monocytogenes (INRS-Institut Armand Frappier, Laval, Qc, Canada) and *S.* Typhimurium SL1344 (INRS-Institut Armand Frappier, Laval, Qc, Canada) were maintained at –80 ± 1°C in Brain-Heart-Infusion (BHI, Difco Laboratories, Detroit, MI) containing 10% (wt/v)

glycerol as a stock culture. Strains were individually subcultured [1.0% (v/v)] in BHI at 35 ± 1°C. Prior the experiment cultures were subcultured twice and incubated for 24h at 35 ± 1°C in BHI broth.

Samples Preparation

Bologna and ham (Olymel Cie, Laval, Qc, Canada) (15 kg), were purchased at a local grocery store (IGA, Laval, Qc, Canada) and transported to INRS-Institut Armand Frappier-Canadian Irradiation Center (CIC) under refrigerated conditions in an ice-filled cooler. Bologna and ham, were cut into 225 pieces (150 pieces for microbiological analysis and 75 pieces for biochemical analysis) of equal thickness (7.5 mm) and diameter (47 mm) using a pastry cutter. The 225 bologna and ham pieces (10 pieces/package) were placed in a sterile bags (0.5 mm mettalized polyester/2 mm EVA copolymer; 305 mm X 210 mm, Winpak, St-leonard, Qc, Canada) and sealed under vacuum. The packages were stored overnight at –80 ± 1°C until sterilization treatment by irradiation. Sterilization was done by irradiation at a dose of 25 kGy and a dose rate of 22.93 kGy/h, using a ^{60}Co source UC-15A (MDS-Nordion Int., Inc., Kanata, Ont, Canada). After irradiation, Bologna and ham samples of each group were placed individually into sterile Petri dishes (50 X 11 mm). The surfaces of 150 pieces of bologna and ham were inoculated with *L. monocytogenes* and *S.* Typhimurium to obtain a final concentration of 10^3 colony-forming units (CFU)/cm^2 of bacteria. For each sample, a volume of 50 µl of inoculum was spread over the entire upper surface of the samples. The other 75 slices of bologna and ham reserved for the biochemical analysis and were not inoculated. Samples of each kind of meat were divided into five separate groups: (i) the control group without film (M); (ii) Samples coated with alginate-based films without essential oil (w); (iii) Samples coated with alginate-based films containing Spanish oregano oils (O); (iv) Samples coated with alginate-based films containing Chinese cinnamon oils (C); and (v) Samples coated with alginate-based films containing savory oils (S). Except for the control; all samples were covered on either side with one of the corresponding films. Petri dishes were sealed hermetically and stored at 4 ± 1°C. A sample was taken at each day of storage during five days for microbiological and biochemical analysis. Day 1 corresponded to the day of sample preparation.

Bacterial Enumeration

Each sample of bologna or ham was homogenized for 2 min in 153 ml of sterile peptone water [0.1% (w/v), Difco Laboratories] using a Lab-blender 400 stomacher (Seward Medical, London, U.K.). From this homogenate, serial dilutions were prepared in peptone solution (0.1% (w/v)), and appropriate ones were spread plated on Tryptic Soya Agar (TSA, Difco laboratories). Then, TSA plates were incubated for 24 h at 35 ± 1°C, and results were expressed as log CFU/cm^2 of bacteria in bologna and ham.

Active Compounds Availability

To evaluate the availability of active compounds in the packaging. Films samples (1 g each) were cut aseptically from the center of the film. The thin strips were placed in 50 ml of ethanol solution and kept under agitation with a magnetic stir bar at room temperature for 30 min. Then, the extract was filtered through a Whatman 0.45 μm filter paper (Whatman Int., Ltd.).
Total phenolic compounds content in each film extract containing Spanish oregano and savory oils were determined according to Folin-Ciocalteu procedure *(31)*. The availability of total aldehydes in films containing cinnamon oils was determined following the Kleber method using phenylhydrazine reagent *(32)*.

Statistical analysis

Experiments for microbiological analysis were done using a 2 X 2 X 5 X 2 X 2 X 5 factorial design [2 kind of RTE (bologna and ham), 2 replicates, 5 treatments (control, film without essential oil, film with Spanish oregano, Chinese cinnamon and savory essential oils), 2 bacteria (*L. monocytogenes* and *S.* Typhimurium), 2 kind of films (insolubilized in $CaCl_2$ solution (2% or 20%)), and 5 storage periods (1, 2, 3, 4, and 5 days)]. Analysis of variance and Duncan's multiple-range tests were used to perform statistical analysis on all results. Differences between means were considered to be significant when $P \leq 0.05$. Stat-Packets Statistical Analysis software (Walonick Associates, Inc., Minneapolis, MN) was used for the analysis. For each measurement, three samples in each replication were tested.

Results and Discussion

Microbiology

Effects of alginate-based films on *L. monocytogenes* and *S.* Typhimurium counts, inoculated on bologna surfaces are presented in Tables 1 and 2, respectively. Results illustrated in Table 1 showed that films containing essential oils allowed a gradual and a significant decrease ($P \leq 0.05$) on *L. monocytogenes* counts during storage as compared to control samples whatever the type of pre-treatment used ($CaCl_2$, 2% or 20%). The level of *L. monocytogenes* present in uncoated bologna samples (M) decreased continuously during storage, showing a significant reduction ($P \leq 0.05$) of 1 log at day 5. The same behaviour was also observed in uncoated samples or samples coated with W-based films (W). At the end of storage, a microbial concentration of 2.00 CFU/cm^2 was observed in bologna without coating, and 1.22 and 2.39 log CFU/cm^2 was respectively observed in W samples treated respectively with $CaCl_2$ (20 and 2%). However, at day 3, the level of *L. monocytogenes* in W samples was significantly ($P \leq 0.05$) higher than the level observed in M samples, whatever the pre-treatment applied (2 or 20% $CaCl_2$). Results also showed that C-based films were able to reduce the content of *L. monocytogenes* by over 2.71 log CFU/cm^2 during the first three days of storage when films were treated with $CaCl_2$ (20%) were applied on bologna. However, only a 1.01 log CFU/cm^2 reduction was observed on samples coated with C-based films treated with $CaCl_2$ (2%). Also, at day 3, the level of *L. monocytogenes,* in bologna coated with S-based films treated with $CaCl_2$ (2%), remained significantly higher ($P \leq 0.05$) than the *L. monocytogenes* content in samples coated with O and C-based films treated with $CaCl_2$ (2%), showing a respective value of 2.83, 2.61 and 2.04 log CFU/cm^2. At the end of the storage period, the cell concentration of *L. monocytogenes* was below detection level (< 10 UFC/ml) in bologna samples coated with O, C and S-based films treated in $CaCl_2$ (20%) as compared to 1.52, 1.74 and 1.96 log CFU/cm^2 in the same respective samples but coated with films treated with $CaCl_2$ (2%) ($P \leq 0.05$).

Results on *S.* Typhimurium content on bologna are presented in Table 2. These results showed that the level of microorganisms in M samples decreased gradually during storage on all samples ($P \leq 0.05$). At the end of the storage period, a cell concentration of 1.86 log CFU/cm^2 of

S. Typhimurium was observed. A content of 2.03 and 2.12 log CFU/cm^2 of *S.* Typhimurium was observed in W samples coated with films treated with Ca Cl_2 (2 and 20%) respectively ($P > 0.05$). During storage, the level of *S.* Typhimurium in W samples was significantly ($P \leq 0.05$) higher than the level of *S.* Typhimurium observed in M samples, whatever the pre-treatment applied ($CaCl_2$, 2 or 20%). Results also showed that C-based films treated with $CaCl_2$ (20%) were able to reduce the content of *S.* Typhimurium by 1.8 log CFU/cm^2 during the first three days of storage. However, at day 3, only a 0.5 log CFU/cm^2 reduction was observed on samples coated with C-based films treated with $CaCl_2$ (2%). The level of microorganisms was relatively stable in samples coated with O and S-based films during the first three days of storage. A mean of 0.6 log CFU/cm^2 reduction was observed independently of the treatment used (2 or 20% $CaCl_2$). Also, for all samples, during storage period, the content of *S.* Typhimurium in bologna coated with O-based films was lower than in samples coated with S-based films. After 5 days of storage, a 1.94 log reduction was observed on samples coated with C-based films treated with $CaCl_2$ (20%) as compared to 0.97 and 0.82 log reduction, respectively, in O and S-based samples. The content of *S.* Typhimurium in M samples was 1.82 log CFU/cm^2. Samples coated with films treated with $CaCl_2$ (2%), showed a *S.* Typhimurium reduction of only 1.19, 0.93 and 0.92 log CFU/cm^2, respectively, in samples coated with O-, C- and S- based films, after five days of storage. At the end of storage, for samples coated with films treated with $CaCl_2$ (20%), bologna coated with C-based films had the lowest level of *S.* Typhimurium count ($P \leq 0.05$). However, in samples coated with films treated with $CaCl_2$ (2%), bologna coated with O-based films was the one which allowed the lower level of *S.* Typhimurium count ($P \leq 0.05$).

Effects of alginate-based films on *L. monocytogenes* and *S.* Typhimurium count on ham surfaces are presented in Tables 3 and 4, respectively. Results showed that *L. monocytogenes* content in ham samples (M) increased continously and significantly ($P \leq 0.05$) during the whole storage period. A value of 4.7 log CFU/cm^2 was observed at day 5. The level of *L. monocytogenes* in ham coated with C-based films treated in $CaCl_2$ (20%) was lower than the content founded in ham coated with O and S-based films at day 3, showing a respective value of 2.91, 3.07 and 3.17 log CFU/cm^2 ($P \leq 0.05$). Between days 2 and 4, the level of *L. monocytogenes* in ham samples coated with O-based films was stable with 3.06 log CFU/cm^2 at day 4, whereas, the content of *L. monocytogenes* in ham coated with C-based films decreased at

day 4 with a value of 2.61 log CFU/cm^2. After a slight increase in the *L. monocytogenes* content with the S-based films at days 3 and 4, the level of microorganisms remained stable until day 5 as compared to day 4. A higher level of *L. monocytogenes* was noted in samples coated with W-based films at the end of storage (3.56 log CFU/cm^2). The application of O- and S-based films did not have any significant effect on the control of *L. monocytogenes* growth during storage. C-based films treated in $CaCl_2$ (20%) were the most effective against *L. monocytogenes* growth, with a value of 2.64 log CFU/cm^2 at the end of storage. Results obtained with films treated in $CaCl_2$ (2%) showed that only C-based films were able to control the level of *L. monocytogenes* during storage. Indeed, a value of 3.12 log CFU/cm^2 was observed at day 2 of storage and this level remained stable during the 4 last days of storage. In ham samples coated with O- and C-based films, the level of *L. monocytogenes* was stable between days 2 and 4, with a respective value of 3.13 and 3.00 log CFU/cm^2 at day 4 of storage. A significant increase ($P \leq 0.05$) was however noted at day 5 in samples coated with O- and S-based films, reaching a respective value of 3.62 and 4.07 log CFU/cm^2. These results showed also that any significant difference ($P > 0.05$) was observed between the samples coated with O- and C-based films during the first 4 days of storage. Moreover, an increase of 0.7 log ($P \leq 0.05$) was noted at day 5 in samples coated with W-based films. This level was significantly lower than the level of *L. monocytogenes* reached in M samples. At the end of the storage period, the level of microorganisms in ham coated with C-based films was significantly lower ($P \leq 0.05$) than the one present in all other samples.

Results on *S.* Typhimurium content on ham surfaces are presented in Table 4. Results showed that in M samples, a decrease of only 0.38 log was observed after 5 days of storage. In samples coated with W-based films treated with $CaCl_2$ (2 and 20%), a decrease of 0.92 and 0.71 log of *S.* Typhimurium were respectively observed at the end of storage. Whereas, the application of O-, C- and S-based films treated in $CaCl_2$ (20%) was able to reduce by 1.38, 1.29, and 0.74 log respectively, the content of *S.* Typhimurium after only one day of storage. Indeed, the level of microorganisms was: 1.58, 1.67 and 2.22 log CFU/cm^2, respectively. The content of *S.* Typhimurium in samples coated with O- based films remained stable between days 2 and 4, showing a value of 1.61 log CFU/cm^2 at day 4, whereas C- and S-based films allowed a decrease of *S.* Typhimurium at day 3, with a content of *S.* Typhimurium of 1.05 and 1.93 log CFU/cm^2,

respectively. Also, on samples coated with S-based films, a significant decrease of *S.* Typhimurium was observed at day 4 ($P \leq 0.05$), with a value of 1.77 log CFU/cm^2. After five days of storage, the level of *S.* Typhimurium in ham coated with O-, C -and S- based films was significantly reduced by 1.85, 1.85 and 1.34 log, respectively, with a value of 1.11, 1.11 and 1.62 log CFU/ cm^2 as compared to 2.25 log CFU/cm^2 in samples coated with W-based films. Moreover, any significant difference ($P > 0.05$) was observed between samples coated with C- and O- based films at day 5. These results also showed that the application of O- and C-based films allowed a significantly better control of *S.* Typhimurium, than the application of S-based films.

Results on *S.* Typhimurium content on ham slices coated with films treated with $CaCl_2$ (2%) showed that after one day of storage, the level of microorganisms was significantly lower ($P \leq 0.05$) in coated samples as compared to M samples. The level of *S.* Typhimurium at day 2, was respectively 2.20, 1.36, 1.95 and 2.11 log CFU/cm^2 in samples coated with W-, O-, C- and S-based films compared to 3.02 log CFU/cm^2 in M samples. Any significant difference ($P > 0.05$) was observed between samples coated with W- and S-based films. After 3 and 4 days of storage, the level of *S.* Typhimurium in samples coated with O- and C-based films was significantly lower ($P \leq 0.05$) than the content of *S.* Typhimurium founded in all other samples. At the end of storage, samples coated with O-, C- and S- based films were able to reduce by 1.59, 1.86 and 1.47 log the content of *S.* Typhimurium, whereas the reduction observed in M and W samples was respectively, of 0.38 and 0.92 log. These results showed also that O- and C-based films were the most effective against the growth of *S.* Typhimurium.

Our study showed that the incorporation of essential oils into alginate based films decreased significantly ($P \leq 0.05$) the level of *L. monocytogenes* and *S.* Typhimurium on bologna slices and *S.* Typhimurium on ham slices, whatever the type of pre treatment used (2% or 20% $CaCl_2$). Moreover, O-, C- and S-based films treated with $CaCl_2$ (20%) were more effective on *L. monocytogenes* and C-based films treated with $CaCl_2$ (20%) were more effective on *S.* Typhimurium on bologna than their respective films treated with $CaCl_2$ (2%). On ham slices, the efficiency of O-, C- and S-based films against *S.* Typhimurium was similar independently the type of the pre-treatment used (2% or 20% $CaCl_2$), whereas, *L. monocytogenes* was not affected

by the presence of essential oils. The ineffectiveness of essential oils–based films against *L. monocytogenes* in ham may be due to a number of factors. It is possible that the antibacterial essential oils components remained partitioned in hydrophobic environments created by the presence of fat in ham. These results can be supported by those of Canillac et al. (*33*), who found that introduction of fat into the test medium decreased the bactericidal effect of *Picea excelsa* essential oil against *L. monocytognes*. Cutter et al. *(34)* suggested that the ineffectiveness of spices treatments in ground beef against *E. coli, L. monocytogenes* and *S.* Typhimurium was related to the partitioning of spices components into meat lipids. The effectiveness of the essential oil from plants can be reduced in foods owing to the presence of components, such as proteins and fats that immobilize and inactivate the essential oil components (*35, 36*). Also, Ceylan et al. (*37*) observed that spices were more effective against *E. coli* O157:H7 in agar than in ground beef or in salami, which contained 20% of lipids. It was reported by Gill et al. *(12)* that microorganisms, which were grown in nutrient-rich environment such as meat, have a greater resistance to different stress by reaching their maximum replication rate and still can repair their cellular components. The growth rate of *L. monocytogenes* is dependant mainly on the water activity, the storage temperature, and to a smaller extent on the amount of nitrite in the product *(7, 38, 39)*. Indeed, RTE meat products contain ingredients that may affect the antimicrobial properties of the tested essential oil, which can explain the results obtained with ham and bologna. Several additives are commonly present in ham and bologna suchas sodium chloride, sodium nitrite, sodium erythrobate and sodium phosphate, whereas monosodic glutamate is present only in bologna. Sodium nitrite and sodium chloride are the chemicals ingredients of cured meat products that are ascribed to growth inhibitory effects *(40)*. Indeed, NaCl and nitrite can improve the antimicrobial activity of EDTA, on *E. coli, S.* Typhimurium, *Serratia grimesii*, and also were effective against *S.* Typhimurium and *Shewanella putrefaciens (40)*, whereas, nitrite, nitrate and sodium chloride do not inhibit the growth of *L. monocytogenes* during storage under refrigerated temperatures *(1)*. Glass and Doyle (*41*) showed that *L. monocytogenes* populations increased by 10^3-10^4 CFU/g on bologna and by 10^3-10^5 CFU/g in chicken and turkey products after 4 days of storage. *Salmonella* numbers declined to undetectable levels in the untreated bologna after 20-30 days, and this decline was more rapid with the combined addition of diactate *(1)*.

The essential oils evaluated in this study were already found as being effective *in vitro* against a wide spectrum on microorganisms. Yuste and Fung *(39),* founded that cinnamon added to the apple juice with nisin contributed greatly to the inactivation of *S.* Typhimurium and *E. coli* O157:H7 after 3 and 7 days of storage, respectively. Chang et al. *(42)* founded that cinnamon essential oil had a minimal inhibitory concentration (MIC) of 500 µg/ml and 250µg/ml against *Salmonella* sp. and *E. coli* O157:H7, respectively. Cinnamon extract mixed with two other extracts such as Chinese chive and *Corni fructus* led to the expected antimicrobial effect in orange juice, pork and milk on *E. coli* and *L. monocytogenes (43).* As observed by Dadalioglu and Evrendilek *(44),* oregano essential oil from Turkia exhibited a very strong antibacterial activity against *E.coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium, and *S. aureus.* A concentration of 0.8 %, was able to reduce by 2 - 3 log the level of lactic acid bacteria and *L. monocytogenes* on beef meat fillets *(45).* These results are in agreements with those obtained by Skandamis et al. *(46)*, who showed that 0.8% of oregano essential oil reduced the majority of the microbial population of lactic bacteria and *S.* Typhimurium on meat. Oregano-cranberry mixture was able to reduce *L. monocytogenes* count by 2.8 log on beef slices and by 2.3 log on fish slices (*35*). Also, Chorianopoulos et al. *(47)* showed that savory species from Greece possess remarkable bactericidal properties against *Salmonella* sp., *E. coli* O157:H7, *Staphylococcus aureus, Bacillus cereus* and *Listeria monocytogenes*. Our results also showed that the antimicrobial properties of essential oils tested were not affected by their incorporation in alginate based films. Indeed, the incorporation of antimicrobial agents in films was effective to prolong their antimicrobial effect. A cilantro oil-based gelatine gel allowed 1.3 log reduction of *L. monocytogenes* population compared to the control on vacuum packaged ham after one week of storage, but any difference was observed with the control after a storage of 2 weeks *(12).* Cutter and Siragusa *(48)* have shown that *Brochothrix thermosphacta* contents was reduced by 2 log after 7 days of storage, using nisin immobilized in a calcium alginate gel. It may be a more effective bacteriocin delivery system to the surface of beef carcasses than direct application. Gill et al. *(49)* observed that lysozyme-nisin-EDTA-based gel inhibited the growth of *B. thermosphacta, Lactobacillus sakei, Leuconostoc mesenteroides* and *L. monocytogenes* and also had a bactericidal effect on the growth of *S.* Typhimurium during 4 weeks. Application of milk protein-based edible films containing 1% (w/v) of oregano essential oil allowed a significant ($P \leq 0.05$) reduction of 0.95 and 1.12 log of *Pseudomonas* sp. and *E. coli* O157:H7 level,

respectively after 7 days of storage *(21)*. The growth of *Enterobacteriaceae* and *S. liquefaciens* on bologna was completely inhibited with chitosan-based films which containing acetic acid and cinnamaldehyde, as a result of its greater antimicrobial activity *(50)*. Pranoto et al. *(51)* also showed that the edible alginate-based films containing garlic oil exhibited an antimicrobial activity on *S. aureus* and *B. cereus*, tested using an agar diffusion assay.

The major components of the essential oils tested were indicated in the chemical analysis provided by the manufacturer. Spanish oregano essential oils were composed essentially of carvacrol (76%) and thymol (4.47%), two phenolic monoterpenes. Tested Chinese cinnamon essential oils were rich in aldehydes: cinnamaldehyde (65%) and methoxy-cinnamaldehyde (21%). Savory essential oils were composed of phenolic monoterpenes such as carvacrol (5.70%) and thymol (42.71%), and nonoterpenic hydrocarbons such as γ-terpinene (8.63%) and *p*-cymene (11.68%). The hydroxyl group present in the structure of phenolic compounds confers its antimicrobial activity, and the relative position of the hydroxyl group has a great importance on the effectiveness of these components *(52)*. This fact can explain the difference in antimicrobial activity of carvacrol and thymol. This hypothesis is consistant with our results showing that oregano essential oil has better antimicrobial activity than savory essential oils. It is known that the antimicrobial activities of essential oils are predominately related to their main components *(53)*. The antimicrobial potential of Spanish oregano and savory essential oils is related to their high concentration in phenolic content *i.e.*, carvacrol and thymol *(54, 55)*. Cinnamaldehyde is one of the most active compounds present in Chinese cinnamon essential oil. Ultee et al. *(56)* founded that 0.5 mM of carvacrol and 0.25 mM of cymene reduced by 50% the viable count of *B. cereus* in rice within 48 min. Indeed, it seems that carvacrol is very effective against food borne pathogens and it showed a strong bactericidal activity on *E. coli* O157: H7, *L. monocytogenes*, *S.* Typhimurium and *Vibrio vulnificus (57)*. Lambert et al. *(55)* observed a high sensitivity of *S. aureus* to a mixture of thymol and carvacrol. Karapinar et al. *(54)* showed that thymol was effective against the growth of *S. aureus* and *V. Parahaemolyticus* with a MIC of 75 µg/ml for both bacteria. Burt *(58)* reports that carvacrol, thymol, eugenol, perillaldehyde, cinnamaldehyde and cinnamic acid have been identified as antibacterial agents against *L.monocytogenes, S.* Typhimurium, *E. coli* O157:H7, *Shigella dysenteria, B. cereus* and

S. aureus. The minimum inhibitory concentrations (MICs) reached 0.05-5 µl/ml *in vitro*, whereas a higher concentration, around 0.5-20 µl/g, is needed to achieve the same effect in foods. Kwon et al. *(59)* have evaluated the antimicrobial activity of cinnamic aldehyde on *B. cereus* and have estimated its bactericidal effect at 0.3 ml/l. Chang et al. *(42)* founded that cinnamaldehyde was effective on *E. coli, P. aeruginosa, K. pneumoniae* and *Salmonella* sp. Cinnamaldehyde has been well known to possess strong inhibitory effects against various micro-organisms as well as against toxin production *(59, 60, 61)*. According to Ultee et al. *(56)*, carvacrol attacks the membrane, while making it permeable to the potassium ion and to protons, thus acidifying the cytoplasm of the bacteria, making impossible the synthesis of ATP and so decreasing of the quantity of intracellular ATP. Also, carvacrol has been reported to disintegrate the outer membrane and increased the permeability of ATP through cytoplasmic membrane *(59)*. Indeed, Helander et al. *(62)* founded that thymol and carvacrol have the ability to disintegrate the membrane of *E. coli* and *S.* Typhimurium at a concentration close to the MIC, whereas cinnamaldehyde, being an electronegative compound *(63)*, rather interfere with the level of electron transfer and while reacting by substitution with the nucleic acids and the proteins *(52)* or by inhibiting amino acid decarboxylase activity *(64,65)*.

Contrary to several antibiotics, the hydrophobic compounds presents in essential oils can reach the periplasmic space of Gram-negative bacteria through the porin proteins *(55, 62)* and inhibit the direct transport of cellular proteins, nutrients and elements which play a role in the Proton Motive Force (PMF, 66*)*. The hydrophobicity of essential oils enables them to partition in the lipids of the cell membrane and mitochondria, rendering them permeable and leading to leakage of cell contents *(58)*.

Availability of active compounds

The availability of active compounds in films containing essential oils and treated with $CaCl_2$ (2% or 20%) during storage of bologna at 4°C is presented in Table 5. Results showed that at day 2 of storage, a significant decrease ($P \leq 0.05$) of the active compounds availability in the film for all films evaluated. Active compounds availability in films treated with $CaCl_2$ (2%), was respectively 66.75, 62.55 and 26.63% in O-, S- and C-based films at day 2. These results also

indicated that after one day of storage, the level of active compounds released from C-based films was 2 times higher than from O- and S-based films. Indeed, a percentage of 73% of active compound released in C-based films was noted in bologna, whereas a release level of 33 and 37% was observed with O and S-based films respectively, between days 1 and 2. Between days 2 and 4, a significant decrease ($P \leq 0.05$) of the active compounds availability in O-, S- and C-based films was also observed, with a respective level of 49.21, 47.36 and 14.06% at day 3, and 20.24, 15.37 and 9.00% at day 4. A respective release level of 59, 68 and 36% was noted between days 3 and 4 in O-, S- and C-based films. As compared to day 4, the active compounds availability decreased significantly ($P \leq 0.05$) in O- and C-based films at day 5, reaching respective values of 18.84 and 7.11%, whereas the availability remained stable in S-based films showing an active compounds availability of 17.08%. During all period of storage, O-based films were the one which maintained the higher quantity of active compounds. At the end of storage, when compared to day 1, a significantly higher ($P \leq 0.05$) release level of 93% was noted with C-based films as compared to 81 and 83% obtained with O- and S-based films, respectively.

The active compounds availability in O-, S- and C-based films treated with $CaCl_2$ (20%) showed a significant decrease ($P \leq 0.05$) of active compounds availability during the first two days of storage, showing respective values of 27.60, 46.72 and 18.95%. These results showed that the release level of active compounds was significantly higher than in their respective films treated with $CaCl_2$ (2%). Also, the release level of active compounds during the first two days and between films treated with $CaCl_2$ (20%) showed that the release level in C-based films was similar to the one observed in O-based films, but 1.5 times higher than in S-based films. Another significant decrease ($P \leq 0.05$) of active compounds availability was also observed at day 3, in O-, S-, and C-based films. Between days 2 and 3, a release level of 20 and 25 % in O- and C-based films was observed as compared to 48% in S-based films, whereas between days 3 and 4, a lower release level was observed in O-based films (7.69%) compared to the one in C- and S-based films, which had respective release level of 65 and 41%. C-based films reached a stable concentration of total aldehydes at day 4. The values observed at days 4 and 5 was respectively 5.04 and 4.14%. During the same period of time, the content of total phenolic compounds in O- and S-based films decreased significantly ($P \leq 0.05$), from 20.28% to 12.58% in O-based films and from 14.57% to 9.59% in S-based films. After five days of storage, the active compounds

availability in O-based films was significantly higher than the one in C- and S-based films, showing a percentage of active compounds of 12.58, 9.59 and 4.14% respectively in O-, S- and C-based films.

Results of active compounds availability in films containing essential oils and treated in $CaCl_2$ (2% or 20%) during storage of ham at 4°C are presented in Table 6. The results showed that the active compounds availabilities in O-, S- and C-based films treated in $CaCl_2$ (2%) were respectively, 83.04, 80.10 and 37.75% at day 2 of storage. Indeed, a release level of 17, 20 and 62% in O-, S-, and C-based films, was respectively observed. These results showed that the release level of total aldehydes in C-based films was 3 and 3.6 times higher than the release of total phenolic compounds observed respectively in S- and O-based films during the two first days of storage. Also, results showed a significant and steady decrease ($P \leq 0.05$) of active compounds availability in all films until the end of storage. At day 3, the active compound availabilities were, respectively, 37.08, 27.61 and 15.56% in O-, S- and C-based films. The results also indicated that the release rate of active compounds in O-, S- and C- samples was lower between days 3 and 4 with a respective release level of 8, 21 and 1% respectively. From day 3 to day 5, the availability of active compounds in O-based films remained stable until the end of storage, with an availability of active compounds of 27.65% at day 5. However, a significant decrease of active compounds availability was observed between days 4 and 5 in S- and C-based films. A decrease from 21.70 to 11.09 and from 15.41 to 4.35 was respectively observed. During all period of storage, the release level of active compounds in C- and S-based films was faster than the one observed in O-based films. Moreover, at the end of storage, as compared to day 1, the highest release level was observed in C-based films with 96%, as compared to 89 and 72% observed respectively in S- and O-based films.

The results of active compounds availability in films treated with $CaCl_2$ (20%) and containing essential oils showed a significant decrease ($P \leq 0.05$) of active compounds availability during storage. Indeed, at day 2, a decrease of 50, 68 and 80% of active compounds availability was observed in O-, S- and C-based films, respectively. Then, the availability of active compounds in all films remained stable until day 3. A significant decrease ($P \leq 0.05$) of the active compounds availability was also noted at day 4 of storage in O-, S- and C-based films as

compared to day 1. A respective value of 32.46, 22.79 and 12.04% was observed. Between days 3 and 4, the release level of active compounds from O-, S- and C-based films was 32, 29 and 37% respectively. At the end of storage, as compared to day 1, the release level of active compounds was 78, 86 and 96% respectively.

According to these results, it seems that active compounds migration in C- based films is higher than in O- and S- based films. Many interactions may occur during oils diffusion from polymer to bologna or ham. In particular, water uptake from meat may cause the polymer to swell *(67)* and migration rates change continuously because of a time-dependent relaxation process resulting from swelling stress *(68)*. The oil diffusion would also be expected to relate to their respective composition, their polarity due to the structural configuration of the constituent compounds and their functional groups. The high release rate of cinnamon oils can be explained by the polar character of trans-cinnamaldehyde and 2-methoxycinnamaldehyde, which are the main components of cinnamon oils, implying a better affinity with water that is contained in bologna and ham, as compared to the main components of oregano and savory oils (carvacrol and thymol respectively). Indeed, Helander et al. *(62)* showed that the solubility of cinnamaldehdye in water at 20°C was higher than that of carvacrol and thymol. Moreover, when films were applied on bologna or on ham, a higher availability of total phenolic compounds occurred in O-based films as compared to the availability observed in S- or in C-based films whatever the treatment used ($CaCl_2$ 2 or 20%). Inversely, the level of active compounds release in C- based films is faster than in the O- and S- based films during storage. It is also interesting to note that there was no significant difference ($P > 0.05$) between mechanical properties of films treated with 2% or 20% of $CaCl_2$. For films treated with 20% $CaCl_2$, puncture resistance of S-based films (68.11 Newton/mm) was significantly higher ($P \leq 0.05$) than of O- and C-based films (57.65 and 49.29 Newton/mm, respectively). These results show that the release profile is not just directly related to the mechanical properties of films, which implies that the diffusion process is more complex.

In summary, the application of O-, C- and S-based alginate films was able to control the growth of pathogenic bacteria such as *L. monocytogenes* and *S.* Typhimurium during the storage of bologna and ham slices. Results showed that, C-based films treated with $CaCl_2$ (20 %) were

the most effective to control at the same time the content of *L. monocytogenes* and *S. typhimurium* in bologna. Moreover, the films used in this study showed a better control of *L. monocytogenes* in bologna than on ham slices. Inversely, *S.* Typhimurium was slightly more controlled on ham than on bologna. Also, the immobilization of the films in $CaCl_2$ (2 or 20%) allows a controlled release of the active compounds from the films to the bologna and ham. When applied on bologna, a lower release level was observed with films treated in $CaCl_2$ (2%). However, when applied on ham, the control release of active compounds was less affected by $CaCl_2$ treatment (2 or 20%).

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Table 1. Effect of film composition and insolubilisation on *L. monocytogenes* during storage at 4°C, in bologna.

		Log CFU/cm$^{2\ a,\ b}$				
Treatment	**Sample[c]**	**day 1**	**day 2**	**day 3**	**day 4**	**day 5**
2% $CaCl_2$	**B**	3.05 ± 0.06 a D	2.74 ± 0.13 c C	2.31 ± 0.14 b B	2.21 ± 0.19 c B	2.02± 0.12 c A
	W	3.05 ± 0.06 a D	3.03 ± 0.09 d D	2.87 ± 0.15 d C	2.60 ± 0.16 e B	2.39 ± 0.18 d A
	O	3.05 ± 0.06 a D	2.62 ± 0.15 b C	2.61 ± 0.15 c C	1.99 ± 0.03 b B	1.52± 0.13 a A
	C	3.05 ± 0.06 a C	2.00 ± 0.19 a B	2.04 ± 0.15 a B	1.73 ± 0.17 a A	1.74 ± 0.28 b A
	S	3.05 ± 0.06 a D	2.99 ± 0.15 d D	2.83 ± 0.13 d C	2.42 ± 0.21 d B	1.96 ± 0.04 c A
20% $CaCl_2$	**B**	3.09 ± 0.13 a D	2.52 ± 0.23 b C	2.27 ± 0.19 b c B	2.15 ± 0.18 c A B	2.00 ± 0.17 c A
	W	3.09 ± 0.13 a E	2.90 ± 0.07 d D	2.59 ± 0.26 d C	2.25 ± 0.13 c B	1.22 ± 0.30 b A
	O	3.09 ± 0.13 a E	2.75 ± 0.04 c D	2.54 ± 0.07 c d C	1.55 ± 0.16 b B	0.00± 0.00 a A
	C	3.09 ± 0.13 a D	2.05 ± 0.11 a C	0.38 ± 0.60 a B	0.00 ± 0.00 a A	0.00± 0.00 a A
	S	3.09 ± 0.13 a E	2.71 ± 0.24 c D	2.06 ± 0.30 b C	1.48 ± 0.25 b B	0.00± 0.00 a A

[a] For each treatment, means in the same column bearing the same lower case letters are not significantly different ($p > 0.05$). [b] During storage time, means in the same row bearing the same upper case letters are not significantly different ($p > 0.05$); [c] B, bologna without film; W, films without essential oil; O, films containing Spanish oregano essential oil; C, films containing Chinese cinnamon essential oil; S, films containing winter savory essential oil.

Table 2. Effect of film composition and insolubilisation on *S.* Typhimurium during storage at 4°C, in bologna.

		Log CFU/cm$^{2\ a,\ b}$				
Treatment	**Sample[c]**	**day 1**	**day 2**	**day 3**	**day 4**	**day 5**
2% $CaCl_2$	**B**	2.99 ± 0.12 a C	2.47 ± 0.10 a B	2.29 ± 0.10 a B	1.99 ± 0.09 a A	1.86 ± 0.31 a b A
	W	2.99 ± 0.12 a E	2.71 ± 0.12 b D	2.41 ± 0.22 a C	2.21 ± 0.18 b c B	2.03 ± 0.10 b c A
	O	2.99 ± 0.12 a D	2.46 ± 0.20 a C	2.28 ± 0.24 a B	2.24 ± 0.05 b c B	1.80 ± 0.21 a A
	C	2.99 ± 0.12 a C	2.47 ± 0.09 a B	2.49 ± 0.29 a B	2.15 ± 0.14 a b A	2.06 ± 0.10 c A
	S	2.99 ± 0.12 a D	2.60 ± 0.12 b C	2.34 ± 0.21 a B	2.34 ± 0.26 c B	2.07 ± 0.15 c A
20% $CaCl_2$	**B**	2.98 ± 0.07 a D	2.47 ± 0.20 b C	2.16 ± 0.09 b B	1.98 ± 0.30 b A B	1.82 ± 0.26 b A
	W	2.98 ± 0.07 a D	2.54 ± 0.21 b C	2.30 ± 0.22 b c B	2.11 ± 0.15 b c A	2.12 ± 0.09 c d A
	O	2.98 ± 0.07 a D	2.45 ± 0.16 b C	2.34 ± 0.23 b c C	2.18 ± 0.11 c B	2.01 ± 0.07 c A
	C	2.98 ± 0.07 a D	2.02 ± 0.12 a C	1.18 ± 0.16 a B	1.10 ± 0.16 a A B	1.04 ± 0.11 a A
	S	2.98 ± 0.07 a C	2.46 ± 0.19 b B	2.38 ± 0.18 c B	2.26 ± 0.16 c A	2.16 ± 0.15 d A

[a] For each treatment, means and in the same column bearing the same lower case letters are not significantly different ($p > 0.05$). [b] Means in the same row bearing the same upper case letters are not significantly different ($p > 0.05$); [c]B, bologna without film; W, films without essential oil; O, films containing Spanish oregano essential oil; C, films containing Chinese cinnamon essential oil; S, films containing winter savory essential oil.

Table 3. Effect of film composition and insolubilisation on *L.monocytogenes* during storage at 4°C, in ham

		Log CFU/cm$^{2\ a,b}$				
Treatment	**Sample[c]**	**day 1**	**day 2**	**day 3**	**day 4**	**day 5**
2% $CaCl_2$	**H**	3.27 ± 0.09 a A	3.40 ± 0.14 c B	3.41 ± 0.09 b B	4.29 ± 0.20 d C	4.70 ± 0.12 d D
	W	3.27 ± 0.09 a A	3.29 ± 0.23 b c A	3.41 ± 0.34 b A	3.70 ± 0.06 c B	3.97 ± 0.06 c C
	O	3.27 ± 0.09 a B	3.11 ± 0.06 a A	3.14 ± 0.11 a A	3.13 ± 0.11 a A	3.62 ± 0.08 b C
	C	3.27 ± 0.09 a B	3.12 ± 0.10 a A B	3.07 ± 0.09 a A	3.00 ± 0.36 a A	3.18 ± 0.17 a A B
	S	3.27 ± 0.09 a A	3.21 ± 0.11 a b A	3.25 ± 0.13 a b A	3.40 ± 0.06 b B	4.07 ± 0.11 c C
20% $CaCl_2$	**H**	3.30 ± 0.04 a A	3.27 ± 0.20 a A	3.46 ± 0.04 c B	4.17 ± 0.07 d C	4.66 ± 0.08 d D
	W	3.30 ± 0.04 a A	3.31 ± 0.08 a A	3.38 ± 0.09 c A B	3.47 ± 0.31 c A B	3.56 ± 0.32 c B
	O	3.30 ± 0.04 a C	3.16 ± 0.11 a A B	3.07 ± 0.15 b A	3.06 ± 0.19 b A	3.25 ± 0.08 b B C
	C	3.30 ± 0.04 a C	3.09 ± 0.39 a B C	2.91 ± 0.34 a B	2.61 ± 0.27 a A	2.64 ± 0.11 a A
	S	3.30 ± 0.04 a C	3.03 ± 0.20 a A	3.17 ± 0.13 b B	3.29 ± 0.07 c C	3.32 ± 0.18 b C

[a] For each treatment, means in the same column bearing the same lower case letters are not significantly different ($p > 0.05$). [b] During storage time, means in the same row bearing the same upper case letters are not significantly different ($p > 0.05$); [c]H, ham whithout film; W, films whitout essential oil; O, films containing Spanish oregano essential oil; C, films containing Chinese cinnamon essential oil; S, films containing winter savory essential oil.

Table 4. Effect of film composition and insolubilisation on *S.* Typhimurium during storage at 4°C, in ham

		Log CFU/cm$^{2\ a,\ b}$				
Treatment	**Sample[c]**	**day 1**	**day 2**	**day 3**	**day 4**	**day 5**
2% $CaCl_2$	**H**	2.96 ± 0.02 a C	3.02 ± 0.0.08 d C	2.84 ± 0.10 c B	2.58 ± 0.06 d A	2.58 ± 0.06 d A
	W	2.96 ± 0.02 a C	2.20 ± 0.28 c B	2.03 ± 0.21 b A B	1.95 ± 0.08 c A	2.04 ± 0.14 c A B
	O	2.96 ± 0.02 a B	1.36 ± 0.23 a A	1.36 ± 0.21 a A	1.31 ± 0.22 a b A	1.37 ± 0.21 b A
	C	2.96 ± 0.02 a D	1.95 ± 0.16 b C	1.34 ± 0.21 a B	1.27 ± 0.20 a A B	1.10 ± 0.16 a A
	S	2.96 ± 0.02 a D	2.11 ± 0.19 b c C	1.91 ± 0.16 b B	1.46 ± 0.20 b A	1.49 ± 0.15 b A
20% $CaCl_2$	**H**	2.96 ± 0.03 a C	2.92 ± 0.07 d C	2.78 ± 0.06 e B	2.53 ± 0.12 e A	2.58 ± 0.08 d A
	W	2.96 ± 0.03 a C	2.54 ± 0.28 c B	2.31 ± 0.06 d A	2.33 ± 0.12 d A	2.25 ± 0.15 c A
	O	2.96 ± 0.03 a C	1.58 ± 0.16 a B	1.66 ± 0.22 b B	1.61 ± 0.17 b B	1.11 ± 0.15 a A
	C	2.96 ± 0.03 a C	1.67 ± 0.17 a B	1.05 ± 0.16 a A	1.09 ± 0.15 a A	1.11 ± 0.16 a A
	S	2.96 ± 0.03 a D	2.22 ± 0.22 b C	1.93 ± 0.25 c B	1.77 ± 0.19 c A	1.62 ± 0.17 b A

[a] For each treatment, means in the same column bearing the same lower case letters are not significantly different ($p > 0.05$). [b] During storage time, means in the same row bearing the same upper case letters are not significantly different ($p > 0.05$); [c]H, ham without film; W, films without essential oil; O, films containing Spanish oregano essential oil; C, films containing Chinese cinnamon essential oil; S, films containing winter savory essential oil.

Table 5 – Effect of essential oils source and $CaCl_2$ treatment on of active compounds availability in films (%), during storage of bologna at 4°C.

		Percentage of active compounds availability[a] [b] (%)				
Treatment	**Film[c]**	**day 1**	**day 2**	**day 3**	**day 4**	**day 5**
2% $CaCl_2$	**O**	100.00 a D	66.75 ± 3.96 c C	49.21 ± 2.06 b B	20.24 ± 1.32 c A	18.84 ± 0.68 c A
	S	100.00 a D	62.55 ± 1.23 b C	47.36 ± 3.27 b B	15.37 ± 1.08 b A	17.08 ± 0.22 b A
	C	100.00 a E	26.63 ± 1.30 a D	14.06 ± 1.47 a C	9.00 ± 0.69 a B	7.11 ± 0.33 a A
20% $CaCl_2$	**O**	100.00 a D	27.60 ± 0.94 b C	21.97 ± 1.86 c B	20.28 ± 0.38 c B	12.58 ± 0.97c A
	S	100.00 a E	46.72 ± 1.78 c D	24.53 ± 1.07b C	14.57 ± 5.73 b B	9.59 ± 0.43 b A
	C	100.00 a D	18.95 ± 0.36 a C	14.26 ± 1.50 a B	5.04 ± 0.33 a A	4.14 ± 0.45 a A

[a] For each treatment, means in the same column bearing the same lower case letters are not significantly different ($p > 0.05$). [b] During storage time, means in the same row bearing the same upper case letters are not significantly different ($p > 0.05$); [c] O, films containing Spanish oregano essential oil; S, films containing winter savory essential oil; C, films containing Chinese cinnamon essential oil.

Table 6 - Effect of essential oils and $CaCl_2$ treatment on active compounds availability in films (%), during storage of ham at 4°C.

		Percentage of active compounds availability[a, b] (%)				
Treatment	**Film[c]**	**day 1**	**day 2**	**day 3**	**day 4**	**day 5**
2% $CaCl_2$	**O**	100.00 a C	83.04 ± 8.62 b B	37.08 ± 3.19 c A	34.25 ± 3.01 c A	27.65 ± 0.54 c A
	S	100.00 a E	80.10 ± 3.01 b D	27.61 ± 3.51 b C	21.70 ± 2.15 b B	11.09 ± 0.29 b A
	C	100.00 a D	37.75 ± 3.92 a C	15.56 ± 0.66 a B	15.41 ± 2.08 a B	4.35 ± 0.28 a A
20% $CaCl_2$	**O**	100.00 a D	49.77 ± 6.02 c C	47.79 ± 5.47 c C	32.46 ± 3.38 c B	21.65 ± 1.59 c A
	S	100.00 a D	32.51 ± 3.00 b C	32.03 ± 3.51 b C	22.79 ± 0.90 b B	14.10 ± 1.61 b A
	C	100.00 a D	20.47 ± 1.27 a C	19.14 ± 2.44 a C	12.04 ± 0.86 a B	3.64 ± 0.06 a A

[a] For each treatment, means in the same column bearing the same lower case letters are not significantly different ($p > 0.05$). [b] During storage time, means in the same row bearing the same upper case letters are not significantly different ($p > 0.05$); [c] O, films containing Spanish oregano essential oil; S, films containing winter savory essential oil; C, films containing Chinese cinnamon essential oil.

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Mechanism of Action of Spanish oregano, Chinese cinnamon and savory essential oils against cell membranes and walls of *Escherichia coli* O157:H7 and *Listeria monocytogenes*

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Résumé

Le mode d'action antimicrobien des huiles essentielles d'origan d'Espagne (*Corydothymus capitatus*), de cannelle de Chine (*Cinnamomum cassia*) et de sarriette des montagnes (*Satureja montana*) a été étudié sur deux types de bactéries pathogènes rencontrées dans les aliments, une bactérie Gram-négatif, *Escherichia coli* O157:H7 et une Gram-positif, *Listeria monocytogenes*. L'effet sur l'intégrité de la membrane, le pH intracellulaire (pH_{in}), la concentration en adénosine triphosphate (ATP), le relargage des constituants cellulaires et les observations en microscopie électronique ont été évaluées sur des cellules incubées en présence des huiles essentielles à leur concentration minimale inhibitrice (CMI). Le traitement avec ces huiles essentielles à leur CMI affecte l'intégrité de la membrane des bactéries et induit l'épuisement de la concentration intracellulaire en ATP. Les huiles essentielles d'origan d'Espagne et de sarriette des montagnes induisent une diminution significativement ($P \leq 0.05$) plus importante que celle induite par la cannelle de Chine. Une augmentation de la concentration extracellulaire en ATP a seulement eu lieu lorsque l'origan d'Espagne et la sarriette des montagnes étaient en contact avec *L. monocytogenes* et *E. coli* O157:H7. Un important relargage des constituants cellulaires a été observé lorsque les cellules de *L. monocytogenes* et d'*E. coli* O157:H7 ont été incubées avec les huiles essentielles de cannelle de Chine et d'origan d'Espagne. L'huile essentielle de cannelle de Chine a permis une réduction significative du pH_{in} d'*E. coli* O157:H7, tandis que la cannelle de Chine et l'origan d'Espagne ont permis une diminution significative du pH_{in} de *L. monocytogenes*. Les observations de la microscopie électronique indiquent que l'enveloppe des deux bactéries traitées a été sensiblement endommagée. Ces résultats suggèrent que la membrane cytoplamique est la cible de l'action toxique des huiles essentielles.

Abstract

The mechanism of the antimicrobial action of Spanish oregano (*Corydothymus capitatus*), Chinese cinnamon (*Cinnamomum cassia*) and savory (*Satureja montana*) essential oils against 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 essential oils at their minimal inhibitory concentration (MIC) were in contact with *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *Escherichia coli* O157:H7 and *Listeria monocytogenes*, two pathogenic food borne bacteria were used as Gram negative and Gram positive bacterial models, respectively. Treatment with these essential oils at their MIC was able to affect the membrane integrity of bacteria and to induce depletion of the intracellular ATP concentration. Spanish oregano and savory essential oils induced however, more depletion than Chinese cinnamon oil. 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 ($P \leq 0.05$) cell constituents release 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 to reduce significantly the pH_{in} of *E. coli* O157:H7, whereas Chinese cinnamon and Spanish oregano decreased more significantly the pH_{in} of *L. monocytogenes*. Electronic microscopy observations revealed that the cells membrane of both treated bacteria was significantly damaged. These results suggest that the cytoplamic membrane is involved in the toxic action of essential oils.

Keywords: cell ATP, cell pH_{in} , cell constituents, Spanish oregano, Chinese cinnamon, savory essential oils, *E. coli* O157:H7, *L.* monocytogenes.

The antimicrobial effect of many herbs and spices used to increase the shelf life of foods are attributed to the essential oil fraction (*33*). A number of authors have mentioned the antibacterial properties of a wide variety of essential oils (*12, 16, 19, 22*). It is known that the antimicrobial activities of essential oils are predominately related to their main components (*26*). The antimicrobial potential of Spanish oregano and savory essential oils are related to their high concentration in phenolic compounds *i.e.* carvacrol and thymol. These compounds exhibited an important antimicrobial activity against a wide range of microorganisms, such as *E. coli* and *L. monocytogenes* (*26, 30*). Also, *Cinnamomum cassia* containing mainly cinnamaldehyde was found effective against these bacteria (*32*). However, the mechanism of action of these preservatives was not been established with details. Essential oils contain terpenes which have the ability to disrupt or penetrate lipid structures (*15*) and accumulate in the membrane and cause a loss of membrane integrity and dissipation of the proton motive force (*15*, *42*). According to Ultee *et al.* (*45*), carvacrol attacks the membrane, while making it permeable with the ions potassium and to protons, thus acidifying the cytoplasm of the bacteria, making impossible the synthesis of ATP and hence decreasing of the quantity of intracellular ATP. Quantitative variations are expected in the activity of essential oils against Gram staining response (*30*). The hydrophobic constituents of essential oils are able to gain access to the periplasm through the porin proteins of the outer membrane of Gram-negative bacteria (*21*). Action of tea tree oil, which has a broader spectrum of antimicrobial activity on Gram-negative and Gram-positive (*12, 15, 39*) is comparable to that of membrane active disinfectants by denaturing proteins and disrupting membrane structure, leading to cytoplasmic leakage, cell lysis and cell death (*15*). Tea tree oil and its components compromise cytoplasmic membrane of methicilin-resistant *S. aureus* (*9*). Carson *et al.* (*11*) also suggested that tea tree oil acts on *S. aureus* until making it sensitive to antibiotics.

The aim of this study is to investigate the mechanism of action of Spanish oregano, savory and Chinese cinnamon essential oils against cell membranes and walls of *L. monocytogenes* and *E. coli* O157:H7 by measuring: (i) the intracellular and extracellular ATP concentrations, (ii) the intracellular pH of cells, (iii) 260 nm absorbing cellular components released and using an electron microscopy to show that these oils attacked the cell wall.

Materials and Methods

Bacterial strains

Escherichia coli O157:H7 EDL 933 (INRS-Institut Armand Frappier, Laval, Québec, Canada) and *Listeria monocytogenes* 2812 1/2a (Health Canada, St-Hyacinthe, Québec, Canada) were maintained at –80 ± 1°C in Brain Heart Infusion broth (BHI, Difco, Becton Dickinson, Sparks, MD) containing 10% glycerol. Prior to the experiment, working culture was prepared by subculturing 100 µl of each stock culture in 9 ml of Tryptic soya broth (TSB, Difco, Becton Dickinson, Sparks, MD) and incubated at 35 ± 1°C through two successive incubations of 24 h. After incubation, 75 µl of *Escherichia coli* O157:H7 and 85 µl of *Listeria monocytogenes* 2812 1/2a from each working culture were individually inoculated in 9 ml of fresh TSB broth and incubated at 35 ± 1°C for 1h and 2h, respectively, in order to obtain working cultures in the exponential growth phase of approximately 10^7 CFU/ml. To evaluate the cell constituent's release, 700 µl of *E. coli* O157:H7 and 820 µl of *L. monocytogenes* 2812 1/2a inoculated in 100 mL of TSB were used to obtain the working culture (10^7 CFU/ml).

Essential oils dispersion

Spanish oregano (*Corydothymus capitatus*), Chinese cinnamon (*Cinnamomum cassia*) and savory (*Satureja montana*) essential oils were provided by Robert et Fils (Montréal, Québec, Canada) and stored at 4°C before use. Three (3) suspensions were prepared with these plant essential oils. The oils were dispersed at room temperature in sterile 10% (vol/vol) modified starch (Food starch-modified, Purity gum Be, National Starch & Chemical Co, Boucherville, Québec, Canada) solution for 2 min using a homogenizator Ultra-Turrax TP18/1059 (Janke & Funkel, Staufen, Germany) at 20000 rpm to obtain a colloidal suspension (10%, vol/vol) (*35*). The presence of modified starch improved suspension stability and could improve the antimicrobial properties (*8*). Each starch-oil suspension was added in experimental medium at the minimum inhibitory concentration (MIC), a concentration equivalent to twice the MIC and a concentration below the MIC. The MIC of Spanish oregano, Chinese cinnamon and savory essential oils was, respectively, 0.025, 0.05 and 0.05% (vol/vol) against *E. coli* O157:H7 and

0.025, 0.05 and 0.05% (vol/vol) against *L. monocytogenes*. The concentration equivalent to twice the CMI of Spanish oregano, Chinese cinnamon and savory essential oils was, respectively 0.05, 0.1 and 0.1% (vol/vol) against *E. coli* O157:H7 and 0.05, 0.1 and 0.1% (vol/vol) against *L. monocytogenes*. The concentration below the CMI was 0.013, 0.025 and 0.025% (vol/vol) for Spanish oregano, Chinese cinnamon and savory essential oils respectively against both of bacteria.

Intra- and extracellular ATP concentrations

The intracellular and extracellular ATP concentrations were measured using methods described by Lee *et al.* (*31*). Working culture of *E. coli* 0157:H7 and *L. monocytogenes* containing approximately 10^7 CFU/mL, was centrifuged for 10 min at 1,000 x g. Cell pellets were washed three times in 0.1 M sodium phosphate buffer (pH 7), and then recentrifuged under the same conditions. A cell suspension (10^7 CFU/mL) was prepared with 9 mL of sodium phosphate buffer (0.1 M, pH 7) and divided into four groups for each essential oil (control, CMI, a concentration equivalent to twice the CMI and a concentration below the MIC). Oil suspensions were placed into Eppendorf tubes containing the cell suspensions (final volume of 0.5 mL) and samples were maintained at room temperature for 30 min (*45*), centrifuged for 5 min at 2000 x g, and then incubated in ice to prevent ATP loss until measurement. The extracellular (upper layer) and the intracellular (lower layer) ATP concentrations were measured by using an ATP assay kit (Calbiochem, EMD Biosciences Inc, San Diego, CA). The ATP concentration of the supernatants, which represents the extracellular concentration, was determined using a luminometer (EG&G Berthold Lumat LB 9507-2, Mandel Scientific Company Inc., Guelph, Ontario, Canada) after the addition of 100 µL of Nuclear Releasing Reagent to 10 µL of supernatant and 1 µL of luciferase. The ATP concentrations representing intracellular content were determined from cell pellets. Cell pellets were washed in 0.85% (wt/vol) sodium chloride solution, centrifuged for 5 min at 1000 x g, and the supernatant was removed. The resulting cell pellets were maintained at room temperature for 15 min in 20 µL of 5 X Passive Lysis Buffer (PLB, Promega, Madison, WI) to disrupt the microorganisms, and then centrifuged directly at 1200 x g for 1 min. Subsequently, 1 µL of luciferase and 100 µL of Nuclear Releasing Reagent were added to 10 µL of resulting supernatants and the ATP concentrations were measured in 1

min with a luminometer. To calculate the intracellular and extracellular ATP concentrations, a standard ATP curve ranging from 10^{-1} to 10^{-9} ng/mL portion was used to obtain a linear relationship between ATP concentration (ng/mL) and relative light unit which resulted in a r^2 (coefficient of determination) value of 0.98.

Intracellular pH measurements

The determination of the intracellular pH was done the method described by Breeuwer *et al.* (*7*). Working cultures of *E. coli* 0157:H7 and *L. monocytogenes* containing approximately 10^7 CFU/mL were treated separately with the essential oil at four studied concentrations (control, CMI, a concentration equivalent to twice the CMI and an intermediate concentration between the MIC and MTC). The treated and untreated (control) cells (9 mL) were incubated at 35 ± 1°C for 1 hour, harvested by centrifugation (15 min at 1,000 x g) and then washed three times in 5 mM HEPES buffer (pH 8.0) containing 50 mM of EDTA. Subsequently, cells were resuspend in 8.8 ml of carboxyfluorescein diacetate succinimydil ester (1.5 µM) solubilized in phosphate buffer (50 mM) and incubated for 10 min at 30 ± 1°C. Carboxyfluorescein diacetate succinimydil ester is hydrolyzed to carboxyfluorescein succinimydil ester in the cell and subsequently conjugated to aliphatic amines. Cells were washed with 50 mM potassium phosphate buffer (pH 5.81), and incubated in presence of 10 mM glucose for 30 min at 30 ± 1°C to eliminate nonconjugated carboxyfluorescein succinimidyl ester. The cells containing fluorescent probe were then washed twice by centrifugation at 1,000 X g for 15 min, resuspended in 50 mM potassium phosphate, and kept on ice until measurement. The cell suspension was introduced in a 3-mL glass quartz cuvette and placed in the stirred and thermostated cuvette holder of a spectrofluorometer (Varian Canada Inc., Mississauga, Ontario, Canada), and stirred continuously. Fluorescence intensities were measured at excitation wavelengths of 490 nm and 440 nm by rapidly alternating the monochromator between both wavelengths. The wavelength emission was 525 nm. The excitation and emission slit widths were set on 5 and 10 nm, respectively. The intracellular pH of bacteria was determined from the ratio of the fluorescence signal at the pH-sensitive wavelength (490 nm) and the fluorescence signal at the pH-insensitive wavelength (440 nm). A calibration curve was determined in buffers with pH values ranging from 4 to 8. Buffers contained 50 mM

citric acid (pH 4 and 5), 50 mM Na_2HPO_4 (pH 6 and 7) and 50 mM tris-HCL (pH 8). The pH_{in} was equilibrated by addition of 1 µM valinomycin and 1 µM nigericin.

Cell constituent's release

The release of cell constituents into the supernatants was measured using method described by Rhayour *et al.* (*40*). Cells from working culture (100 mL) of *E. coli* 0157:H7 and *L. monocytogenes* were collected by centrifugation (3000 X g for 15 min.), washed three times and resuspended in PBS (Passive Buffer Saline, Difco, Becton Dickinson, Sparks, MD). One hundred (100) mL of cell suspension were incubated under agitation for one hour at 35 ± 1°C in presence of each essential oil at four different concentrations (control, CMI, a concentration equivalent to twice the CMI and an intermediate concentration between the MIC and MTC), in a G24 Environmental Incubator Shaker (New Brunswick, Edison, NJ). Then, samples of 10 ml were collected and filtered through a 0.2-µm-pore-size filter (Sarstedt Inc., Montréal, Québec, Canada) (*11*). The concentration of the constituents released was determined by UV absorption measurements of each filtrate using a spectrophotometer Cary 1 (Varian Canada Inc., Mississauga, Ontario, Canada) at 260 nm. Correction was made for the absorption of the suspending liquids with PBS containing the same concentration of oil filtrated after 2 min of contact with bacteria. The untreated cells (control) were corrected with PBS.

Transmission electron microscopy

Spanish oregano, Chinese cinnamon and savory essential oils were added at their respective MIC to working cultures (9 mL) of *E. coli* 0157:H7 and *L. monocytogenes*. The control without oil and samples containing essential oils were incubated at room temperature for 30 minutes. After incubation, cells were fixed in phosphotungstic acid (1% (wt/vol), pH 6), and samples were placed on a 200-mesh copper grid and observed with a Hitachi model 7100 transmission electron microscope at an accelerating voltage of 75 kV. The electron micrographs were obtained with a magnification of 24,800.

Statistical analysis

The experiment was done in triplicate. For each measurement, three samples in each replicate were tested. Analysis of variance and Duncan's multiple-range tests were used to analyze statistically all results. Differences between means were considered significant when $P \leq 0.05$. Stat-Packets Statistical Analysis software (SPSS Base 10.0, SPSS Inc., Chicago, IL) was used for the analysis.

Results and Discussion

Intracellular and Extracellular ATP Concentrations

The effect of Spanish oregano, Chinese cinnamon and savory essential oils on the ATP concentration in *E. coli* O157:H7 and *L. monocytogenes* cells were measured and presented in Figures 1 and 2, respectively. The intra- and extracellular ATP concentrations in the untreated cells showed a respective value of 1.84 and 0.03 ng/mL of ATP in *E. coli* O157:H7. In *L. monocytogenes*, the intra- and extracellular ATP concentrations were 6.26 and 0.03 ng/ml respectively.

When *E. coli* O157:H7 was treated with Spanish oregano at concentrations $\geq$ 0.013% (vol/vol), a significant reduction of the intracellular ATP concentration ($P \leq 0.05$) and a significant increase of the extracellular ATP concentration ($P \leq 0.05$) was observed. When cells were treated with 0.013 % (vol/vol), a respective ATP concentration of 0.10 and 0.88 ng/mL, was observed and no significant difference ($P > 0.05$) was observed by increasing the concentration of Spanish oregano (Fig. 1-A). A treatment with 0.025% (vol/vol) Chinese cinnamon essential oil allowed a significant decrease ($P \leq 0.05$) of the intracellular ATP concentration from 1.84 ng/mL to 1.09 ng/ml (Fig. 1-B). However, any significant difference ($P > 0.05$) of the intracellular ATP concentration was observed by increasing the concentration of Chinese cinnamon oil (> 0.025%, vol/vol). A very light increase of the extracellular ATP concentration was observed after incubation of *E. coli* O157:H7 cells in presence of 0.025% (vol/vol) Chinese cinnamon oil. The ATP level increased from 0.03 to 0.05 ng/ml and any significant difference ($P > 0.05$) was observed by increasing the concentration of Chinese

cinnamon essential oil at concentrations ≥ 0.025%. When *E. coli* O157:H7 cells were in presence of 0.025% (vol/vol) savory essential oil, a significant decrease ($P \leq 0.05$) of the intracellular ATP concentration was observed (Fig. 1-C). The intracellular ATP decreased from 1.84 to 0.08 ng/mL. Then, the intracellular ATP levels remained stable by increasing the concentrations level of savory at concentrations > 0.025% (vol/vol). At a concentration of 0.025% (vol/vol), an increase of the extracellular ATP was observed, showing a level of 0.54 ng/mL as compared to 0.03 ng/mL observed for the untreated cells (control). Moreover, a significant increase ($P \leq 0.05$) of the extracellular ATP level was observed when cells were treated with 0.05 and 0.1% (vol/vol) savory essential oil, reaching respective values of 0.67 and 0.71 ng/mL.

Addition of Spanish oregano essential oil to *L. monocytogenes*, allowed a significant decrease ($P \leq 0.05$) of the intracellular ATP concentration (Fig. 2-A). The intracellular ATP concentration decreased from 6.26 to 0.36 ng/mL by addition of 0.013% (vol/vol) Spanish oregano essential oil. An increase of the Spanish oregano essential oil concentration had a significant effect ($P \leq 0.05$) on the extracellular ATP levels. An increase of the extracellular ATP from 0.03 ng/mL for the control to 0.22 ng/mL of the extracellular ATP concentration was observed in presence of 0.05% (vol/vol) Spanish oregano essential oil. When cells were treated with Chinese cinnamon oil, a gradual and continuous decrease of the intracellular ATP level was observed (Fig 2-B). The level of the intracellular ATP of *L. monocytogenes* decreased significantly ($P \leq 0.05$) from 6.26 for the control to 4.09, 1.60 and 0.22 ng/mL in presence of 0.025, 0.05 and 0.1% (vol/vol) Chinese cinnamon oil, respectively. Under the same conditions, a very light increase of the extracellular ATP level was observed with increasing the concentration of Chinese cinnamon oil. The concentration of the extracellular ATP was at 0.09 ng/mL in presence of 0.1% (vol/vol) Chinese cinnamon oil as compared to 0.03 ng/mL for the control. When *L. monocytogenes* was treated with savory oil, a significant decrease ($P \leq 0.05$) of the intracellular ATP concentration was also observed. The concentration of the intracellular ATP decreased from 6.26 for the control to 0.067 ng/mL in presence of 0.025% (vol/vol) savory oil (Fig 2-C). However, any significant difference ($P > 0.05$) of the intracellular ATP concentration was observed by increasing the concentration of savory oil at concentrations > 0.025% (vol/vol). The concentration of the intracellular ATP was respectively 0.045 and 0.037 ng/mL in the presence of 0.05 and 0.1% (vol/vol) essential oil. The same treatments increased significantly (*P*

$\leq$ 0.05) the extracellular ATP level to 2.11, 2.65 and 3.59 ng/mL in the presence of 0.025, 0.05 and 0.1% (vol/vol) savory oil, respectively.

These results showed that these essential oils had the same behavior on both bacteria. However, results showed that Spanish oregano oil allowed a higher increase of the extracellular ATP levels when applied on *E. coli* O157:H7 as compared to *L. monocytogenes*. On the contrary, savory oil allowed a higher increase of the extracellular ATP levels when applied on *L. monocytogenes*. Also, after incubation with Chinese cinnamon, the intracellular ATP concentration decreased gradually to a value of 0.22 ng/mL in presence of 0.1% (vol/vol) Chinese cinnamon when applied on *L monocytogenes*. Whereas a maximal reduction to a value of 1.09 ng/mL was observed in presence of 0.025% (vol/vol) Chinese cinnamon on *E. coli* O157:H7 cells. Our results showed also that after one hour of incubation, the intracellular ATP concentration dropped to very low levels when Spanish oregano or savory essential oils was added to *E. coli* O157:H7 cells and when savory oil was added to *L. monocytogenes* while the levels of external ATP increased. This indicates that the induction of ATP depletion by the addition of essential oils was partially due to the release of ATP from the cells. This phenomenon could be due to a consequence of envelope damage induced by the antibacterial agents. The antibacterial activity of essential oils is probably due to the ability of their compounds to disrupt the membranes of bacterial cells causing lysis of the cell (*39*). According to Helander *et al.* (*21*), carvacrol and thymol are able to inhibit *E. coli* O157:H7 at a concentration between 1 - 3 mM by disintegration of the outer membrane and by the release of outer membrane-associated materials from the cells to the external medium. Moreover according to the same authors, these components decrease the intracellular ATP pool of *E. coli* O157:H7 and also increase extracellular ATP pool, indicating a disruptive action on the cytoplasmic membrane. Similarly, Han (*20*) reported that antimicrobial agent increased the extracellular ATP concentration of *L. monocytogenes*, and the phenomenon of decreasing the intracellular ATP concentration causes the destruction of cell membrane and produce an abnormality in cell membrane. The antimicrobial effect is observed through the extracellular elution of intracellular ATP. According to Gonzalez *et al.* (*17*), the release of ATP and its unabated hydrolysis by the proton-pumping ATPase result in a rapid depletion of the intracellular ATP pool. Lee *et al.* (*31*) founded that lipophilic compounds can exert an antimicrobial effect on *Bacillus cereus* and *Salmonella*

enteritidis by causing cell surface or intracellular abnormality and then eluting intracellular ATP to outside of cells. Depletion of ATP upon addition of lipophilic compounds is a result of their stimulation on the activity of proton and/or ion translocating ATPase, which may originate from the removal of the ion motive force that limits ATP hydrolysis by ATPase in intact cells (*42*).

However, our results showed any correlation between the intra- and extracellular ATP concentrations when *L. monocytogenes* was treated with Spanish oregano or when Chinese cinnamon essential oil was added to both bacteria. This effect has been also reported for several antibacterial agents, such as bacteriocins (*14, 23*). The molecular weight of ATP (Mw 507) may play a role in determining its inability to pass through the damaged pore by essential oil treatment. Moreover, the fact that internal ATP may be strongly reduced in the presence of a weak ATP efflux, suggests that it is hydrolyzed inside the sensitive cells. Two mechanisms could explain the hydrolysis: a shift in the equilibrium of the ATP hydrolysis reaction as a consequence of inorganic phosphate loss through the membrane with impaired permeability (*1*) or accelerated hydrolysis due to the attempt of cells to regenerate the electrochemical gradient by proton extrusion driven by the ATPase energy-consuming pump (*14*). According to Chen and Montville (*14*), this second mechanism was concomitant with cell death in *L. monocytogenes*.

Cell constituents' release

The cell constituents' release was observed by the measurement of the absorbance at 260 nm of the filtrates of the *E. coli* O157:H7 and *L. monocytogenes* culture. Table 1 shows the results of the cell constituent's release when *E. coli* O157:H7 and *L. monocytogenes* were treated with essential oils. Results showed that after adding Spanish oregano, Chinese cinnamon and savory oils to *E. coli* O157:H7 and *L. monocytogenes*, the cell constituent's release increased gradually with the increase of the essential oils concentration.

A significant increase ($P \leq 0.05$) of the cell constituents release was observed when *E. coli* O157:H7 was treated with Spanish oregano at a concentration of 0.013% (vol/vol). An increase of the absorbance from 0.150 to 0.278 was observed when compared to the control. The release of the cell constituents remained stable when cells were treated with 0.025% (vol/vol)

Spanish oregano, whereas a significant increase ($P \leq 0.05$) occurred at 0.05% (vol/vol), reaching an absorbance of 0.471. The treatment by Chinese cinnamon caused a significant increase ($P \leq 0.05$) of the cell constituent's release at a concentration of 0.025% (vol/vol), showing an absorbance value of 0.559. However, any significant difference ($P > 0.05$) of the cell constituents release was observed by increasing the concentration of Chinese cinnamon at concentrations > 0.025% (vol/vol). A significant increase ($P \leq 0.05$) of the absorbance was observed when *E. coli* O157:H7 was treated treated with 0.025 and 0.1% (vol/vol) savory oil, showing a respective value of 0.327 and 0.508.

When *L. monocytogenes* cells are treated with Spanish oregano a significant increase ($P \leq 0.05$) of the cell constituents release was observed after a treatment with 0.013% (vol/vol) Spanish oregano showing an increase of the absorbance from 0.155 to 0.380. The cell constituents' release in the supernatants also increased significantly ($P \leq 0.05$) when cells were treated with Spanish oregano oil at a concentration of 0.05% (vol/vol), showing a value of 0.650. Chinese cinnamon caused a significant release ($P \leq 0.05$) of cell constituents at a concentration of 0.025% (vol/vol), showing an absorbance of 0.559. The absorbance of the filtrates remained stable when cells where treated at 0.05 and 0.1% (vol/vol), showing respective values of 0.549 and 0.584. Moreover, the treatment of *L. monocytogenes* with savory essential oil allowed also a significant increase ($P \leq 0.05$) of the cells constituents' release. When cells are treated with savory essential oils at 0.025 and 0.1% (vol/vol), the absorbance increased to 0.439 and to 0.587 respectively.

These results showed that Chinese cinnamon was the most effective essential oil on both bacteria when the concentration used was 0.025%(vol/vol). Moreover, at a concentration of 0.05% (vol/vol), Spanish oregano essential oil allowed a higher release of *L. monocytogenes* constituents than the release observed for *E. coli* O157:H7, showing an absorbance of 0.650 and 0.471, respectively. An important loss of cytoplasmic constituents means that an important and irreversible damage to the cytoplasmic membrane occurred (*11*, *24*). Chlorhexidine (*24*), hexachlorophene (*25*), α-pinene (*3*), tea tree oil (*11*) and lemongrass oil (*34*) are considered such as antimicrobial compounds and have all a same target on bacteria, which is cytoplasmic membrane and so, act by inducing a loss of 260 nm absorbing material. Carson *et al.* (*11*)

founded that tea tree oil or its components allowed a loss of 260 nm absorbing material and increased susceptibility to NaCl of *S. aureus* and what brings to suggest that the cytoplasmic membrane is compromised. Also, among the 260 nm absorbing material, there are nucleic acids, which were leaked through a damaged cytoplasmic membrane (*11*). Cox *et al.* (*15*) demonstrated that a treatment with tea tree oil on *E. coli* and *S. aureus* allowed a loss of potassium ions, inhibition of respiration and the uptake of promidium iodide. Hada *et al.* (*18*) showed that the total amount of K^+ ions lost from *S. aureus* cells increased with the increase in the concentration of tea tree oil. According to Rhayour *et al.* (*40*), oregano and clove oils or their major components (thymol and eugenol) were able to release from *E. coli* a 260 nm absorbing material cells reaching a slightly value more than double than that released with polymyxin B. In our study, the comparison of the cell constituents released of both bacteria tested under the same conditions after one hour of incubation, suggests that Spanish oregano caused a more important cytoplasmic membrane alteration and permeability perturbation in *L. monocytogenes* than in *E. coli* O157:H7. Savory oil showed a slightly higher permeability perturbation in *L. monocytogenes* than in *E. coli* O157:H7. On the other hand, it appears that Chinese cinnamon had the same effect on both bacteria. The present study demonstrated that Spanish oregano, Chinese cinnamon and savory essential oils are very strong antimicrobial agents. Recent studies showed that antimicrobial action of some essential oils is related to the disruption of the bacterial and fungal membrane (*5*, *15*, *30*). Tassou *et al.* (*44*) showed effect of concentration of mint essential oil on the release of ribose and material absorbing at 260 nm of *S. aureus* and *S. enteritidis*. The changes observed in the absorbance were correlated with the amount of mint essential oil added. Bennis *et al.* (*5*) showed that the release of cellular content absorbing at 260 nm increased in terms of the concentration of thymol and eugenol, from a respective concentration of 3 mM and 6 mM. Chemical antimicrobial agents showed a similar action on the cell constituents' release of microorganims. When treated with dendrimer biocides, a release of 260 nm absorbing materials of *E. coli* quickly increased and reached a plateau afterwards, while the release of *S. aureus* cell constituents increased monotonically with the concentration due to the difference in cell structures (*13*). Moreover, Chen and Cooper (*13*) suggested that the principal mechanism of the antimicrobial dendrimer biocide action is the damage to the cell membranes. Similarly, Koga *et al.* (*28*) showed that the bactericidal plaunotol led to an increase in permeability of the membrane, as evidenced by measurement of the leakage of 260 nm

absorbing-material from *H. Pylori*. A 260-nm-absorbing material leaked from *Candida albicans* cells when incubated with 6 mM of antimicrobial carrot phytoalexin 6-methoxymellein resulted of its interaction with their membranes and disturbance of membrane-associated functions (*2*). Anaesthetics agents caused a lost of substantial amounts of compounds absorbing at 260 nm from *E. coli* ML30 and thus a greatest increases in cell permeability was observed and permeability changes generally decreased as the concentrations of anaesthetics were reduced (*38*).

Intracellular pH

Depletion of intracellular ATP in presence of essential oils suggests on ion gradient variation across the cellular membrane. Therefore, the pH_{in} of *E. coli* O157:H7 and *L. monocytogenes* was investigated after one hour of incubation in presence of essential oils and the results are presented in Table 2, respectively. The pH_{in} of *E. coli* O157:H7 and *L. monocytogenes* was 7.25 and 6.23, respectively. Addition of 0.025% (vol/vol) Spanish oregano, 0.025% (vol/vol) Chinese cinnamon and 0.05% (vol/vol) savory essential oils caused a significant decrease ($P \leq 0.05$) of the pH_{in} of *E. coli* O157:H7. The pH_{in} decreased from 7.25 to 6.68, 5.16 and 5.94, respectively. In presence of 0.05% (vol/vol) Spanish oregano, the intracellular pH decreased to 6.13. In presence of 0.05 and 0.1% (vol/vol) Chinese cinnamon oil, the intracellular pH decreased to 5.07 and 4.87, respectively ($P \leq 0.05$). Also, it seems that addition of savory essential oil at 0.05% (vol/vol) was the maximal concentration which affects the pH gradient of *E. coli* O157:H7 cells. Results obtained with *L. monocytogenes*, showed that exposure of cells to essential oils decreased significantly ($P \leq 0.05$) the pH_{in}, whatever the essential oils used. The pH_{in} decreased from 6.23 to 5.99 and 5.11 and 5.10 in presence of Spanish oregano at 0.013, 0.025 and 0.05% (vol/vol), respectively. In presence of other essential oils, a concentration of 0.025, 0.05 and 0.1% (vol/vol) decreased the pH_{in} from 6.23 to 5.34, 5.12 and 5.13 for cells treated with Chinese cinnamon oil and to 6.13, 5.32 and 5.24 when treated with Savory oil. It appears that addition of 0.025% (vol/vol) Spanish oregano and 0.05% (vol/vol) Chinese cinnamon were the maximal concentrations which affects the pH gradient of *L. monocytogenes* cells. Also, any significant difference ($P > 0.05$) was observed between pH_{in} of *L. monocytogenes* treated with Spanish oregano and Chinese cinnamon oils at 0.05% (vol/vol).

The results obtained for both bacteria showed a significant decrease ($P \leq 0.05$) of the pH_{in} by increasing the concentration of essential oils. Also, Chinese cinnamon was the oil that affected more the pH_{in} of *E. coli* O157:H7 while *L. monocytogenes* was more affected by the presence of Spanish oregano. The pH_{in} is critical for the control of many cellular processes, such as DNA transcription, protein synthesis, and enzyme activity (*7*). It is known that different bacteria (acidophiles, neutrophiles and alkalophiles) can exhibit a wide range of pH_{in} values, from 5.6 to 9 (*6, 7*), so the depletion of this parameter can be a perfect indicator of a changes occurred in cells bacteria. The decrease of pH_{in} was shown to correlate with the accumulation of the compounds in the cytoplasmic membrane (*42*). Loss in the barrier function of the membrane to protons impairs pH homeostasis (*6*). The hydrophobic compounds such as carvacrol dissolve in the membrane, and their activity was closely correlated with the membrane fluidity and their concentration (*45*). According to Salmond *et al.* (*41*), the effects of food preservatives as cinnamic, propionic, benzoic and sorbic acids is related to their capacity to reduce specifically the intracellular pH. Cardoso and Leao (*10*) reported that the inactivation of cells of *S. cerevisiae* by monocarboxylic acid and ethanol is related to the decrease in intracellular pH. Warth *et al.* (*46*) found that at concentration of benzoic acid near the MIC, there was lowered ATP and intracellular pH in *S. cerevisiae*. Also, Krebs *et al.* (*29*) showed that at low external pH, acid benzoic entering *S. cerevisiae* cells caused a large reduction in pH_{in}. A depletion of intracellular pH of *B. cereus* was observed after addition 0.5 and 0.1 mM of carvacrol (*45*). The pH gradient of *P. aeruginosa* and *S. aureus* was rapidly dissipated after addition of 18 µl of oregano essential oil (*30*). According to these studies, the hypothesis about essential oils which disrupted the cell membrane and then caused a decrease in intracellular pH is assumed. It can be concluded that these essential oils provoke an increase of the membrane permeability for both bacteria used.

Electronic microscopy

Electron micrographs of *E. coli* O157:H7 and *L. monocytogenes* cells untreated or treated with Spanish oregano, Chinese cinnamon and savory oils are presented in Figures 3 and 4. Cells of *E. coli* O157:H7 and *L. monocytogenes* in exponential phase of growth had the typical structure of Gram-negative and Gram-positive bacteria, respectively showing an irregular and striated wall for *E. coli* O157:H7 (Fig. 3-A) and a smoothly envelope which point to intracellular

constituents for *L. monocytogenes* (Fig. 4-A). The electron micrographs of treated cells showed an important morphological damage and a disrupted membrane in both bacteria which allowed an increase in the permeabilization of cells to the dye (Fig. 3-B, 3-C, 3-D and Fig. 4-B, 4-C, 4-D). We have previously demonstrated that the essential oils used in this study killed bacteria cells (*36, 37*). In addition, the results present in this paper showed that the content of *E. coli* O157:H7 and *L. monocytogenes* cells treated with essential oils appeared depleted, indicating that the structure of both bacteria envelope was severely affected by the antimicrobial agent. *E coli* O157:H7 treated with Spanish oregano (Fig. 3-B) showed holes or white spots on cellular envelope. When *L monocytogenes* are treated with Spanish oregano (Fig. 4-B) an incomplete and deformed shape of the cells is observed. When both cells are treated with Chinese cinnamon, this oil did not affect the appearance of the cells (Fig. 3-C and 4-C). When both cells are treated with savory essential oils (Fig. 3-D and 4-D), a more important deformation is observed with *L. monocytogenes* than that observed with *E. coli* O157:H7. Skandamis *et al.*, (*43*) observed that the minimum bacteriocidal concentration of oregano essential oil on cells of *E. coli* O157:H7 affected the dense cellular materials and coagulated the cytoplasmic constituents, while the membrane structure tended to be irregular and rough. According to Rhayour *et al.* (*40*), the type of damage observed by scanning electronic microscope could be different from one bacterium to another even when treated with the same essential oils. Their results showed that when *Bacilus subtilis* or *E. coli* cells were treated with oregano and clove oils, different comportments were observed. The presence of holes in the envelope was observed on *E. coli*, whereas damage as cell deformity was observed with *B. subtilis*. Anh *et al.* (*4*) observed that when *L. monocytogenes* is treated with an antibacterial agent as allyl isothiocyanate, destruction of cell wall and at the same time an elution of intracellular ATP occurred. Recent investigations about antimicrobial effect of thymol and eugenol on *S. cerevisiae* showed important membrane damage by scanning electronic microscopic and this was accompanied by an important surface alteration (*5*). According to Knoblock *et al.*, (*27*), the presence of terpenes in essential oils acts on the cytoplasmic membrane or on enzyme-dependant reaction.

This work reported a variety of morphological and physiological changes in *E. coli* and *L. monocytogenes* induced by the action of essential oils. Our study showed that Spanish oregano, Chinese cinnamon and savory essential oils creates permeability in the cell membrane, provoking

a release of the cell constituents, a decrease of the ATP concentration in the cells and a decrease of the intracellular pH. The originality of this study is the relation established between the evaluation of the membrane integrity by electronic miscroscopy and the measurement of the intracellular pH and ATP concentration. Many of the studies on the mechanism of essential oils have been focused on release of material absorbing at 260 nm, while few studies dealt with the measurement of the intracellular pH and ATP concentration. Further work is undergoing to understand the mechanisms of action involved.

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Table 1. Effect of Spanish oregano, Chinese cinnamon and savory essential oils on the Cell constituents'release of *E. coli* O157:H7 and *L. monocytogenes.*

[a]The values are the average of three experiments. Means for the same column bearing the same lower case letters are not significantly different (P>0.05). Means for the same raw bearing the same upper case letters are not significantly different (P>0.05).

[b]Not determined

Essential oil concentrations (%, vol/vol)	Cell constituents'release (OD 260 nm)[a]		
	Spanish oregano	Chinese cinnamon	Savory
***E. coli* O157:H7**			
0	0.150 ± 0.021 a	0.150 ± 0.021 a	0.150 ± 0.021 a
0.013	0.278 ± 0.018 b	ND[b]	ND
0.025	0.319 ± 0.035 bA	0.559 ± 0.004 bB	0.327 ± 0.031 bA
0.05	0.471 ± 0.029 cB	0.581 ± 0.041 bC	0.328 ± 0.029 bA
0.1	ND	0.606 ± 0.017 Bb	0.508 ± 0.081 cA
L. monocytogenes			
0	0.155 ± 0.01a	0.155 ± 0.01 a	0.155 ± 0.01 a
0.013	0.380 ± 0.028 b	ND[b]	ND
0.025	0.382 ± 0.031 bA	0.559 ± 0.053 bB	0.439 ± 0.026 bA
0.05	0.650 ± 0.027 cC	0.549 ± 0.026 bB	0.449 ± 0.023 bA
0.1	ND	0.584 ± 0.038 bA	0.587 ± 0.025 cA

Table 2. Effect of Spanish oregano, Chinese cinnamon and savory essential oils on pH_{in} of *E. coli* O157:H7 and *L. monocytogenes*.

Essential oil concentrations (%, vol/vol)	pH_{in}		
	Spanish oregano	Chinese cinnamon	Savory
***E. coli* O157:H7**			
0	7.25 ± 0.20 c	7.25 ± 0.20 d	7.25 ± 0.20 b
0.013	7.06 ± 0.24 c	ND[b]	ND
0.025	6.68 ± 0.37 bB	5.16 ± 0.05 cA	7.25 ± 0.08 bC
0.05	6.13 ± 0.21 aC	5.07 ± 0.04 bA	5.94 ± 0.12 aB
0.1	ND	4.87 ± 0.02 aA	5.98 ± 0.11 aB
L. monocytogenes			
0	6.23 ± 0.03 c	6.23 ± 0.03 c	6.23 ± 0.03 d
0.013	5.99 ± 0.05 b	ND[b]	ND
0.025	5.11 ± 0.08 aA	5.34 ± 0.03 bB	6.13 ± 0.04 cC
0.05	5.10 ± 0.03 aA	5.12 ± 0.01 aA	5.32 ± 0.04 bB
0.1	ND	5.13 ± 0.01 aA	5.24 ± 0.08 aA

[a]The values are the average of three experiments. Means for the same column bearing the same lower case letters are not significantly different (P>0.05). Means for the same raw bearing the same upper case letters are not significantly different (P>0.05).

[b]Not determined

FIGURE LEGENDS

Figure 1. ***Effect of Spanish oregano, Chinese cinnamon and savory essential oils on intra- and extracellular ATP concentrations of E. coli O157:H7.*** Intracellular (■) and extracellular (◇) ATP concentrations; Spanish oregano (A), Chinese cinnamon (B), savory (C).

Figure 2. ***Effect of Spanish oregano, Chinese cinnamon and savory essential oils on intra- and extracellular ATP concentrations of L. monocytogenes.*** Intracellular (■) and extracellular (◇) ATP concentrations; Spanish oregano (A), Chinese cinnamon (B), savory (C).

Figure 3. ***Electron micrographs of E. coli 0157:H7 cells***: untreated (A), treated with Oregano oil (B), treated with Cinnamon oil (C), treated with Savoy oil (D). Magnification X24,800.

Figure 4. ***Electron micrographs of L. monocytogenes cells:*** untreated (A), treated with Oregano oil (B), treated with Cinnamon oil (C), treated with Savoy oil (D). Magnification X24,800

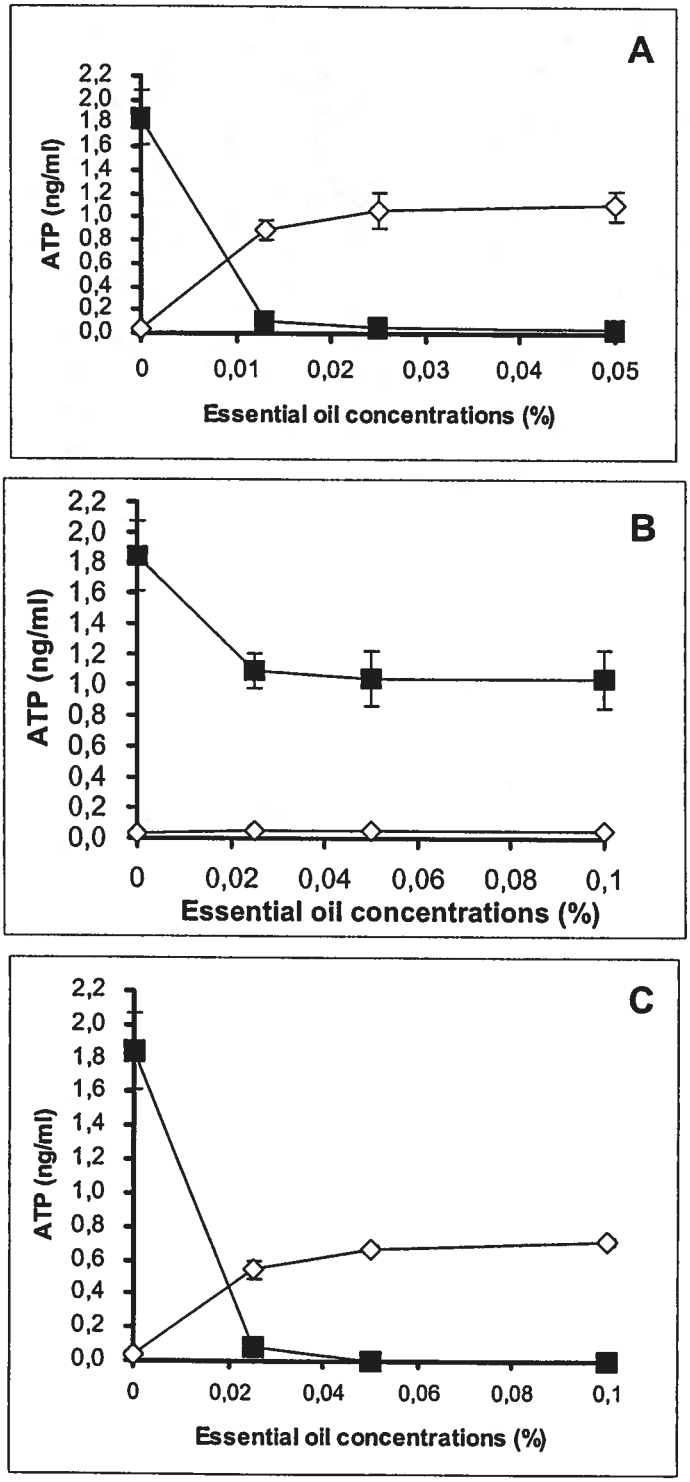
A
ATP (ng/ml)
2,2
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
0,01
0,02
0,03
0,04
0,05
Essential oil concentrations (%)
B
ATP (ng/ml)
0
0,02
0,04
0,06
0,08
0,1
Essential oil concentrations (%)
C
ATP (ng/ml)
Essential oil concentrations (%)

Figure 1.

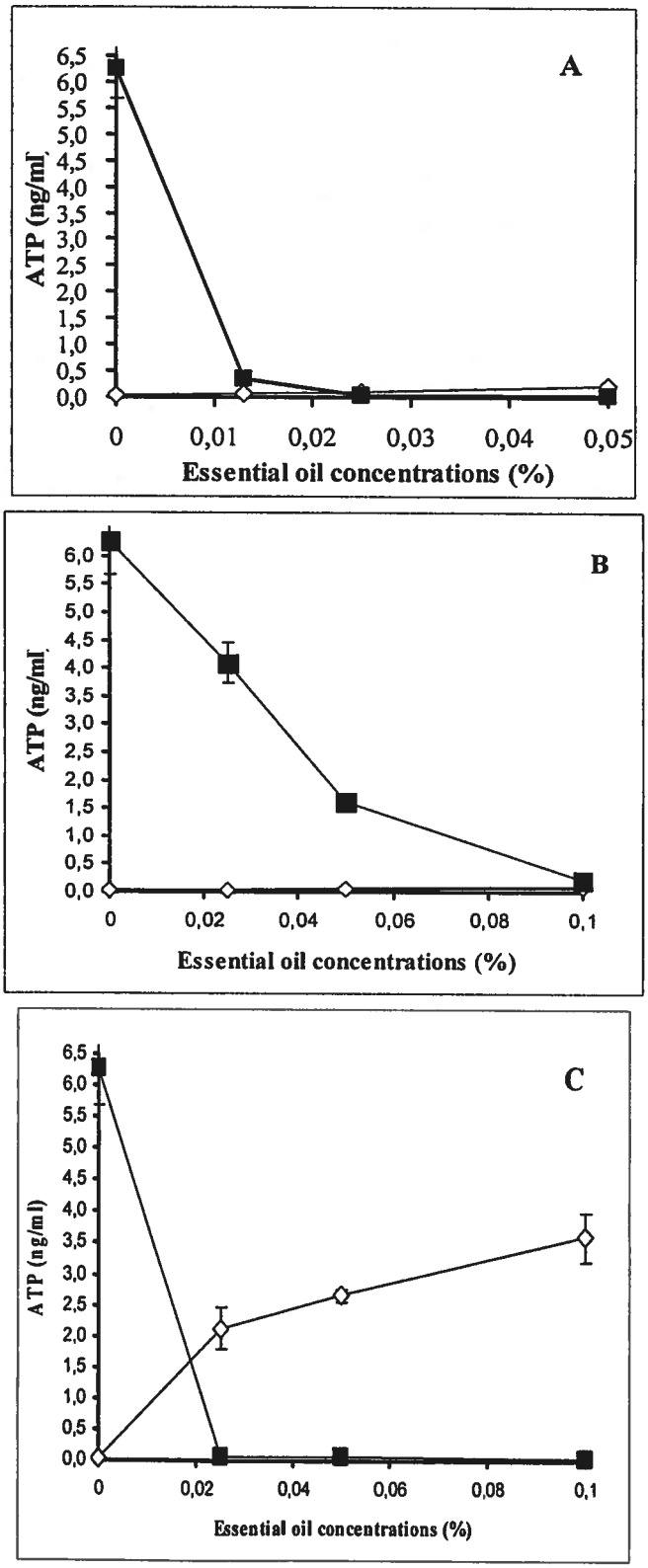
A
ATP (ng/ml)
6,5
6,0
5,5
5,0
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
0,01
0,02
0,03
0,04
0,05
Essential oil concentrations (%)
B
ATP (ng/ml)
6,0
5,5
5,0
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
0,02
0,04
0,06
0,08
0,1
Essential oil concentrations (%)
C
ATP (ng/ml)
6,5
6,0
5,5
5,0
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
0,02
0,04
0,06
0,08
0,1
Essential oil concentrations (%)

Figure 2.

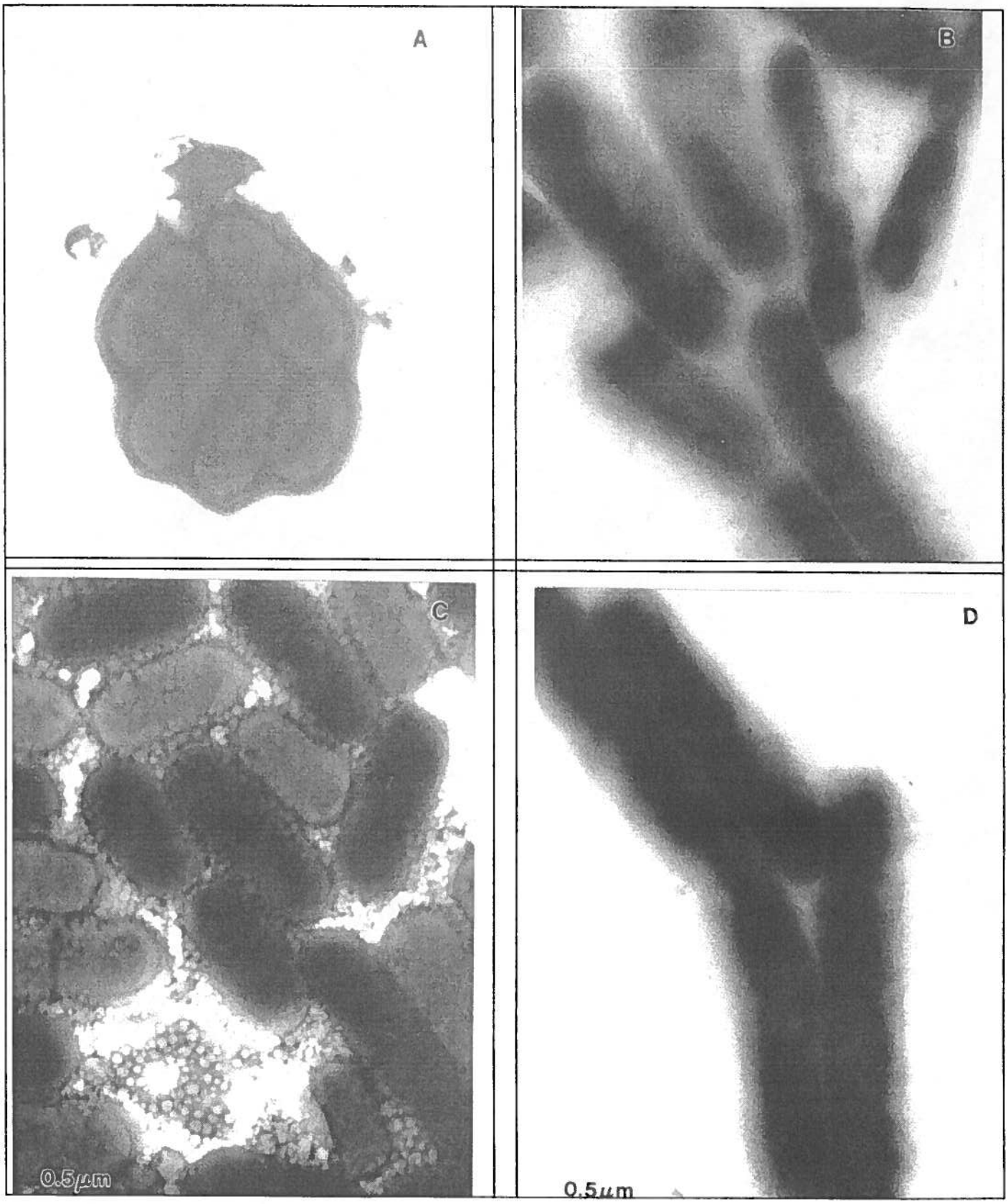

Figure. 4

De : John Sofos

À : Lacroix, Monique

Objet : **ACCEPTANCE**

Dr. Monique Lacroix
Canadian Irradiation Centre
Laval, Quebec, Canada H7V 1B7

Dear Dr. Lacroix:

I have received your revised manuscript JFP-05-353, titled "**Mechanism of action of Spanish oregano, Chinese cinnamon and savory essential oils against cell membranes and walls of Escherichia coli O157:H7 and listeria monocytogenes.**"

I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Food Protection.

The manuscript has been sent to IAFP headquarters for further handling. You will receive page proofs of the article and information for ordering reprints following completion of copy-editing and typesetting. Should you have any questions concerning the manuscript, its projected publication date, ordering reprints, or other matters, please contact the Administrative Assistant, Didi Loynachan (Telephone: 515-276-3344 or 800-369-6337, Fax: 515-276-8655, E-mail: dloynachan@foodprotection.org). If special assistance is required, or if you need a hard copy of this notification printed on letterhead, please contact me directly.

Sincerely,

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Chapitre III

Discussion

Les propriétés antimicrobiennes de plusieurs plantes et d'épices sont exploitées depuis très longtemps pour la conservation des produits alimentaires (Farag et al., 1989). Leur effet antimicrobien est principalement attribué aux principes actifs retrouvés dans les huiles essentielles extraites de ces plantes et ce à des concentrations très élevées (Farag et al., 1989). L'analyse chimique des huiles essentielles, effectuée par chromatographie phase gazeuse et fournie par le manufacturier, montre que la plupart des composés présents dans les huiles essentielles testées sont des monoterpènes phénoliques tel que le carvacrol, le thymol ou l'eugénol, des monoterpènes hydrocarbonés tel que le *p*-cimène, le γ-terpinène, l'α-pinène ou le limonène, des alcools tels que le bornéol, le linalool, le 1,8-cinéole ou le géraniol, des aldéhydes tel que le cinnamaldéhyde, le géranial ou le citronnéllal et enfin des cétones tel que le pipéritone ou le carvone.

Le cinnamaldéhyde et l'eugénol sont les constituants majeures antimicrobiens présents dans la cannelle et le clou de girofle, ces composés représentent 65-75% et 93-95% respectivement des composés volatils totaux (Bullerman et al., 1977). Dans l'origan et le thym, les constituants majeures sont le carvacrol (62-79%) et le thymol (42%), respectivement (Farag et al., 1989; Sivropoulou et al., 1996). Pour la sarriette, seulement certaines espèces contiennent des composés phénoliques tels que le carvacrol et le thymol (Tümen et al., 1998). L'huile essentielle de la sarriette des montagnes (*Satureja montana*) et la sarriette des jardins (*Satureja hortensis*) utilisées dans cette étude contiennent une quantité importante de thymol (42.71%) et de carvacrol (40.89%), respectivement. L'activité antimicrobienne est aussi liée à la configuration structurale des principes actifs, à leurs groupements fonctionnels et à l'effet synergétique entre les composés (Dorman et Deans, 2000).

Afin de contrôler la contamination bactérienne qui engendre d'importants problèmes tels que l'altération des viandes et les toxi-infections alimentaires, une technologie récente a fait son apparition dans le domaine des viandes. Cette technologie consiste à immobiliser des composés

antimicrobiens et antioxydants dans des polymères biodégradables qui diffusent lentement et permettent de maintenir des concentrations élevées, préalablement ajoutées, sur la surface des viandes (Oussalah et al., 2004). L'incorporation d'huiles essentielles ou d'acides organiques dans des films d'emballages ont déjà fait l'objet de quelques études afin de contrôler la croissance bactérienne au cours de l'entreposage de la viande (Ouattara et al., 1997; Giroux et al., 2001) ou des crevettes (Ouattara et al., 2001). Pranoto et al. (2004) ont montré, par le test de diffusion sur milieux gélosés, que les films d'alginate à base d'huile essentielle d'ail possédaient une activité antimicrobienne contre *S. aureus* et *B. cereus*. L'immobilisation de la nisine dans un gel d'alginate et de calciu ı a aussi permis une diminution de 2 log les comptes de *Brochothrix thermosphacta* après sept jours de conservation sur des surfaces de carcasses de boeuf (Cutter et Siragusa, 1996).

Le mode d'action de toxicité bactérienne de ces huiles essentielles antimicrobiennes n'est pas très bien compris. Les seules hypothèses qui existent reposent sur le fait que ces composés ont un caractère hydrophobe et que ces huiles peuvent ainsi interagir avec les structures hydrophobes de la cellule bactérienne tel que leurs membranes (Sikkema et al., 1995).

1. Détermination de la CMI et la CMT

Dans la présente étude, un nombre important d'huile essentielles, reconnues comme étant antimicrobiennes a été testé sur des bactéries jamais encore étudiées et que l'on retrouve sur la viande rouge ou dans les charcuteries. L'objectif de cette étude consistait à déterminer les concentrations minimales inhibitrices et les concentrations maximales tolérées des huiles essentielles contre une bactérie d'altération, *P. putida* et quatre bactéries pathogènes, *E. coli* O157:H7, *S. aureus, L. monocytogenes* et *S.* Typhimurium. La méthodologie utilisée a permis de sélectionner les huiles essentielles capables d'inhiber la croissance bactérienne à une concentration minimale inhibitrice (CMI) inférieure ou égale à 0.8% (v/v). La CMT permet d'évaluer le niveau de sensibilité de la bactérie envers cet agent antibactérien. La concentration maximale tolérée (CMT) est la concentration maximale qui est sans effet sur la concentration cellulaire. Ainsi, les huiles essentielles ayant les plus petites CMI sont celles qui, lorsque rajoutées aux aliments, auront le moins d'impact sur la qualité organoleptique de l'aliment tout en exercant des activités antimicrobiennes intéressantes.

Cependant, il est très difficile de comparer l'efficacité des huiles essentielles rapportée dans les nombreuses recherches. Ceci est principalement dû à différents paramètres externes qui sont incontrôlables comme la composition des huiles qui peut varier en fonction des conditions climatiques et environnementales (Janssen et al., 1987; Sivropoulou et al., 1996). La méthode utilisée et le choix des microorganismes à tester sont aussi des paramètres qui font varier l'activité antimicrobienne des huiles essentielles (Janssen et al., 1987). La technique de la diffusion dans des disques d'Agar est très fréquemment utilisée lors de la sélection des huiles essentielles (Smith-Palmer et al., 1998). La concentration bactérienne, le temps d'exposition avec les huiles essentielles, la solubilité de l'huile essentielle dans le milieu de culture utilisé et enfin le moyen utilisé pour l'homogénéiser sont autant des facteurs responsables de la variabilité entre des résultats rapportés. Dans notre étude, la dispersion des huiles essentielles dans le TSA a été obtenue grâce à l'ajout de l'amidon, formant ainsi une suspension colloïdale.

1.1. Effet des huiles essentielles sur *P. Putida*

Plusieurs bactéries d'altération (ex. *Pseudomonas* sp.) sont reconnues pour leur nature psychotrophe qui leur permet de croître à des températures aussi basses que 4°C et ont montré une grande sensibilité envers certaines huiles essentielles testées. Dans d'autres recherches, l'inhibition *in vitro* de *P. aeruginosa* a été observée avec des huiles essentielles d'origan, de citronnelle et de piment à une même concentration de 1.0% (v/v) (Hammer et al., 1999). Delaquis et al. (2002) ont déterminé des CMI de 0.30%, 0.15% et de 0.30% (v/v) respectivement pour les huiles essentielles d'eucalyptus, de coriandre et d'aneth contre *P. fragi*. Les huiles essentielles de cannelle, de clou de girofle, de piment et de romarin ont démontré aussi une grande efficacité contre *P. fluorescens* (Ouattara et al., 1997).

Les résultats obtenus dans cette première section ont permis de confirmer l'activité antimicrobienne d'au moins 28 huiles essentielles sur un milieu solide contre *P. putida*. Il a été possible de conclure que les huiles essentielles d'origan (*Corydothymus capitatus*, *Origanum compactum* et *O. heracleoticum*), de sarriette (*Satureja hortensis* et *S. montana*), de cannelle de Chine (*Cinnamomum cassia*), de thym (*Thymus vulgaris thymoliferum*) et de thym à carvacrol (*Thymus vulgaris carvacroliferum*) avaient une très importante activité antimicrobienne contre

cette bactérie. Deux types d'huile essentielle de cannelle (*Cinnamomum verum*) ont été testées, l'une provenait de l'écorce et l'autre des feuilles. *P. putida* a montré une sensibilité deux fois plus grande à l'huile de cannelle de Chine (*Cinnamomum cassia*), avec une CMI de 0.05% (v/v) et une CMT de 0.025% (v/v), qu'à l'huile de cannelle (*Cinnamomum verum*). Il est à noter que dans une autre étude, ces deux types d'huiles ont montré la même efficacité contre 20 espèces de *L. monocytogenes* (Lis-Balchin et Deans, 1997).

Trois types d'origan ont été testé au cours de notre étude, *Corydothymus capitatus*, *Origanum compactum* et *O. heracleoticum*, ces huiles ont été très efficaces contre *P. putida* et ce malgré leur différence d'espèce et d'origine géographique. Une recherche effectuée par Bagamboula et al. (2003) a démontré que la CMI de l'origan (*Origanum vulgare*) était de 0.5% (vol/vol) contre *Shigella sonnei* , *Shigella sonnei* CIP 82.49, *Shigella flexneri*, *Shigella flexneri* CIP 82.48 et *E. coli* LMG 8223. Ces résultats sont en parfait accord avec ceux obtenus par Paster *et al.* (1990) où deux types d'huiles essentielles d'origan (*Origanum vulgare* and *Coridothymus capitatus*) ont été efficaces contre *P. aeruginosa, S.* Typhimurium, *S. aureus*, *C. jejuni* et *Clostridium sporogenes* à une concentration de 0.025% (vol/vol).

Même si une CMI identique a été trouvée pour les deux types d'huiles essentielles de sarriette testées (*Satureja hortensis* et *Satureja montana*), *P. putida* a été deux fois plus sensible à *Satureja hortensis* avec une CMT de 0.013% (v/v). Cette dernière possède un large spectre antimicrobien contre des bactéries pathogènes pour l'homme et les plantes, des bactéries d'altération et certaines moisissures (Gülluce et al., 2003; Nevas et al., 2004).

Parmi huit espèces d'huile essentielles de thym testées contre *P. putida*, il n'y a eu que quatre qui se sont démarquées. *Thymus vulgaris thymoliferum* et *Thymus vulgaris carvacroliferum* ont été les plus efficaces avec une CMI de 0.05% (v/v), suit le *Thymus serpyllum* et le *Thymus satureoides* avec une CMI de 0.1 et 0.2% (v/v), respectivement.

Il est à noter que des huiles essentielles de sauge (*Salvia* species), d'aneth (*Anethium graveolens)*, de citron (*Citrus limon*), d'orange (*Citrus aurantium, C. sinensis*), de bergamote (*Citrus bergamia*), de tarragon (*Artemisia dracunculus*), de niaouli (*Melaleuca quinquenervia*), de thé (*Melaleuca alternifolia*), de turmeric (*Curcuma longa*), de basilic (*Ocimum basilicum*), de

céleri (*Apium graveolens*) et de romarin (*Rosemarinus* species), dont l'activité antimicrobienne a déjà été prouvée (Bagamboula et al., 2003; Cox et al., 2000; Jirovetz et al., 2003; Kivanç et Akgül, 1986; Lis-Balchin et Deans, 1997; Negi et al., 1999; Shelef et al., 1984; Suppakul et al., 2003; Tassou et al., 1996) montrent soit une absence ou une activité antimicrobienne faible contre *P. putida*. Hammer et al. (1999) ont montré que ces huiles étaient très efficaces contre *S. aureus*, *Enterococcus faecalis* et *E. coli*, et moins efficaces contre *Pseudomonas aeruginosa* dont la CMI était supérieure à 2.0% (v/v). Notre étude a montré aussi que les huiles essentielles qui contiennent plus de composés phénoliques comme le carvacrol, le thymol et l'eugénol ont été plus efficaces contre *Pseudomonas putida*. Ces résultats sont en parfait accord avec ceux déjà retrouvés dans une autre étude qui démontre que l'activité antimicrobienne de plusieurs huiles essentielles est relié à leur important taux de composés phénoliques (Sivropoulou et al., 1996). En effet, dépendamment de la concentration utilisée, les composés phénoliques démontrent une activité bactéricide ou bactériostatique (Pelczar et al., 1988). Parmi les huiles essentielles qui ne contenaient pas de composés phénoliques mais des aldéhydes tel que le geranial, le néral, le citronnéllal ou le cuminal, on retrouve des huiles essentielles de *Cymbopogon citratus*, de *Cymbopogon winterianus* et de *Cuminum cyminum*, ces huiles n'ont pas eu un important effet inhibiteur sur *P. putida*.

1.2. Effet des huiles essentielles sur *E. coli* O157:H7, *S.* Typhimurium, *S. aureus* et *L. monocytogenes*

L'objectif de cette étude était de sélectionner les huiles essentielles pouvant inhiber plusieurs bactéries pathogènes à la fois et à de faibles concentrations. Parmi les 28 huiles essentielles testées, huit d'entre elles (*Satureja hortensis, Corydothymus capitatus, Thymus vulgaris carvacroliferum, Origanum heracleoticum, Cinnamomum cassia, Origanum compactum, Satureja montana* and *Cinnamomum verum*) ont une CMI $\leq$ 0.1% et une CMT $\leq$ 0.025% (v/v) contre les quatre bactéries testées. Notre étude montre aussi que la croissance d'*E. coli* O157:H7, de *S.* Typhimurium et de *S. aureus* a été réduite en présence de toutes les huiles essentielles testées à des concentrations $\leq$ 0.4% (v/v). La croissance bactérienne de *L. monocytogenes* a été ralentie avec vingt sept huiles essentielles. Dix sept, vingt six et vingt six huiles essentielles ont inhibé la croissance d'*E. coli* O157:H7, de *S.* Typhimurium et de *S. aureus*

à des concentrations ≤ 0.8%, 0.8% et 0.4% (v/v), respectivement. Et enfin, il n'y a eu que vingt qui ont inhibé la croissance de *L. monocytogenes* à une concentration ≤ 0.8% (v/v).

Sept espèces de thym ont été testées et chacune d'entre elles a montré une activité antimicrobienne différente par rapport aux autres et ce sur la même bactérie. Le *Thymus vulgaris* carvacroliférum contenant 32.90% de carvacrol a été deux fois plus efficace contre les quatre bactéries testées comparativement au *Thymus vulgaris* thymoliferum contenant 11.85% de carvacrol et 38.12% de thymol. Ceci pourrait être expliqué par le fait que la première espèce a plus de carvacrol que la deuxième. Une étude réalisée par Rasooli et al. (2003) a démontré que le *Thymus kotschyanus* et le *T. persicus* composés respectivement de thymol (7% et 27%), de carvacrol (23% et 38%) et de *P*-cymène (8% et 11%) ont été bactéricides à une très faible concentration contre *E. coli*, *S. aureus* et *P. aeruginosa*.

Trois espèces d'origan ont été testées, *Origanum majorana* contenant 9% de carvacrol a été le moins efficace avec une CMI ≥ 0.8% (v/v) pour *E. coli* O157:H7 et *L. monocytogenes*. Des résultats similaires ont été obtenus par Hammer *et al.* (1999). En présence de cette huile, des CMI de 0.2% et 0.4%, respectivement, ont été obtenues pour *S. aureus* et *S.* Typhimurium. À l'inverse, l'*Origanum heracleoticum* contenant 54.38% de carvacrol et l'*O.compactum* contenant 30.53% de carvacrol ont été plus efficaces avec une CMI de 0.025%, 0.05% et de 0.013% contre *E. coli* O157:H7, *S.* Typhimurium et *S. aureus*, respectivement. *L. monocytogenes* a été plus sensible à l'*Origanum heracleoticum*, avec une CMI de 0.05%, par rapport à une CMI de 0.1% pour l'*O. compactum*. La même explication que celle donnée avec les huiles essentielles de thym s'applique soit qu'un pourcentage élevé en carvacrol confère à l'huile une plus grande activité antimicrobienne. Ceci a été même confirmé par les travaux d'Aligiannis et al. (2001), qui ont trouvé que l'*Origanum scabrun* contenant 74.86% de carvacrol avait un effet antibactérien très élevé montrant des CMI de 0.35 et 0.28 mg/ml contre *S.aureus* et *E. coli* respectivement et que l'*Origanum. microphyllum* avec une concentration ≤ 50% de carvacrol avait une MIC de 6.35 et 3.35 mg/ml contre ces mêmes bactéries.

Corydothymus capitatus, plus connu sous le nom de l'origan d'Espagne, contenant 75.53% de carvacrol et 4.47% de thymol, et *Cinnamomum cassia* contenant 74% de cinnamaldéhyde, connu sous le nom de la cannelle de Chine, ont eu une très grande efficacité

contre les quatre bactéries testées. En effet l'inhibition de la croissance bactérienne a été observée à des concentrations ≤ 0.025% et ≤ 0.05% pour les deux huiles, respectivement. Les mêmes observations ont été faites pour le deuxième type de cannelle (*Cinnamomum verum*) testé. Là encore, l'activité antimicrobienne des huiles est due au pourcentage élevé de leurs principes actifs. Ces deux variétés de cannelle contiennent principalement du cinnamaldéhyde, un principe actif déjà retrouvé comme étant bactéricide contre *Bacillus cereus* (Kwon et al., 2003).

Leuschner et Lelsch (2003) ont démontré que l'huile essentielle du clou de girofle, à une concentration de 1%, possédait un effet bactéricide sur *L. monocytogenes*, un effet inhibiteur de la croissance aussi observé dans notre étude. Toutefois, cette bactérie est celle qui a manifesté le plus de résistance envers cette huile (*Eugenia caryophillus*) avec une CMI de 0.2% (v/v). Pour les huiles essentielles de sarriette (*Satureja montana* et *Satureja hortensis*), une CMI ≤ 0.1% (v/v) a été obtenue avec les quatre bactéries testées, les huiles essentielles de cette espèce ont aussi été considérées comme ayant un large spectre antimicrobien (Gülluce et al., 2003; Deans et Svoboda, 1989; Sahin et al., 2003). *L. monocytogenes* a encore été la plus résistante en présence d'huile essentielle de cilantro (*Coriandrum sativum*) montrant une CMI > 0.8%, où avec *E. coli* O157:H7, S. Typhimurium et *S. aureus*, cette huile avait une même CMI de 0.2%.

Les recherches réalisées à ce jour montrent que les bactéries Gram-positif sont plus sensibles que les Gram-négatif à la présence d'huiles essentielles (Hammer et al., 1999). Cette hypothèse est basée sur le fait que ces dernières ne possèdent pas de membrane externe dont les lipopolysaccharides (LPS) empêcheraient l'accumulation des huiles essentielles dans la membrane cytoplasmique. Ceci n'est pas vraiment en accord avec nos résultats puisque *L. monocytogenes*, une bactérie Gram-positif, a été la plus résistante en présence des huiles testées. Cependant, *S. aureus*, une bactérie Gram-positif est celle qui a été la plus sensible en présence de toutes les huiles essentielles testées. Cette bactérie a déjà été trouvée comme étant sensible à un mélange de thymol et de carvacrol (constituants majeures de l'origan) (Lambert et al., 2000; 2001). Les deux bactéries de type Gram-négatif ont aussi montré un certain niveau de sensibilité aux huiles testées. Ces huiles essentielles hydrophobes peuvent atteindre l'espace périplasmique à travers les protéines porines (Helander et al., 1998; Lambert et al., 2001).

2- Immobilisation des huiles essentielles dans les films

2-1 Films à base de protéines laitières

Des huiles essentielles d'origan, de piment et un mélange d'origan et de piment ont été incorporées dans un film à base de caséinate de calcium. Le mélange des deux huiles a été aussi testé afin de savoir si il y a un effet synergétique ou additif entre ces huiles. Notre étude montre que les films à base d'huiles essentielles à une concentration de 1% (p/v) ont permis de réduire significativement le niveau d'*E. coli* O157:H7 et de *Pseudomonas* sp. inoculées sur des tranches de boeuf pendant sept jours d'entreposage à 4°C. Cependant, les comptes microbiens sont restés élevés avec les films sans huile essentielles. Ceci pourrait être expliqué par le fait que les constituants des films ont été utilisés comme substrat par les bactéries. Les films à base d'origan ont été nettement plus efficaces que ceux à base de piment avec les deux types de bactéries. Ces résultats rejoignent ceux obtenus par Burt et Reinders (2003), où l'huile essentielle d'origan a été plus efficace que celle du piment sur *E. coli* O157:H7. Cette différence d'efficacité pourrait être expliquée par la différente composition chimique de ces huiles. En effet, l'huile essentielle d'origan contient 61.75% de carvacrol, 4.32% de thymol et le reste est sous forme de terpènes hydrocarbonés alors que l'huile essentielle de piment contient 74.40% d'eugénol, de 5.98% de méthyleugénol et le reste est sous forme de monoterpènes hydrocarbonés. Il est connu que les composés phénoliques tel que le carvacrol et le thymol présentent un important effet inhibiteur (Karapinar et al., 1987; Lambert et al., 2001). Un effet synergétique entre le carvacrol et l'eugénol a été rapporté par Gülluce et al. (2003) sur les microorganisms. Cet effet n'a pas été observé avec le film à base d'origan et de piment testé dans notre étude. Cependant le film à base d'origan et de piment a été meilleur que celui qui contenait juste le piment.

Une conclusion majeure de cette étude était que les films obtenus n'étaient pas très résistants à l'eau. Toutefois, l'utilisation des films à base d'huiles essentielles reste un moyen de préservation très prometteur ou la formulation et les propriétés mécaniques restent à améliorer pour contrôler la teneur en eau dans le film et le taux de diffusion des huiles des films vers la matrice alimentaire.

2-2- Films à base d'alginate

La complexité des polymères naturels utilisés représente un inconvénient majeur pour la comparaison des méthodes utilisées avec des polymères synthétiques. Afin d'améliorer les propriétés mécaniques des films, des films à base d'alginate, un polysaccharide naturel et de polycaprolactone (PCL), un polyester synthétique biodégradable qui a une bonne résistance à l'eau, ont été mis au point. De plus, le PCL joue le rôle d'agent hydrophobe et d'émulsifiant. Lu et Lin (2002) ont même montré que le PCL permettait un système de diffusion contrôlée. L'alginate, aussi un polymère, est composé de séquences de résidus d'acides β-D mannuronique et α-L gluronique liés par des liaisons glycosidiques (Haug et al., 1967). Puisque l'alginate devient insoluble par gélification ionotropique, ce polymère a souvent été étudié comme matrice pour la fabrication des films (Pavlath et al., 1999). Cette gélification a été obtenue après immersion des films dans une solution de $CaCl_2$, une solution à 2% et une autre à 20% (p/v). Deux concentrations ont été utilisées afin d'observer l'impact des propriétés mécaniques du film sur la diffusion contrôlée des huiles essentielles vers le substrat.

De la sélection des huiles effectuée tout au début de notre étude, trois huiles essentielles à très petite CMI ont été choisis et incorporées dans ces films. Les huiles essentielles sélectionnées sont les huiles essentielles d'origan d'Espagne, de cannelle de Chine et de sarriette des montagnes.

Ces films ont été utilisés pour vérifier le contrôle de la croissance bactérienne lorsque des bactéries telles qu'*E. coli* O157:H7, *L. monocytogenes* et *S.* Typhimurium sont inoculées sur des tranches de boeuf, de bologne ou de jambon.

2.2.1. Test sur les tranches de boeuf

Les films à base d'alginate et d'huiles essentielles traités dans 2 ou 20% de $CaCl_2$ ont permis de diminuer significativement les comptes cellulaires d'*E. coli* O157:H7 et de *S.* Typhimurium inoculés sur des tranches de boeuf. En absence d'huiles essentielles, ces deux bactéries semblent utiliser les ingrédients du film comme nutriments pour soutenir leur croissance. Indépendemment du traitement reçu (2 ou 20% de $CaCl_2$), les films à base d'huiles

essentielles ont permis la même diminution de *S.* Typhimurium sur les tranches de boeuf. Même si le taux de libération des huiles essentielles d'origan d'Espagne, de cannelle de Chine et de sarriette des montagnes a été plus important lorsque les films ont été traités dans du $CaCl_2$ (20%), les films traités avec du $CaCl_2$ (2%) ont été légèrement plus efficaces contre *E. coli* O157:H7 que ceux traités avec du $CaCl_2$ (20%). En effet, au dernier jour d'entreposage, les taux de libération étaient de 65, 90 et de 62% respectivement lorsque les films ont été traités dans du $CaCl_2$ (20%) et de 40, 77 et de 60% respectivement lorsque les films ont été traités dans du $CaCl_2$ (2%). Ces résultats nous laisse déduire que le calcium a un effet protecteur sur *E. coli* O157:H7, ceci pourrait être expliqué par le fait que le calcium induit une résistance au stress éxterieur chez certaines bactéries (Woegerbauer et al., 2002). Le $CaCl_2$ induit même la synthèse de la phosphoéthanolamine transférase, qui est une des causes de la résistance bactérienne au stress environnemental chez certaines éspèces d'*E. coli* (Kanipes et al., 2000).

Des recherches antérieures effectuées sur les propriétés antimicrobiennes de l'origan se trouvent être en parfait accord avec nos résultats. En effet, à une concentration de 0.8%, l'origan a permis une diminution de 2 à 3 log les comptes bactériens des bactéries lactiques et de *L. monocytogenes* sur des fillets de boeuf (Tsigarida et al., 2000) et la réduction de la majorité de la population microbienne des bactéries lactiques et de *S.* Typhimurium sur la viande (Skandamis et al., 2002).

2.2.2. Test sur les charcuteries

Les résultats montrent que les films d'alginate à base d'huiles essentielles d'origan d'Espagne, de cannelle de Chine et de sarriette des montagnes ont permis une diminution significative les comptes bactériens de *L. monocytogenes* et de *S.* Typhimurium sur la bologne et de *S.* Typhimurium seulement sur le jambon et ce indépendemment du traitement reçu (2% ou 20% de $CaCl_2$).

Les films à base d'huiles essentielles traités dans 20% de $CaCl_2$ ont été plus efficaces que ceux traités dans 2% de $CaCl_2$ contre *L. monocytogenes* et *S.* Typhimurium sur la bologne. Cette différence d'efficacité pourrait être expliquée par le fait que la disponibilité des huiles essentielles d'origan d'Espagne, de cannelle de Chine et de sarriette des montagnes dans les films dépend du

type de traitement de gélification reçu. En effet, après cinq jours d'entreposage, la disponibilité de ces huiles était de 12, 4.14 et de 9.59% respectivement dans les films traités dans 20% de $CaCl_2$ et de 18.84, 7.11 et de 17.08% respectivement dans les films traités dans 2% de $CaCl_2$.

Sur le jambon, l'efficacité des films à base d'huiles essentielles a été la même contre *S.* Typhimurium et ce indépendamment du traitement de gélification ionotropique reçu alors que *L. monocytogenes* n'a pas été très sensible à ces films. En effet, il n'y a pas eu une grande différence entre la disponibilité des huiles essentielles dans les deux types de film. Après cinq jours d'entreposage, la disponibilité des huiles essentielles d'origan d'Espagne, de cannelle de Chine et de sarriette des montagnes était de 21.65, 3.64 et de 14.10% respectivement dans les films traités dans 20% de $CaCl_2$ et de 27.65, 4.35 et de 11.09% respectivement dans les films traités dans 2% de $CaCl_2$. L'inefficacité retrouvée avec *L. monocytogenes* pourrait être dûe au fait que les huiles essentielles se soient accumulées dans la partie grasse du jambon. D'autres recherches ont expliqué l'inefficacité des épices dans du boeuf haché contre *E. coli, L. monocytogenes* et *S.* Typhimurium par le fait que ces épices se répartissent plus dans la partie lipidique de la viande (Cutter et al., 2000). Il a même été suggéré par Gill et al. (2002) que les microorganismes qui poussent dans un milieu aussi riche que celui de la viande, deviennent résistantes alors à différents stress.

Dans ces deux types de charcuterie, il existe des additifs chimiques tel que le chlorure de sodium, le sodium nitrite, l'érythrobate de sodium et le phosphate sodium qui ont pu interférer avec l'activité antimicrobienne des huiles essentielles testées. Le chlorure de sodium et le nitrite ont été très efficaces contre *S.* Typhimurium et *Shewanella putrifaciens* et ont amélioré l'effet antimicrobien de l'EDTA contre *E. coli, S.* Typhimurium et *Serratia grimesii* (Gill et Holley, 2003). Cependant, le nitrite et le chlorure de sodium n'ont pas inhibé la croissance de *L. monocytogenes* (Mbandi et Shelef, 2002). Notre étude révèle qu'il existe un effet synergétique entre les huiles essentielles testées et les additifs chimiques présents dans la bologne. Ceci a aussi été observé par Mbandi et Shelef (2002), cette étude a montré que le niveau de *Salmonella* sur de la bologne non traitée a diminué significativement après 30 jours de conservation et que cette diminution a été plus rapide lorsque le lactate et le diacétate ont été ajoutés.

3. Mécanisme d'action des huiles essentielles

Le mécanisme d'action des huiles essentielles d'origan d'Espagne, de cannelle de Chine et de sarriette des montagnes a été étudié sur une bactérie Gram-positif (*L. monocytogenes*) et une bactérie Gram-négatif (*E. coli* O157:H7) à des concentrations inférieures de deux fois, égales et supérieures de deux fois à la CMI.

On a observé chez les deux bactéries à l'étude que les huiles essentielles d'origan et de sarriette ont induit une diminution de l'ATP intracellulaire, une diminution supérieure à celle observée avec l'huile essentielle de cannelle. L'ATP extracellulaire n'a augmenté que lorsque ces bactéries ont été incubées avec l'origan et la sarriette. Cette diminution de l'ATP intracellulaire pourrait être expliquée par le fait qu'il y a eu libération de cet ATP à l'extérieur de la cellule causé par l'endommagement de l'enveloppe bactérienne par ces huiles et par conséquent, c'est ce qui fait augmenter l'ATP extracellulaire. Selon Gonzalez et al. (1996) la libération de l'ATP ainsi que son hydrolyse par l'ATPase induisent une dimunution de l'ATP intracellulaire. Ceci suggère donc qu'avec la cannelle, il y a eu juste une hydrolyse de l'ATP sans pour autant qu'il y est une libération à l'extérieur de la cellule. Dans une étude éffectuée par Helander et al. (1998), le carvacrol et le thymol, les principaux composés phénoliques de l'origan et de la sarriette ont permis de diminuer l'ATP intracellulaire et d'augmenter l'ATP extracellulaire d'*E. coli* O157:H7, un effet causé principalement par l'altération de l'intégrité de la membrane cytoplasmique.

La mesure du pH intracellulaire est une analyse qui nous informe sur le niveau de contrôle de divers processus dans une cellule tels que la transcription de l'ADN, la synthèse des protéines et l'activité enzymatique (Breeuwer et al., 1996). Lorsque ce paramètre varie dans une cellule bactérienne, il est clair que cette dernière réagit à l'ajout de l'agent antibactérien testé. L'incubation d'*E. coli* O157:H7 et de *L. monocytogenes* en présence de ces huiles essentielles a causé une diminution significative du pH_{in}. Cette diminution a plus été marquée avec la cannelle de Chine sur les deux bactéries et ce à la plus faible concentration testée (0.025% (v/v)). Une diminution du pH_{in} est directement liée à l'accumulation de certains composés dans la membrane cytoplasmique (Sikkema et al., 1995). Cependant, les bactéries testées ont eu des comportements différents en présence de ces huiles. À une concentration de 0.05% (v/v), la sarriette des

montagnes diminue le pH_{in} d'*E. coli* O157:H7 que l'origan d'Espagne et le contraire est observé à 0.025% (v/v). Lorsque *L. monocytogenes* a été incubée avec les huiles essentielles d'origan d'Espagne et de cannelle de Chine à une concentration de 0.05%, aucune différence significative n'a été observée entre les pH intracellulaire. Encore une fois, ces résultats montrent que le comportement de chaque huile essentielle est principalement dû à sa composition et à sa concentration en principes actifs. La diminution du pH intracellulaire est aussi l'effet de certains agents de conservation alimentaires tel que les acides propionique, benzoiques et cinnamique (Salmond et al., 1984). D'autres études ont aussi prouvé l'activité antimicrobienne du carvarol et de l'origan par une diminution du pH intracellulaire chez *B. cereus* et de *P. aeruginosa*, respectivement (Ultee et al., 1999; Lambert et al., 2001).

La mesure de l'absorbance des constituants intracellulaires est encore une autre étape qui nous a permis de comprendre le mécanisme d'action des huiles essentielles. Diverses études ont démontré un relargage important des constituants intracellulaires des bactéries après incubation avec certains composés antibactériens tel que le chlorhexidine, l'hexachlorophene, l'α-pinene et l'huile du thé (Hugo et Longworth., 1964; Joswick et al., 1971; Andrews et al., 1980; Carson et al., 2002). Ces études ont montré que la principale cible de ces agents est l'enveloppe cellulaire dont l'intégrité est alors très compromise. Koga et al. (1996) ont mis en évidence l'activité bactéricide du plaunotol sur *Helicobacter pylori* qui induisait une perméabilité accrue de la membrane selon les résultats de la mesure du relargage des constituants absorbants à 260 nm. Les résultats obtenus dans notre étude rejoignent aussi ces précédentes recherches. En effet, les huiles essentielles d'origan d'Espagne, de cannelle de Chine et de sarriette des montagnes ont induit un relargage des constituants intracellulaires qui absorbent à 260 nm. Cependant, l'origan d'Espagne a permis un relargage plus important chez *L. monocytogenes* que chez *E. coli* O157:H7, ce qui suggère que cette huile a affecté d'une façon plus importante la membrane plasmique de *L. monocytogenes* et par la même occasion sa perméabilité. De plus, la membrane externe des bactéries Gram-négatif (absente chez les Gram-positif) est un moyen de protection comme c'est le cas avec beaucoup d'agents antimicrobiens. Un important relargage a été obtenu avec la cannelle dès la plus petite concentration testée (0.025%) contre les deux bactéries. La sarriette des montagnes a induit un relargage proportionnel aux concentrations appliquées avec les *E. coli* O157:H7 et *L. monocytogenes*. Toutefois, un relargage légèrement plus important chez *L. monocytogenes* a été observé. Ces résultats montrent que les huiles composés essentiellement de

phénols (origan et sarriette) agissent différement sur les deux types d'enveloppes cellulaires (Gram-positif et Gram-négatif) alors que les aldéhydes tel que le trans-cinnamaldéhyde présent dans la cannelle semblent avoir le même mode d'action.

Afin de confirmer l'hypothèse émise sur la cible des huiles essentielles comme étant l'enveloppe cellulaire des bactéries, des analyses sur microscopie électronique ont été effectuées afin de comparer l'effet de ces différentes huiles sur ces deux types de bactéries. La comparaison entre les photos des bactéries traitées et non traitées par les huiles essentielles révèle très bien la différence qui existe entre elles. L'origan d'Espagne et la sarriette des montagnes provoquent des trous et des tâches distinctes sur la paroi d'*E. coli* O157:H7 et une importante déformation de la paroi bactétienne de *L. monocytogenes*. Cette différence de comportement a déjà été observée par microscopie à balayage lors des travaux de Rhayour et al. (2003), où l'origan et le clou de girofle ont causé des trous sur *E. coli* (Gram-négatif) et une déformation sur *B. subtilis* (Gram-positif). Quant à l'effet de la cannelle, les résultats obtenus montrent qu'il n'y a aucune différence apparente entre les deux types d'enveloppes cellulaires des bactéries testées. Il semblerait même que la cannelle ait un mécansime d'action différent que celui des deux autres huiles essentielles.

Tous ces résultats confirment que la différence qui existe entre le comportement de ces huiles essentielles serait dû aux différents mécanismes d'action de leur principes actifs et de leur quantité dans l'huile. Ceci est même observé avec des principes actifs de la même famille. En effet, il y a une différence de degré d'efficacité entre des composés phénoliques tel que l'eugénol, le carvacrol et le thymol, cette différence est liée à la position du groupement hydroxyle sur le cycle benzoique (Dorman et Deans, 2000). Selon Ultee et al. (1999), le carvacrol attaque la membrane, la rendant ainsi perméable aux ions potassium et aux protons, ce qui acidifie le cytosol bactérien et inhibe la synthèse de l'ATP. Même que le carvacrol et le thymol causent un changement de la composition de la bicouche phospholipidique (Burt et Reinders, 2003).

Pour les composés non phénoliques comme le cinnamaldéhyde, le principe actif de la cannelle, il s'agirait plus d'une inhibition de l'activité des acides aminés décarboxylases (Didry et al., 1993; Wendakoon et Sakaguchi, 1995). Les aldéhydes sont des composés électronégatifs qui agissent par interférance avec le transfert d'électrons et par substitution avec les acides nucleiques et les protéines de la cellule bactérienne (Dorman et Deans, 2000).

Conclusion

Les résultats obtenus dans cette recherche montrent qu'un bon nombre d'huiles essentielles a été efficace contre les bactéries testées. Cependant, une différence d'efficacité existe entre ces huiles, du fait de leur différence de composition. Les huiles essentielles composées essentiellement de composés phénoliques et d'aldéhyde sont ceux qui ont été les plus efficaces à de très faibles CMI. Il a été aussi démontré que deux bactéries du même type se sont démarquées par le fait que l'une ait été la plus sensible tel que *S. aureus* et que l'autre ait été la plus résistante tel que *L. monocytogenes* à toutes ces huiles testées.

L'objectif de trouver la formulation d'un film qui pouvait immobiliser des huiles essentielles antimicrobiennes et qui leur permettrait une diffusion contrôlée au cours de l'entreposage a été amplement satisfait au cours de cette étude. Les résultats obtenus démontrent que l'immobilisation de l'huile essentielle d'origan dans un film à base de caséinate de calcium a été plus efficace que l'immobilisation de l'huile du piment, permettant ainsi une réduction significative des comptes de *Pseudomonas* sp. et d'*E. coli* O157:H7 inoculés sur les tranches de boeuf, tout au long de l'entreposage. Même que la migration des composés phénoliques des films à base d'origan a été plus importante que celle survenue dans les films à base de piment.

Cette étude a aussi démontré qu'il était possible de conserver des tranches de boeuf, de la bologne et du jambon à une température de 4°C en contrôlant les bactéries pathogènes en présence des huiles essentielles. En effet, les films à base d'alginate et d'origan d'Espagne immobilisés dans les films traités dans du $CaCl_2$ (2%) ont permis une diminution significative du niveau d'*E. coli* O157:H7 inoculé sur des tranches de boeuf, ce qui n'a pas été observé lorsque ces mêmes films ont été insolubilisés dans 20% de $CaCl_2$. Il a aussi été démontré que les films à base d'alginate contenant des huiles essentielles de cannelle de Chine et d'origan d'Espagne, indépendement du traitement de gélification ionotropique reçu, ont été très efficaces contre *S.* Typhimurium inoculé sur des tranches de boeuf. De plus d'importants taux de libération des huiles essentielles d'origan d'Espagne, de cannelle de Chine et de sarriette des montagnes ont été enregistrés au cours de l'entreposage. Même qu'un taux de libération plus important est survenu lorsque les films ont été traité dans 20% de $CaCl_2$ que ceux qui ont été traités dans 2% de $CaCl_2$.

Les études effectuées sur la bologne montrent que le niveau de *S.* Typhimurium a été significativement réduit par la présence de films à base d'alginate et d'origan d'Espagne immobilisés dans du $CaCl_2$ (2%) et de cannelle insolubilisé dans du $CaCl_2$ (20%). De plus, il a aussi été possible de réduire le niveau de *L. monocytogenes* avec les films d'alginate à base d'origan d'Espagne et de cannelle de Chine indépendamment du traitement de gélification reçu (2 ou 20% de $CaCl_2$). Toutefois, il a été observé que le niveau de *L. monocytogenes* sur le jambon n'a pas été diminué en présence de ces films bioactifs, à l'exception du film qui contenait de la cannelle immobilisé dans 20% de $CaCl_2$ qui a permis une réduction de 0.66 log après 5 jours d'entreposage. Sur ces deux types de charcuterie, le taux de libération des huiles essentielles a été plus important lorsque les films ont été traités dans 20% de $CaCl_2$. De plus, le taux de libération de l'huile essentielle de cannelle de Chine a été plus important que celui des huiles essentielles d'origan d'Espagne et de sarriette des montagnes avec les deux types de films.

Le dernier objectif de cette étude était de faire quelques analyses sur le mécanisme d'action de ces huiles essentielles sur deux types différents de bactéries (*E. coli* O157:H7 et *L. monocytogenes*). Il a été démontré que l'ATP intracellulaire a diminué lorsque les deux bactéries ont été incubées avec les huiles essentielles testées (origan d'Espagne, cannelle de Chine et sarriette des montagnes) alors que l 'ATP extracellulaire n'a augmenté qu'avec la sarriette des montagnes sur les deux bactéries et avec l'origan sur *E. coli* O157:H7. Il a aussi été prouvé qu'un relargage des constituants cellulaires de ces deux bactéries survient lorsque ces dernières sont incubées avec ces huiles. Néanmoins, un relargage plus important a eu lieu lorsque la cannelle de Chine a été ajoutée. Par la suite, il a été démontré que le pH intracellulaire de ces bactéries diminuait significativement après ajout de ces huiles et qu'une fois encore, la cannelle est celle qui a permis la diminution la plus élevée par rapport aus deux autres huiles essentielles. Et en finalité, l'analyse en microscopie électronique des cellules bactériennes incubées avec ces huiles a été effectuée. De cette observation, il en ressort clairement que les huiles affectent l'intégrité de l'enveloppe cellulaire des bactéries. En effet, les huiles essentielles d'origan d'Espagne et de sarriette des montagnes provoquaient des trous au niveau de l'enveloppe cellulaire d'*E. coli* O157:H7 et des déformations sur l'enveloppe cellulaire de *L. monocytogenes* alors que l'effet de la cannelle a été similaire sur les deux bactéries.

Tous ces résultats nous révèlent qu'effectivement les huiles essentielles possèdent un potentiel antimicrobien très important qu'il faut exploiter afin de contrôler la croissance de bactéries dites pathogènes. Le choix des huiles essentielles à large spectre antimicrobien serait en effet la suite logique à faire, suivie d'une meilleure compréhension du mécanisme d'action de ces huiles afin de bien cibler leur utilisation dans divers domaines tel que le bio-emballage.

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