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DÉVELOPPEMENT DES SYSTÈMES D'ENCAPSULATION DES COMPOSÉS NATURELS BIOACTIFS POUR APPLICATION ALIMENTAIRE

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RÉSUMÉ

L'industrie de transformation des aliments est très florissante. La recherche de nouveaux produits est en constante évolution, parfois dans le but d'augmenter la diversité de la gamme offerte, de satisfaire un besoin des consommateurs, de réduire la quantité des ingrédients utilisés ou simplement de minimiser le coût des aliments. Dû au changement du rythme de vie dans la société Nord-Américaine et Européenne, plusieurs nouveaux produits tels que les aliments prêts-à-manger et prêts-à-cuire sont de plus en plus présents sur les rayons de l'épicerie. Cependant, ces produits ont une durée de conservation très courte et peuvent facilement être contaminés, principalement à cause des différentes étapes de transformation. De plus, avec la mondialisation de l'industrie alimentaire, les distances parcourues par ces produits sont augmentées, ce qui augmente les risques de contamination et de dégradation. De ce fait, les antimicrobiens d'origine naturelle peuvent être une solution. Ils sont efficaces et ont une bonne réputation chez les consommateurs. En revanche, plusieurs défis gouvernent leur utilisation; d'une part, leur arôme fort et d'autre part, leur sensibilité et instabilité au cours du temps. Dans ce contexte, le premier but de ce projet est de développer des formulations antimicrobiennes à base d'un mélange de différents antimicrobiens naturels. De cette façon, nous pouvons créer une synergie entre les antimicrobiens et nous pouvons également réduire la dose nécessaire de chacun d'entre eux. Le deuxième but de ce projet est de développer des formes d'encapsulation permettant une utilisation adéquate des antimicrobiens naturels afin d'améliorer leur stabilité pour une application alimentaire (carottes précoupées et pain).

Afin d'atteindre ces buts, nous avons d'abord évalué l'activité antibactérienne et antifongique de 17 antimicrobiens naturels, y compris 15 huiles essentielles (HE) et dérivés et 2 extraits de végétaux contre *Listeria monocytogenes*, *Staphylococcus aureus*, *Enterococcus faecium*, *Bacillus subtilis*, *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Aspergillus flavus*, *Penicillium chrysogenum* et *Candida albicans*. À partir des résultats obtenus, nous avons pu concevoir des formulations à base d'HE d'origan, de cannelle, de deux espèces de citronnelle (*Cymbopogon winterianus Cymbopogon flexuosus* nommées lemongrass et citronnelle) et d'un mélange commercial d'extraits de *citrus aurantium amara* et *citrus reticulata* (Biosecur F440D) pour les tests d'encapsulation. Nous avons ensuite développé différentes matrices d'encapsulation des formulations antimicrobiennes développées précédemment incluant nanoémulsion, nanoliposome et émulsion gélifiée et nous avons également caractérisé les capsules obtenues.

Les résultats ont montré une amélioration de l'activité antibactérienne *in vitro* lorsque la formulation antimicrobienne est encapsulée dans une nanoémulsion ou dans une émulsion gélifiée. Lorsqu'appliquée sur la matrice alimentaire, l'émulsion gélifiée et le nanoliposome semblent être les plus efficaces à contrôler la qualité microbienne du pain. Toutefois, l'utilisation de la pectine comme matrice d'encapsulation était particulièrement efficace à améliorer l'activité antimicrobienne de l'émulsion contre *L. monocytogenes* et *P. chrysogenum* sur les carottes précoupées et contre *A. flavus* et *B. subtilis* sur le pain. La nanoémulsion est également efficace ; cependant, l'activité antimicrobienne est rapidement perdue due principalement à une libération rapide et à une dégradation des composés bioactifs. De plus, nous avons démontré que le mécanisme d'encapsulation varie selon le caractère hydrophile ou hydrophobe des composés encapsulés. D'un côté, l'encapsulation d'une émulsion à base de Biosecur F440D (hydrophile) dans une matrice de pectine a été conduite principalement par des liaisons hydrogène et par une élongation –CH. D'un autre côté, l'encapsulation d'une émulsion à base d'HE (lipophile) dans la matrice de pectine a été conduite par une forte élongation asymétrique $–CH_3$ dans les groupes alkyles. En outre, les nanoliposomes avaient plus d'affinité pour encapsuler les composés hydrophiles que ceux lipophiles. L'incorporation de ces deux composés dans les nanoliposome a été réalisée notamment par une déformation antisymétrique éthylique, une déformation symétrique méthyle, une élongation P-O-C et une élongation symétrique PO_2.

L'application de tels systèmes d'encapsulation sur les matrices alimentaires était efficace à augmenter la durée de conservation des aliments testés et à contrôler les microorganismes d'altération sans affecter négativement les propriétés physicochimiques des aliments.

En résumé, cette thèse a été entreprise pour développer des systèmes d'encapsulation comestibles pour une application dans le domaine alimentaire. Compte tenu de l'amélioration de la stabilité des antimicrobiens naturels, de leur activité biologique et de leur impact sur l'augmentation de la durée de conservation et de la salubrité du pain et des carottes précoupées, il devrait être intéressant de considérer ces méthodes d'encapsulation comme techniques permettant la commercialisation de ces antimicrobiens au niveau industriel. Ceci devrait être d'un grand intérêt pour remplacer les antimicrobiens synthétiques hautement controversés.

Mots-clés :

Microencapsulation, nanotechnologie, nanoémulsion, nanoliposome, biopolymère, pathogènes alimentaires, qualité des aliments, Légumes prêts à manger, produit de boulangerie, contamination, durée de conservation.

ABSTRACT

The food processing industry is booming. The search for new products is constantly evolving, sometimes to increase the diversity of the offered range, to satisfy a consumer need, to reduce the quantity of ingredients used or simply to minimize the cost of food. Due to the change in the lifestyle in North American and European society, several new products such as ready-to-eat and ready-to-cook dishes are put on the shelves of the grocery stores. However, these products have a very short shelf-life and can easily be contaminated mainly due to the different processing steps. On the other hand, with the globalization of the food industry, the distances traveled by these products are increased, which increases the risks of contamination and degradation. Natural antimicrobials may be a solution. They are effective and well perceived by consumers. However, several challenges govern their use; especially their strong aroma and their sensitivity and instability. Therefore, the first goal of this work is to develop antimicrobial formulations based on a mixture of different natural antimicrobials. In this way, we can create a synergy between the antimicrobials and we can also reduce the necessary dose of each. The second goal of this work is to develop encapsulation forms allowing an adequate use of natural antimicrobials to improve their stability for food application (pre-cut carrots and bread).

The antibacterial and antifungal activities of 17 natural antimicrobials including 15 essential oils (EO) and derivatives and 2 plant extracts were evaluated against *Listeria monocytogenes*, *Staphylococcus aureus*, *Enterococcus faecium*, *Bacillus subtilis*, *Escherichia coli* O157: H7, *Salmonella* Typhimurium, *Aspergillus flavus*, *Penicillium chrysogenum* and *Candida albicans*. From the obtained results, formulations based on EO of oregano, cinnamon, two lemongrass species (*Cymbopogon winterianus Cymbopogon flexuosus* and named citronella and lemongrass respectively) and commercial mixture of *citrus aurantium amara* and *citrus reticulata* (Biosecur F440D) were developed for the encapsulation tests. The encapsulation of the antimicrobial formulations previously developed in different matrices including nanoemulsion, nanoliposome and gelled emulsion has been developed and the obtained capsules have also been characterized. The results showed an improvement in antibacterial activity *in vitro,* when the antimicrobial formulation was encapsulated in a nanoemulsion or in a gelled emulsion. When applied to the food matrix, the gelled emulsion and the nanoliposome seem to be the most effective for controlling microbial quality on bread. The use of pectin as an encapsulation matrix was particularly efficient to improve the antimicrobial activity of the emulsion against *L.*

monocytogenes and *P. chrysogenum* on pre-cut carrots and against *A. flavus* and *B. subtilis* on bread. The nanoemulsion was also effective; however the antimicrobial activity was rapidly lost mainly due to rapid release and degradation of the bioactive compounds. Indeed, we demonstrated that the mechanism of encapsulation varies according to the hydrophilic or hydrophobic nature of the encapsulated compounds. On the one hand, the encapsulation of an emulsion based on Biosecur F440D (hydrophilic) in a pectin matrix was carried out mainly by hydrogen bonds and -CH stretching. On the other hand, when the emulsion is based on essential oils (lipophilic), encapsulation was carried out by a strong asymmetric stretching - CH_3 in the alkyl groups. Moreover, nanoliposomes had more affinity to encapsulate hydrophilic compounds than lipophilic ones. The incorporation of both compounds in nanoliposome was carried out in particular by ethyl antisymmetric deformation, methyl symmetrical deformation, P-O-C stretch and PO_2 symmetrical stretch.

The application of such encapsulation systems to food matrices was effective in increasing the shelf-life of food and controlling spoilage microorganisms without adversely affecting the physicochemical properties of the commodity.

In summary, this thesis was undertaken to develop edible encapsulation systems for food applications. Given the valuable improvement in the stability of natural antimicrobials, their biological activity and their impact on the application of shelf-life and the safety of bread and pre-cut carrots, it should be interesting to consider these encapsulation methods as techniques allowing the commercialization of these antimicrobials at the industrial level. This should be of great interest to replace the highly controversial synthetic antimicrobials.

Keywords:

Microencapsulation, nanotechnology, nanoemulsion, nanoliposome, biopolymer, food pathogens, food quality, Ready-to-eat vegetables, bakery product, contamination, shelf-life.

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A

A. flavus : Aspergillus flavus
a_w : water activity

B

B. subtilis : Bacillus subtilis
BCG : Boston Consulting Group

C

C. albicans : Candida albicans
C. jenuni: *Campylobacter jnuni*
CDC : Centres pour le contrôle et la prévention des maladies
CMI: Concentration minimale inhibitrice
CRIBIQ : Consortium de Recherche et Innovations en Bioprocédés Industriels au Québec
CRSNG: Natural Sciences and Engineering Research Council of Canada
^{60}CO : Cobalt 60

D

DJA: Dose Journalière Admissible

E

E. coli: *Escherichia coli*
EE: Encapsulation efficiency
EFSA : European Food Safety Authority
EO: Essential oil

F

FAO: Food and Agriculture Organization of the United Nations
FDA: Food and Drug Administration
FIC: Fractional inhibitory coefficient
FTIR: Fourier Transform InfraRed spectroscopy

G

g: Gramme
GAE: Gallic acid equivalent

H

HACCP: Hazard Analysis and Critical Control Point
HE: Huile essentielle
HPP: High Pressure Processing

K

kg: Kilogramme
kGy: kilogray
kGy h^{-1}: Kilogray par heure

L

L. monocytogenes: *Listeria monocytogenes*
LAB: Lactic acid bacteria
LBL : Layer by layer

M

MAP : Emballage sous atmosphère modifiée
MAPAQ: Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec
MBC: Minimal bactericidal concentration
MFC: Minimal fungicidal concentration
MIC: Minimal inhibitory concentration
Min: minutes
mV: millivolt
nm: nanometer

P

P. chrysogenum: *Penicillium chrysogenum*
PDA: Potato Dextrose Agar
PDI: polydipersity index
pH: Potentiel hydrogène
POD : Peroxidase
p/v: poids sur volume

R

RPM : Revolutions per minute
RTE : Ready to eat
RTC : Ready to cook

S

S. aureus: Staphylococcus aureus
S. Typhimurium : *Salmonella enterica* serovar Typhimurium

T

TS: Tensile strength
TM : Tensile Modulus
TFC: Total flavonoids content
TMF : Total mesophilic flora
TP : Total phenol
TPC : Total phenol content
TSA : Tryptic Soy Agar

U

UFC : Unité Formatrice de Colonies
USDA: United States Department of Agriculture

V

v/v: Volume/Volume

W

WVP: Water Vapour Permeability
WHO : Wolrd health organization

Z

ζ-potential: Zeta potential

CHAPITRE 1: INTRODUCTION

La conservation des aliments a toujours été une préoccupation de l'être humain. Les moyens de conservation développés et utilisés sont divers et se basent principalement sur le traitement thermique (le froid et la chaleur), la diminution de l'activité de l'eau (a_w) (séchage et la salaison) et l'utilisation de procédés tels que la fermentation et l'ajout d'épices. Plusieurs variétés de plantes ont été utilisées par les anciennes civilisations comme épices, agents de conservation, arômes et colorants. Cependant, avec le début de l'industrialisation de l'industrie agroalimentaire au 19ème siècle, on a assisté à une remontée rapide des additifs synthétiques, réputés pour leur faible coût et leur efficacité. Beaucoup de découvertes concernant les additifs synthétiques ont ainsi été réalisées. La confiance que cette branche a prise durant ces deux siècles a fait reculer dans l'oubli les alternatives naturelles que nos ancêtres utilisaient dans le passé, surtout que les produits naturels dans la plupart des cas étaient instables en les comparants à ceux synthétiques. On trouve les produits synthétiques dans la vie de tous les jours comme les cosmétiques et les détergents mais aussi dans des formes pharmaceutiques et même dans des aliments.

Actuellement, au 21ème siècle, les impacts négatifs des produits synthétiques ne cessent de faire surface incluant des risques à moyen et à long terme. Ceci a mis en question la sécurité de l'utilisation de ce genre de produit. On assiste, de plus en plus, au retour des consommateurs vers les produits d'origine naturelle. Ces produits ont déjà prouvé leur efficacité, mais aussi les doses nécessaires sont généralement sures. Dans le domaine de la santé, on trouve une branche dans la médecine moderne appelée la phytothérapie et en industrie alimentaire, nous assistons à une vraie tendance de changement vers les additifs naturels comme les antimicrobiens d'origine naturelle. Ces antimicrobiens ont l'avantage d'avoir une efficacité similaire et parfois meilleure par rapport aux antimicrobiens synthétiques mais aussi avec beaucoup moins d'effets toxiques et allergènes. En plus de leurs propriétés antimicrobiennes, ils possèdent souvent des propriétés nutraceutiques et

fonctionnelles, utilisés déjà comme anticancéreux, radioprotecteurs et hypoglycémique. Dans ce projet de thèse, nous avons exploité des antimicrobiens d'origine végétale dans le but de remplacer les agents de conservation synthétiques dans le domaine alimentaire. Afin d'améliorer et de protéger leurs activités antimicrobiennes, ces antimicrobiens ont été encapsulés dans différentes matrices. L'évaluation de ces méthodes d'encapsulation a été réalisée *in vitro* et *in situ* sur une matrice alimentaire (le pain et les carottes précoupées).

Le mémoire de thèse est présenté dans un format de manuscrit conforme aux directives de préparation des thèses à l'INRS et est organisé comme suit. Les chapitres 2, 3 et 4 sont consacrés à la revue de littérature détaillant les différents concepts utilisés dans la présente thèse. Le chapitre 2 introduit les enjeux reliés à la qualité des aliments, les notions et les causes de la détérioration de la qualité des aliments. Nous nous sommes intéressés plus particulièrement à la famille des aliments: les légumes et les fruits transformés et les produits de boulangerie sur lesquels s'appuient nos travaux. Le chapitre 3 est consacré à la revue de littérature décrivant les différents antimicrobiens utilisés pour la préservation des aliments, leur mode d'action et les inconvénients de leur utilisation. Le chapitre 4 est consacré à l'introduction du concept de microencapsulation et à définir les différentes formes récurrentes dans le domaine alimentaire. Le chapitre 5 présente la première publication qui est la première étape du projet et qui consiste à la sélection de la formulation antimicrobienne efficace. Les chapitres suivants exposeront en détail les différentes formes d'encapsulation de cette étude. Le chapitre 6 consiste en notre seconde publication et qui détaillera notre première approche d'encapsulation de la formulation antimicrobienne dans une matrice polymérique. Ensuite, on propose, au chapitre 7, la deuxième technique d'encapsulation développée et qui consiste en nanoliposome. Le chapitre 8 détaille l'encapsulation sous la forme de nanoémulsion. Le chapitre 9 présente la dernière publication qui consiste à une comparaison des trois approches développées dans les chapitres précédents. Finalement, le chapitre 10 présente la conclusion, la discussion générale et les perspectives du projet.

CHAPITRE 2 : QUALITÉ DES ALIMENTS

La nourriture est une partie essentielle de notre vie. Elle contient les nutriments nécessaires pour le bon fonctionnement du corps humain tels que l'eau, les protéines, les glucides, les vitamines, les lipides et les minéraux. De récentes études ont démontré qu'une mauvaise qualité d'alimentation peut engendrer des problèmes de santé physique et mentale (Morris *et al.*, 2019, Mussttaf *et al.*, 2019). C'est pourquoi, la façon dont la nourriture est cultivée, transformée et transportée mérite d'être bien comprise et améliorée. Il existe plusieurs produits alimentaires (produits de charcuterie, les légumes et les fruits, les céréales, etc.). Ces produits vont être le plus souvent transformés afin de faciliter leur commercialisation et améliorer leur acceptabilité par le consommateur.

Avec l'augmentation de la population mondiale et les prédictions d'évolution au cours du 21ème siècle, le monde a besoin de plus en plus d'aliments. Ceci implique une augmentation de la production, mais aussi une meilleure gestion des flux alimentaires et une amélioration de la durée de vie des aliments (Hashemi *et al.*, 2017). A cause de leur teneur élevée en nutriments, la qualité des aliments se détériore assez rapidement. Cette détérioration est souvent plus rapide lorsque les aliments sont transformés. Ceci engendre des pertes économiques énormes. Selon l'Organisation des Nations Unies pour l'Alimentation et l'Agriculture, environ un tiers des aliments produits dans le monde pour la consommation humaine chaque année – l'équivalent de 1.3 milliard de tonnes - sont perdus ou gaspillés (FAO, 2019). Les pertes et le gaspillage alimentaires s'élèvent à environ 680 milliards de dollars dans les pays industrialisés et à 310 milliards de dollars dans les pays en développement. Les pays industrialisés et les pays en développement dissipent, à peu près, les mêmes quantités de denrées alimentaires - respectivement 670 et 630 millions de tonnes. Les fruits et légumes et les tubercules ont les taux de perte les plus élevés parmi tous les aliments. Le Boston Consulting Group (BCG) estime que d'ici 2030, la perte et le gaspillage alimentaires annuels atteindront 2.1 milliards de tonnes d'une valeur de 1.5 billion de dollars (BCG, 2018). Ce chapitre présentera les différents types d'altérations des aliments notamment les légumes, les fruits et les produits de boulangerie qui seront étudiés dans cette thèse ainsi que les méthodes souvent utilisées pour maintenir leur qualité.

2.1. LÉGUMES ET FRUITS

Les fruits et les légumes frais sont des éléments importants d'une alimentation saine et équilibrée; leur consommation est encouragée dans de nombreux pays pour se protéger contre plusieurs maladies telles que les cancers et les maladies cardio-vasculaires. Beaucoup de recherches sur les agents pathogènes humains d'origine alimentaire ont mis l'accent sur la transmission des aliments d'origine animale. Cependant, des études récentes ont identifié des fruits et des légumes qui sont la source de nombreuses épidémies (Jackson *et al.*, 2013, Stephan *et al.*, 2015).

En effet, les fruits et les légumes sont, de plus en plus, reconnus comme des vecteurs importants de transmission des agents pathogènes humains qui étaient traditionnellement associés aux aliments d'origine animale et le nombre de rappels ne cesse d'augmenter (**Figure 2.1**). Malgré l'impact important de ce problème sur la santé humaine, les connaissances sont toujours limitées pour les méthodes permettant de préserver les fruits et les légumes contre ces contaminants tout en gardant leur fraîcheur.

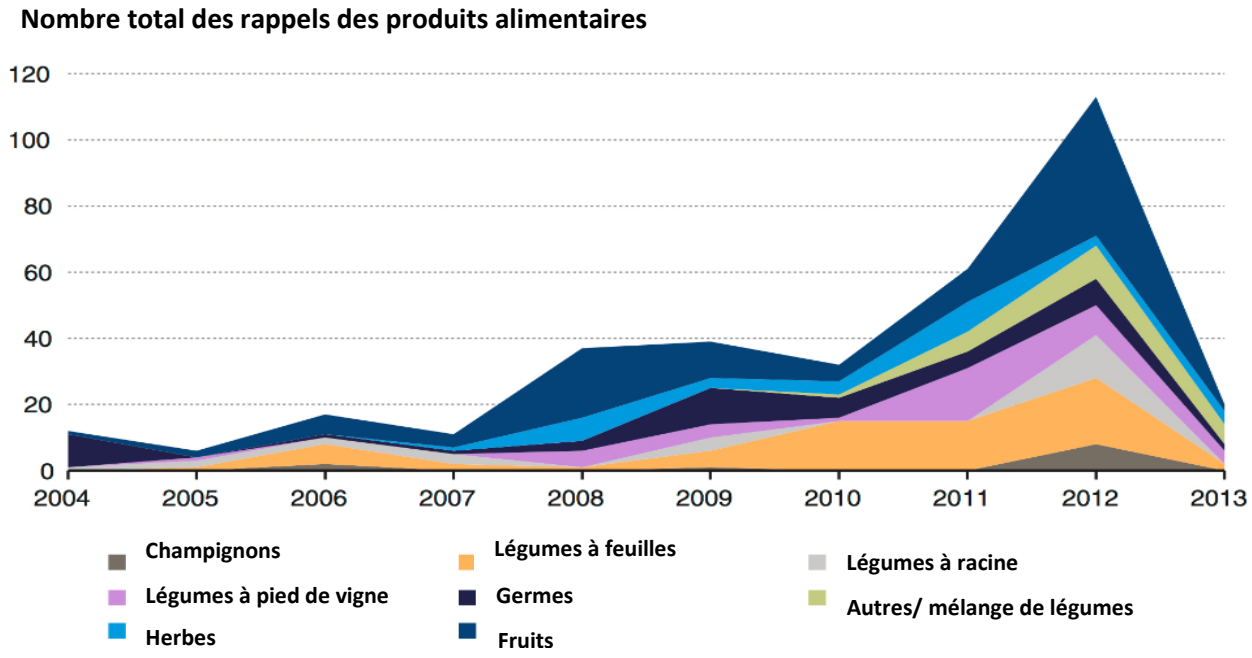

Figure 2.1. Evolution du nombre des rappels aux USA (Source: ERS)

2.1.1. Flore microbienne des fruits et légumes

Les fruits et légumes abritaient plusieurs espèces bactériennes qui varient selon le produit. Cependant, Leff *et al.* (2013) ont démontré que certains types de produits tels que les germes, les épinards, la laitue, les tomates, les poivrons et les fraises avaient tendance à partager des communautés bactériennes plus similaires appartenant à la famille des Enterobacteriaceae par rapport aux autres types de produits comme les pommes, les pêches, les raisins et les champignons qui sont plutôt dominés par les Actinobacteria, Bacteroidetes, Firmicutes et Proteobacteria phyla. Cette flore bactérienne est égalemnt affectée par des facteurs tel que les pratiques agricoles entre autres l'agriculture convetionnelle et biologique. Ces différences étaient souvent attribuables à une abondance des Enterobacteriaceae dans l'agriculture conventionnelle par rapport à celle biologique (Leff *et al.*, 2013).

Les fruits et les légumes peuvent également être contaminés par plusieurs microorganismes pathogènes. Les plus répandus sont généralement *E.coli*, *Listeria* et *Salmonella* (Moreno *et al.*, 2012, Sant'Ana *et al.*, 2012). Toutefois, d'autres pathogènes peuvent aussi être isolés des légumes comme *Clostridium difficile* (Metcalf *et al.*, 2010).

Avec l'émergence des fruits et légumes prêts-à-manger sur le marché Canadien, le risque de contamination bactérienne augmente vue les procédés industriels à savoir la coupe, l'épluchage et le broyage qui peuvent enlever ou endommager la surface de protection de la plante ou des fruits et pourraient permettre la contamination croisée et la croissance microbienne. L'étape de lavage des fruits et des légumes permet dans certains cas de propager la contamination aux autres surfaces du végétal. Il est à noter aussi, que le manque général d'efficacité des désinfectants à enlever ou éliminer les agents pathogènes sur les fruits et les légumes crus est attribué, en partie, à leur inaccessibilité aux surfaces abritant les pathogènes.

Par exemple, *Listeria* est une bactérie Gram positif qui peut se développer sur les produits de charcuterie mais aussi sur les légumes. Elle a été identifiée dans les choux, les germes de soja, céleri, laitue, tomate, etc. *Listeria* a été impliquée dans plusieurs éclosions de maladies d'origine alimentaire. La listériose engendre un taux de mortalité d'environ 24% principalement chez les femmes enceintes, leurs fœtus et les personnes immunodéprimées, avec des symptômes d'avortement, de décès néonatal, de septicémie et de méningite (Farber *et al.*, 1991). Selon le MAPAQ (2009a), pour les légumes prêts à l'emploi, aucune colonie n'est tolérable dans 25 g de produit (Tableau 2.1).

E. coli est une bactérie Gram négatif qui a été détectée dans les choux, céleri, coriandre avec une fréquence respective de 25%, 17.6% et 19.5% (Beuchat, 2002). Au Canada, de 2001 à 2009, trois épidémies dues à *E. coli* O157 ont été associées à la laitue contaminée (Kozak et al., 2013). Une contamination à *E. coli* O157:H7 peut engendrer des douleurs abdominales, de la fièvre, une fatigue générale, des selles sanglantes et des diarrhées et peut causer des hospitalisations, des décès et l'apparition du syndrome hémolytique et urémique (Swerdlow *et al.*, 1992, Watanabe *et al.*, 1999). Selon le MAPAQ (2009a), la limite d'acceptation pour le compte d'*E. coli* est de 10 UFC g^{-1} (Tableau 2.1).

Salmonella est une bactérie Gram négatif qui peut aussi contaminer les fruits et les légumes avec une incidence de 18 à 20 % dans les aliments pourris et 9 à 10 % dans les aliments sains. Les fruits et les légumes peuvent être contaminés dans les champs par l'utilisation d'eau d'irrigation contaminée et l'utilisation de fumier animal à des fins de fertilisation mais également pendant la récolte et au stade post-récolte en raison de la mauvaise hygiène des travailleurs et du manque d'hygiène dans l'usine de transformation (Golberg *et al.*, 2011). Les centres pour le contrôle et la prévention des maladies (CDC) estiment que la bactérie *Salmonella* cause chaque année environ 1.35 million d'infections aux États-Unis causées principalement par la nourriture. Cette contamination cause la diarrhée, la fièvre et des crampes d'estomac. La plupart des gens se rétablissent sans traitement spécifique. Cependant, *Salmonella* peut causer dans certaines situations des cas d'hospitalisations (26 500) et des décès (420) aux États-Unis (CDC, 2020). *Salmonella* dans les légumes prêts à l'emploi doit être absente dans 25 g (Tableau 2.1).

Tableau 2.1. Normes d'acceptabilité des légumes (MAPAQ, 2009a)

	Critères UFC g^{-1}	
	m	**M**
Bactéries aérobies mésophiles	1×10^7	1×10^8
E. coli	10	1×10^2
S. aureus coagulase positive	1×10^2	1×10^4
Salmonella	Non détecté/25 g	-
L. monocytogenes	Non détecté/25 g	-
Levures ou moisissures	1×10^4	1×10^5

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

D'autre part, les fruits et les légumes sont aussi très souvent contaminés par des levures et moisissures. Les levures sont des organismes les plus fréquemment trouvés, avec des concentrations allant de moins de 100 à 4.0×10^8 UFC g^{-1} (Tournas, 2005a). Il est important de contrôler les moisissures dans les aliments car elles peuvent se développer même dans des conditions où de nombreuses bactéries ne peuvent pas se développer, comme un pH acide, une faible activité de l'eau (a_w) et une forte pression osmotique (Ray *et al.*, 2013). De nombreuses souches produisent également des mycotoxines et ont été impliquées dans l'intoxication d'origine alimentaire (Ray *et al.*, 2013). Une identification des souches montre que les moisissures les plus communes trouvées dans les légumes fraîs et peu transformés sont *Cladosporium*, *Alternaria* et *Penicillium*, et moins fréquemment *Geotrichum* (Tournas, 2005a). Selon le MAPAQ (2009a), la norme attribuée aux légumes prêts à l'emploi est de 1.0×10^4 pour les levures et les moisissures (Tableau 2.1).

Afin de mieux contrôler la contamination des légumes et des fruits, plusieurs techniques sont utilisées. Le lavage est la première étape de base utilisée dans toutes les industries agroalimentaires. Cette étape pourra être combinée à l'usage d'un désinfectant comme l'ajout d'un antioxydant, du calcium ou de cations divalents (Yosra Ben-Fadhel *et al.*, 2018, Rahman, 2007). L'enrobage comestible incorporant des antimicrobiens permet également de contrôler certains microorganismes et de prolonger la durée de conservation des légumes et des fruits (Yosra Ben-Fadhel *et al.*, 2017). Le traitement thermique permet aussi de contrôler certaines souches de *Penicillium*, *Alternaria*, *Rhizopus,* etc. (Rahman, 2007). L'irradiation est une méthode nouvellement utilisée pour traiter les produits alimentaires. Cette technique est capable d'inhiber la sporulation à des doses ≤ 0.15 kGy, à éliminer les insectes à des doses ≤ 0.5 kGy, à retarder la maturité et la scenescence à des doses ≤ 1 kGy et à augmenter la durée de conservation à des doses ≤ 3 kGy (Annexe VI) (Rahman, 2007).

Récemment, la méthode du plasma froid a également prouvé son efficacité pour la décontamination des aliments et l'augmentation de leur durée de conservation. Dépendamment de l'aliment évalué et des conditions de transformation, la méthode est capable de réduire jusqu'à > 5 log de *Salmonella*, *Escherichia coli* O157: H7, *Listeria*

monocytogenes et *Staphylococcus aureus* avec un temps de traitement qui varie de 120 s à 3 s. Cependant la technologie est très récente et une étude plus approfondie est nécessaire avant une éventuelle commercialisation (Pankaj *et al.*, 2018).

2.1.2. Détérioration physicochimique

Les fruits et légumes peuvent voir leur qualité se détériorer à cause du brunissement enzymatique qui survient principalement lors du procédé du découpage ou de l'épluchage. Ce facteur présente la cause la plus importante de la perte de qualité des salades prêtes à l'emploi (Jeantet *et al.*, 2007). Ce phénomène implique des enzymes comme les polyphénols oxydases et les peroxydases. Il peut être observé dans des produits tels que les fruits (abricots, poires, bananes, raisins) et les légumes (pommes de terre, champignons, laitue).

Il est possible de réduire l'impact du brunissement enzymatique en utilisant la technique de découpe sous jet d'eau ou en utilisant des inhibiteurs chimiques tels que l'acide ascorbique, l'acide citrique, ou les composés soufrés (Jeantet *et al.*, 2007). L'emballage sous atmosphère modifiée peut également réduire les réactions d'oxydation enzymatique. Par exemple, la diminution de la teneur en oxygène (1 à 5 %) et l'augmentation de la teneur en dioxyde de carbone (10 à 15 %) a permis de réduire significativement le brunissement de la laitue (Jeantet *et al.*, 2007). D'autre part, l'application de brefs chocs thermiques > 45 °C a également permis de réduire le brunissement de la laitue iceberg et ceci en inhibant les enzymes responsables.

2.2. PRODUITS DE BOULANGERIE

La détérioration des produits de boulangerie peut être reliée à la détérioration physique (perte d'humidité), la détérioration chimique (rancissement), et l'altération microbiologique (levures, moisissures et bactéries).

2.2.1. Détérioration physique

Parmi les facteurs influençant la détérioration physique, on trouve :

- L'absorption ou la perte de l'humidité : ce qui induit le changement de la texture et le développement de certains microorganismes. Ce genre de détérioration est souvent remédié par l'emballage sous atmosphère modifiée (MAP) (comme le polyéthylène à faible densité) (Kilcast *et al.*, 2011).
- Les changements pendant ou après la cuisson : redistribution de l'humidité, rétrogradation de l'amidon, augmentation de la fermeté et perte des arômes et de la saveur. Pour retarder ce genre de changement, les industriels reformulent souvent leurs recettes et intègrent des lipides (matières grasses, mono- et diglycérides), des tensioactifs, des gommes et même des enzymes (Wrigley *et al.*, 2015).

2.2.2. Détérioration chimique

Les produits de boulangerie riches en lipides risquent de voir leur qualité se détériorer principalement à cause du rancissement. Le rancissement est caractérisé par la dégradation des lipides qui résulte en des mauvaises odeurs et saveurs et la réduction de la durée de vie du produit. Le rancissement peut être oxydatif ou hydrolytique. Il est toutefois possible de retarder le rancissement des produits de boulangeries par l'utilisation d'ingrédients naturels à base d'herbes (Iorgachova *et al.*, 2019).

2.2.3. Détérioration microbiologique

La flore qui contamine les produits de boulangerie dépend principalement de l'activité de l'eau (a_w), l'humidité et le pH du produit en question. Les produits de boulangerie ont une a_w qui varie entre 0.8 et 0.9. Les *Lactobacillus* composent la flore microbienne la plus dominante dans le pain. La présence de ces bactéries a permis l'amélioration du processus de fermentation (Lues *et al.*, 1993).

Cependant, les produits de boulangeries ont été particulièrement impliqués dans des maladies d'origine alimentaire impliquant *Salmonella* spp., *Listeria monoctyogenes* et *Bacillus cereus*, tandis que *Clostridium botulinum* est une préoccupation dans les produits de boulangerie à haute teneur en humidité emballés dans des MAP (Smith *et al.*, 2004).

Toutefois*, Bacillus subtilis*, malgrè son non pathogénicité, reste le problème majeur de la détérioration de la qualité microbiologique des produits de boulangerie. Ce microorganisme, qui est généralement présent dans les matières premières telles que la farine, le sucre et la levure, survit à l'opération de cuisson, se termine lors du refroidissement et se développe dans les deux conditions de conditionnement aérobiques et anaérobiques. Les spores de *Bacillus* sont capables de survivre à un traitement thermique (100 °C, 10 min) ce qui correspond au processus de cuisson (Rosenkvist *et al.*, 1995). *Bacillus subtilis* est responsable du phénomène de « ropiness ». Ce problème peut généralement être surmonté par l'utilisation de conservateurs synthétiques (ie. propionates, acide acétique) (Tarar *et al.*, 2010).

Les levures peuvent également provoquer la détérioration de la surface du pain. On trouve, principalement, *Pichia burtonii* ("craie moule») et, d'une façon moindre, *Candida guilliermondii*, *Hansenula anomala* et *Debaryomyces hansenii* (Saranraj et al., 2016). Des conservateurs, tels que le sorbate, le benzoate et les parabènes sont efficaces à inhiber la détérioration par les levures (Magan *et al.*, 2006).

En boulangerie, le type d'altération microbienne le plus courant est la croissance des moisissures et, dans de nombreux cas, c'est le facteur principal régissant la durée de conservation (Saranraj et al., 2016). Parmi les moisissures impliquées dans la détérioration du pain, on trouve *Rhizopus*, *Mucor*, *Penicillium*, *Eurotium*, *Clodosporium*, *Aspergillus* et *Monilia*. La contamination par *Penicillium* sp. constitue entre 90 et 100 % (Saranraj et al., 2016).

Plusieurs chercheurs ont essayé de développer de nouvelles méthodes pour prolonger la durée de vie des produits de boulangerie. On trouve des films antimicrobiens et le MAP (Lopes *et al.*, 2014). Le MAP permet d'augmenter la durée de conservation du pain. Cependant, des études antérieures ont démontré que le MAP n'est pas efficace à contrôler la croissance des moisissures sur certains produits tels que le pain, les petits pains, le pain tranché et les pains à hamburger (Rodriguez et al., 2000). En effet, l'élimination de l'oxygène des produits avec des structures poreuses tel que le pain est empêchée par la structure rigide de la surface du pain ce qui engendre le développement des moisissures dans des atmosphères même à des concentrations élevées de CO_2 (Rodriguez *et al.*, 2000).

CHAPITRE 3 : LES AGENTS DE CONSERVATION EN AGROALIMENTAIRE

Le parlement Européen et du Conseil de l'Union définit les additifs alimentaires comme « des substances qui ne sont normalement pas utilisées en tant qu'aliments, mais qui sont délibérément ajoutées à des denrées alimentaires à des fins technologiques décrites dans le présent règlement, comme leur conservation, par exemple » (Parlement européen et du conseil, 2008).

Parmi les additifs alimentaires on trouve les colorants, les anti-agglomérants, les agents de conservation et certains édulcorants. Cependant, Santé Canada (2016a) exclut de la liste des additifs alimentaires les (1) substances qui sont les ingrédients d'aliments, comme le sel, le sucre et la fécule; (2) les vitamines, les minéraux et les acides aminés; (3) les épices, les assaisonnements et les arômes; (4) les produits chimiques agricoles; (5) les médicaments vétérinaires et (6) les matériaux d'emballage des aliments.

On trouve une liste des différents agents de conservation autorisés sur les sites gouvernementaux (Santé Canada, 2019). Cependant, cette liste contient principalement les agents de conservation synthétiques.

Dans ce chapitre, nous avons présenté les agenst de conservation les plus utilisés en industrie alimetaire. Nous avons également classé ces agents de conservation en deux principales sous classes : agents de conservation synthètiques et naturels.

3.1. LES AGENTS DE CONSERVATION SYNTHÉTIQUES

Plusieurs agents de conservation synthétiques sont utilisés actuellement dans le but d'assurer la salubrité des aliments mis sur le marché. Leur usage dépend, à la fois, des propriétés de l'aliment voulu préserver et des microorganismes cibles.

3.1.1. Acides faibles et leurs sels

On trouve dans cette catégorie l'acide lactique, propionique, citrique, benzoïque, sorbique etc. Ricke (2003) et Theron *et al.* (2010) ont confirmé l'efficacité antimicrobienne de certains acides organiques tels que l'acide lactique, propionique et acétique et leurs sels respectifs. Cette efficacité varie selon le type de l'acide. L'activité antimicrobienne des acides organiques et de leurs sels dépend principalement du pH. En effet, les acides organiques sont plus efficaces à un pH < pKa lorsqu'ils sont dans leur forme non dissociée, où ils seront capables de pénétrer dans la cellule microbienne. L'activité antimicrobienne a été associée à une succession de réactions commençant par la pénétration de l'acide organique sous sa forme associée dans les cellules bactériennes provoquant une acidification des composants internes des membranes cellulaires et leur perturbation. Ceci engendre la perte du transport actif des substances nutritives, une augmentation de la perméabilité membranaire, une réduction de l'a_w, l'inhibition des

réactions métaboliques essentielles et l'accumulation d'anions toxiques (Theron *et al.*, 2010).
Cependant, il existe dans la nature des bactéries naturellement tolérantes aux acides. Certains microorganismes peuvent même développer une résistance. En effet, pour les bactéries Gram positif, la résistance est relativement faible due à l'absence de la membrane externe (Brul *et al.*, 1999). Pour les bactéries Gram négatif, la présence de la membrane externe engendre, dans certains cas, l'augmentation de la résistance bactérienne. Plusieurs acides organiques sont jugés inoffensifs tels que l'acide ascorbique (E 300) et l'ascrobate de sodium (E 301) dont l'innocuité a été prouvée sur les animaux. L'EFSA (2015) ne juge pas nécessaire la fixation des niveaux d'utilisation et d'une dose journalière admissible.

3.1.2. Dioxyde de soufre et sulfites E220-228

Les sulfites sont utilisés, en agroalimentaire, pour leur pouvoir à contrôler le brunissement enzymatique et non enzymatique, leur pouvoir antimicrobien, leur pouvoir antioxydant et comme agent de blanchiment. Il est présent sous la forme de sulfite de sodium et le disulfite de sodium. La controverse des sulfites est reliée principalement à leur rôle, dans certains cas, à initier des réactions asmathiques.

Les sulfites peuvent affecter les principaux processus métaboliques essentiels à la viabilité des micro-organismes tels que la production d'énergie (cas des cellules de levure), la biosynthèse des protéines, la réplication de l'ADN et l'intégrité de la membrane (Russell *et al.*, 2003, Schimz *et al.*, 1979). La concentration du sulfite de sodium maximale autorisée est de 500 ppm dans les pâtes biscuit et 300 ppm pour les conserves de thon émietté (Santé Canada, 2016c).

3.1.3. Nitrite

Les nitrites sont utilisés généralement pour préserver la viande transformée contre le *Clostridium botulinum*, la viande de volailles et ses sous-produits (Cammack et al., 1999). Tout comme les sulfites, les nitrites sont capables de stabiliser la couleur des aliments. Ils ont une grande activité antimicrobienne jugée bactériostatique. Leur activité antimicrobienne dépend du pH et elle est plus élevée à pH acide. La concentration maximale autorisée dans les produits de charcuterie au Canada est de 200 ppm, sauf pour le porc où seulement 120 ppm sont permises (Santé Canada, 2016b). Leur pouvoir antimicrobien est dû principalement à 4 dérivés du nitrite : l'acide nitreux, le peroxynitrite, les complexes Fe-S-NO, Nitrosothiols et les composés N-nitroso. L'acide nitreux et les composés de N-nitroso sont capables de diazoter et de désaminer des groupes amines comme les nucléotides des bactéries (Cammack *et al.*, 1999).

3 mécanismes d'action sont suggérés par Davidson et al. (2005):

- Blocage des sites sulfhydryles dans les cellules bactériennes (cas du *Clostridium perfringens* et de *Staphylococcus aureus*).

- Inhibition du transport actif, l'absorption d'oxygène et la phosphorylation oxydative (Cas de *Pseudomonas aeruginosa*).

- Réaction entre l'oxyde nitrique et un composé essentiel contenant du fer (exp, ferrédoxine) dans les cellules germées (cas du *C. botulinum*).

3.1.4. Parabènes

Les parabènes sont utilisés comme conservateurs afin de prévenir la prolifération des moisissures et des levures. Ils sont chimiquement stables et supportent une large gamme de températures et de pH (pH de 4.5 à 7.5). Ils ont également un faible coût de production, pas d'odeur ou de goût discernable et une toxicité faible (Jagne *et al.*, 2016). La concentration maximale autorisée du méthyle et propyle parabène au Canada est de ≈ 1,000 ppm. Les parabènes provoquent la rupture de la membrane cytoplasmique et la fuite des composés intracellulaires. Les moisissures sont également plus sensibles aux parabènes (Davidson *et al.*, 2005).

3.1.5. Hydroxytoluène butylé (BHT) et hydroxyanisole butylé (BHA)

BHT et BHA sont des antioxydants reconnus comme sûrs pour une utilisation dans les produits alimentaires contenant de la graisse et dans les produits pharmaceutiques (Yehye *et al.*, 2015). Ils se comportent comme des analogues du tocophérol et empêchent la peroxydation des huiles et des lipides insaturés. On les trouve dans les aliments riches en matières grasses ou en huiles, les céréales, les gommes à mâcher, la viande, la margarine, les croustilles, les soupes déshydratées, etc.

L'Autorité européenne de sécurité des aliments EFSA (2012) a attribué une DJA de 0.25 mg/kg de poids corporel /jour de BHT sur la base des effets sur la reproduction et l'induction enzymatique hépatique chez le rat. Le BHT n'était pas préoccupant en ce qui concerne la génotoxicité et il est considéré à la limite de toute cancérogénicité.

3.1.6. Inconvénients associés à l'usage des additifs synthétiques

L'utilisation des agents de conservation synthétiques pose une controverse vis-à-vis du consommateur et aussi des organisations environnementales. En effet, à cause de l'utilisation excessive de ces antimicrobiens, on les trouve à des concentrations supérieures aux réglementations dans les rejets domestiques et dans les stations d'épuration des eaux et peuvent ainsi se trouver dans les sédiments et dans les plantes. Malgré la biodégradabilité de la plupart, certains antimicrobiens peuvent réagir avec des composés de l'eau comme le chlore et former ainsi des complexes dont la toxicité est encore inconnue (Haman *et al.*, 2015).

Plusieurs études ont évalué l'impact des additifs chimiques sur la santé humaine à court, moyen et long termes. Vu que l'utilisation de ces agents de conservation est variée (cosmétiques, alimentaires, cutanée, etc.), l'impact sur la santé n'est pas le même. Les parabènes par exemple sont considérés comme des perturbateurs endocriniens. Aux États-Unis, l'ingestion alimentaire moyenne des parabènes dans les produits alimentaires est estimée à 470 et 307 ng / kg de poids corporel / jour chez les enfants et les adultes, respectivement (Liao *et al.*, 2013). Malgré le dosage assez faible, l'impact sur la santé est beaucoup plus important. En effet, les parabènes sont reliés à des problèmes hormonaux mais aussi ils sont soupçonnés de contribuer à l'épidémie de l'obésité (Kolatorova *et al.*, 2018). Les additifs alimentaires synthétiques peuvent également provoquer l'anaphylaxie tels que les sulfites et les benzoates de sodium (Royal *et al.*, 2016). Les incidents reliés à l'ingestion des sorbates sont rares, mais des cas d'urticaire et de dermatite de contact ont été rapportés (Sanjay Sharma, 2015). Les nitrates et les nitrites sont soupçonnés de causer le cancer de l'estomac. Des études antérieures ont étudié la résistance de certaines souches aux nitrites comme *Salmonella*, *Lactobacillus*, *Clostridium* et *Bacillus* (Pierson et al., 1983).

3.1.7. Préoccupation des consommateurs

Le vieillissement de la population a entraîné un nombre croissant de patients atteints de maladies telles que le cancer, le diabète et l'hypertension. De plus, le nombre de cas d'allergies alimentaires qui touchent même les jeunes enfants a augmenté (Trevathan, 2018). À mesure que l'intérêt du public pour le bien-être grandit, l'inquiétude des consommateurs vis-à-vis des additifs alimentaires augmente également. Les préoccupations des consommateurs concernent principalement les additifs alimentaires, les résidus de pesticides agricoles, les aliments génétiquement modifiés et les injections d'hormones de croissance et d'antibiotiques destinées aux bétails. Certains soutiennent que l'utilisation d'additifs synthétiques comporte des risques techniques associés aux additifs. D'autres, craignent également que les risques des additifs synthétiques, actuellement approuvés, pour la santé ne soient révélés qu'à l'avenir et se méfient donc d'eux. Par conséquent, les consommateurs prévilègent, de plus en plus, avoir une alimentation saine, sans une longue liste d'additifs inconnus pour la plupart, avec plus de transparence. Ils souhaitent, ainsi, recevoir des informations plus détaillées sur les ingrédients alimentaires plus qu'elles ne le sont actuellement, que l'aliment soit naturel, transformé ou préparé au restaurant (Freeman, 2015).

3.2. LES AGENTS DE CONSERVATION NATURELS

Les agents de conservation naturels peuvent être une alternative à l'usage des additifs synthétiques. Ils peuvent être d'origine végétale (ie. extrait de végétaux, essence, huiles essentielles, sous-produits végétaux), animale (ie. lactoferrine, chitosane, lysozyme et les peptides dérivés du lait), bactérienne (ie. bactériocine et reuterin), virale (ie. bactériophage) et algale et fongique (Gyawali et al., 2014, Oliveira et al., 2014). Ces

composés naturels ont l'avantage d'être efficaces et biodégradables. Dans cette section, certains de ces agents de conservation utilisés dans l'industrie agroalimentaire seront détaillés.

3.2.1. Nisine

La nisine est un peptide contenant de la lanthionine produite par certaines souches de *Lactococcus lactis* (Hui Zhou *et al.*, 2014). Elle agit sur les cellules végétatives en s'insérant dans la membrane, en formant des pores, et en dissipant la force motrice protonique. Cela inhibe l'absorption des acides aminés et favorise la fuite rapide de petits métabolites, des ions ou des solutés cytoplasmiques tels que les acides aminés et les nucléotides (Davidson et al., 2005).

Cependant, l'exposition prolongée à la nisine permet de produire des bactéries tolérantes. L'apparition de souches résistantes est rare, mais ces souches sont caractérisées surtout par des changements au niveau de leur membrane avec l'augmentation de la température de transition de phase, plus de chaînes droites d'acides gras et moins d'acides gras à chaîne ramifiée entraînant une diminution de l'efficacité de la formation de pores et une augmentation de l'hydrophobicité et de l'épaisseur de la paroi cellulaire (Davidson *et al.*, 2005).

3.2.2. Natamycine

Natamycine est un antibiotique polyène produit durant la fermentation par *Streptomyces* natalensis, utilisé comme antifongique en raison de son large spectre d'activité et l'absence de développement d'une résistance. Son mode d'action est lié au blocage de la croissance fongique en se liant spécifiquement à l'ergostérol que contiennent les membranes (Te Welscher *et al.*, 2008). Il est appliqué principalement dans des produits de boulangerie et les fromages. Cependant, cet antifongique est très faiblement soluble dans l'eau et migre difficilement dans les matrices alimentaires. La pulvérisation de la surface, le trempage, l'enrobage avec une émulsion ou l'addition directe peuvent améliorer sa biodisponibilité (Pisoschi *et al.*, 2018).

3.2.3. Reuterin

La reuterin est un antimicrobien à large spectre produit par certaines souches de *Lactobacillus reuteri* lors de la fermentation anaérobique du glycérol. Comme la nisine et la pédiocine, la reuterin est active contre les cellules bactériennes végétatives à Gram positif par un processus de liaison, d'insertion, d'agrégation et de formation de pores dans la membrane cellulaire des bactéries, provoquant des fuites du contenu cellulaire et la mort cellulaire (Fan *et al.*, 2018). Elle a également une activité antifongique et inhibe certaines levures et moisissures telles que *Candida*, *Torulopsis*, *Saccharomyces*, *Aspergillus* et *Fusarium* (Jørgensen *et al.*, 2017, Schnürer *et al.*, 2005).

3.2.4. Les huiles essentielles et les extraits de végétaux

Les huiles essentielles sont généralement extraites par distillation (y compris l'hydrodistillation), le pressage à froid ou la macération. Bien que connues pour leurs propriétés aromatiques, certaines huiles essentielles ont démontré diverses activités antivirales, antifongiques, antibactériennes, anti parasitaires, antioxydantes, et même des propriétés insecticides (Hashemi *et al.*, 2017).

Elles doivent leurs propriétés antimicrobiennes à leur nature hydrophobe qui leur permet de réagir avec la couche lipidique de la membrane ce qui rend la structure plus perméable induisant des fuites d'ions et du contenu cellulaire, la lyse et la mort de la bactérie (**Figure 3.1**). Les études sont allées encore plus loin et ont isolé les composés actifs majoritaires des huiles essentielles comme le carvacrol, le citral, l'eugenol, le thymol qui ont un puissant pouvoir antimicrobien (Hashemi *et al.*, 2017).

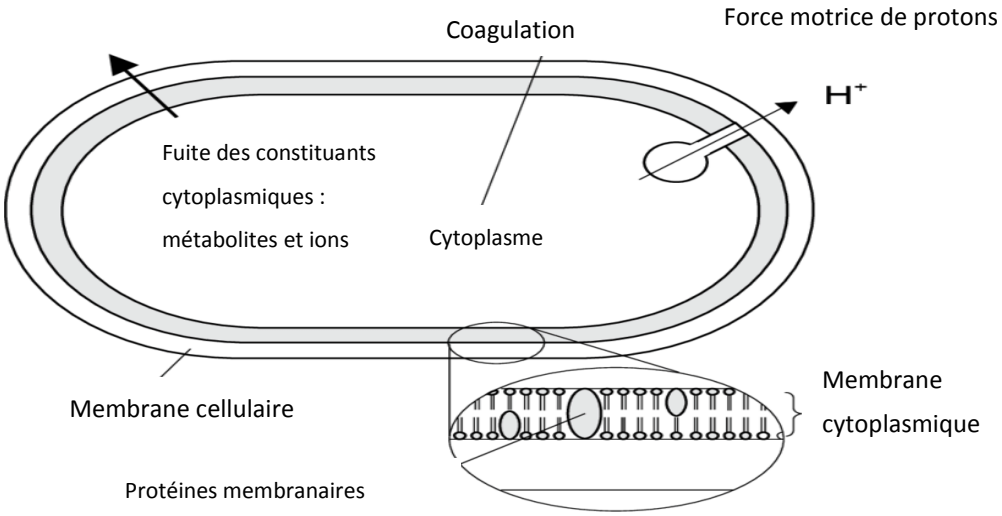

Figure 3.1. Cibles ou mécanismes d'action possibles des composés antimicrobiens des composants des huiles essentielles (Arjin *et al.*, 2014).

Les extraits de végétaux ont aussi un pouvoir antimicrobien mais ils sont moins populaires car leur activité est souvent jugée inférieure à celle des huiles essentielles (**Tableau 3.1**). Cependant, leur richesse en composés nutraceutiques fait des extraits de végétaux de bons candidats pour être incorporés dans les aliments fonctionnels. Certains extraits de végétaux, comme le jus d'ail et du thé, ont la capacité d'inhiber les bactéries mêmes lorsqu'elles sont résistantes aux antibiotiques comme le ciprofloxacine, methicilline et vancomycine (Yee-Lean Lee *et al.*, 2003b, Naveed *et al.*, 2013).

Tableau 3.1. L'effet de quelques extraits végétaux

Extrait	Effet	Molécules actives	Références
Ail	Inhibition à faible dose de *S. aureus, E. faecalis, E. coli 0157 H7, P. aeruginosa, S. enteritidis*	Réaction chimique entre l'allicine et les groupes thiol de différentes enzymes.	(Yee-Lean Lee *et al.*, 2003b)
Thé		Cathechine du thé vert (epigallocatechin gallate)	
Citrus	Effet bactéricide contre *S. enterica, E.coli, B. hyodysenteriae.*	Acide ascorbique, acide citrique, naringine, hesperidin, quercetin, rutin, naringenin, saponines	(Álvarez-Ordóñez *et al.*, 2013)
Aloe vera	Plus efficace contre Gram positif	Pyrocatechol, acide cinnamique, acide p-coumarique, acide ascorbique	(Lawrence *et al.*, 2009)

Les extraits de citrus par exemple, sont très riches en polyphénols totaux (3524 mg acide gallique /100 g d'extrait) (de Lourdes Mata Bilbao *et al.*, 2007). Cette teneur en polyphénol est sensible à un traitement à une haute pression, par contre, il n'y a aucun effet sur l'activité antimicrobienne (Casquete *et al.*, 2015). Les extraits d'agrumes possèdent de fortes propriétés antioxydantes, antimicrobiennes, des arômes et des saveurs agréables, spécialement dû à la présence de flavonoïdes. La classe des flavonoïdes comprend les flavanones, les flavones et leurs dérivés. Les flavonoïdes d'agrumes ont de nombreuses activités biologiques, telles qu'une activité antioxydante, antimicrobienne et anti-inflammatoire. Les flavanones des agrumes comprennent la naringinine, l'hespéridine, l'hespéritine, la prunine et ont un large spectre d'action contre de nombreuses bactéries Gram négatif. Plusieurs études ont essayé d'expliquer l'activité antimicrobienne des extraits de citrus et les composants qui sont mis en œuvre. La plupart ont expliqué cette activité à la présence de phénols, acides phénoliques, quinones, saponines, flavonoïdes, tannins, coumarines, terpenoïdes, et alcaloïdes. Pour atteindre une réduction bactérienne significative, il est préférable d'augmenter le temps d'exposition ou d'utiliser des concentrations 2 à 4 fois plus élevées que la CMI (Álvarez-Ordóñez *et al.*, 2013). L'extrait de citrus agit principalement sur la membrane. Il provoque des dommages conformationnels ou/et compositionnels dans quelques ou tous les composants des cellules membranaires. Il affecte principalement les groupes carboxyliques des acides gras membranaires et altère ainsi la structure macromoléculaire de la membrane bactérienne. A des concentrations plus élevées que la CMI, la formation des pores dans la membrane cytoplasmique est observée induisant une fuite des acides nucléiques (Álvarez-Ordóñez *et al.*, 2013)

3.2.5. Avantages et enjeux de l'utilisation des antimicrobiens naturels

Utiliser des antimicrobiens naturels comme agents de conservation remplaçant les agents de conservation synthétiques est très recherché vu leur biodégradabilité. Ils pourraient agir

comme bioconservateurs, réduisant ou éliminant les bactéries pathogènes et augmentant la qualité globale des produits alimentaires.

Les agents de conservation naturels présentent une bonne activité antibactérienne, mais à cause du caractère hydrophobe, instable et volatile de certains agents, la commercialisation des produits alimentaires incorporant ces antimicrobiens est plus difficile. Comme la plupart des composés bioactifs, les agents de conservation naturels sont chimiquement réactifs et peuvent causer des problèmes considérables lorsqu'ils sont intégrés dans un système alimentaire complexe. Les effets négatifs concernent l'intégrité de la chimie des aliments et la dégradation de l'activité biologique des composés bioactifs (Weiss *et al.*, 2008). En effet, la nécessité d'utiliser des concentrations élevées pour inhiber la croissance microbienne dans les limites imposées par les réglementations gouvernementales implique également une altération de la qualité des aliments (leur goût et leur saveur prononcés qui affectent les propriétés sensorielles du produit final). Leur application est donc limitée aux produits alimentaires dans lesquels le goût ou l'arôme spécifique est apprécié ou non remarqué.

De plus, la solubilité limitée dans l'eau des composés antimicrobiens réduit leur efficacité et l'homogénéité de leur distribution dans les matrices alimentaires qui est nécessaire pour assurer l'inhibition de la croissance microbienne dans les produits alimentaires (Bhavini Shah *et al.*, 2012).

3.2.6. Réglementation

L'apparition de nouveaux additifs sur le marché des produits alimentaires a engendré des recherches intensives afin de s'assurer de leur innocuité et sécurité pour le consommateur. Les organismes de réglementation du monde entier utilisent la science et l'évaluation des risques pour déterminer la sécurité sanitaire des aliments et protéger les consommateurs : U.S. Food and Drug Administration, USDA Agricultural Marketing Service / National Organic Program, USDA Foreign Agricultural Service,Health Canada Food Directorate, European Food Safety Authority, China National Center for Food Safety Risk Assessment, Food Safety and Standards Authority of India, Codex Alimentarius (IFAC, 2019).

CHAPITRE 4 : MICRO-ENCAPSULATION

4.1. APERÇU

L'encapsulation en agroalimentaire consiste à piéger des molécules bioactives généralement sensibles à la température, l'humidité, aux micro-organismes ou d'autres composants du système alimentaire, dans une matrice permettant (i) une meilleure protection de leur activité biologique (les protéger des interactions avec les ingrédients alimentaires), (ii) une libération plus contrôlée de ces molécules au cours du temps (et donc accroître leur bioactivité au cours du temps), (iii) une augmentation de la stabilité physique des composés bioactifs (la taille subcellulaire augmente leur bioactivité), (iv) une amélioration du ciblage des ingrédients encapsulés et (v) une amélioration de la solubilité et de la biodisponibilité (Donsì *et al.*, 2011, Fathi *et al.*, 2012b). Cette technologie a été souvent utilisée dans le domaine pharmaceutique ou les molécules bioactives lorsqu'elles sont ingérées à l'état libre sont libérées ou même dégradées avant d'atteindre la cible due à l'environnement du système digestif (acidité gastrique, enzymes salivaires etc.). Encapsuler ces molécules a permis donc de les protéger contre la dégradation et contre une libération précoce. Actuellement, cette technologie est, de plus en plus, utilisée en agroalimentaire avec l'encapsulation des antimicrobiens sensibles ou des composés aromatiques ou des composés ayant des propriétés nutraceutiques (KGH Desai *et al.*, 2005a, Donsì *et al.*, 2011). Des aromatisants, des antioxydants, des colorants, des édulcorants, des agents antimicrobiens, des molécules bioactives et nutraceutiques, des probiotiques et des enzymes sont actuellement encapsulés (Lakkis, 2008). Les recherches dans ce domaine se sont intensifiées ces dernières décennies. La base de données Pubmed (**Figure 4.1**) montre que le nombre de publications par an considérant «food, encapsulation et nano*» comme mots clés est passé de 3 à 238 entre 2005 et 2019.

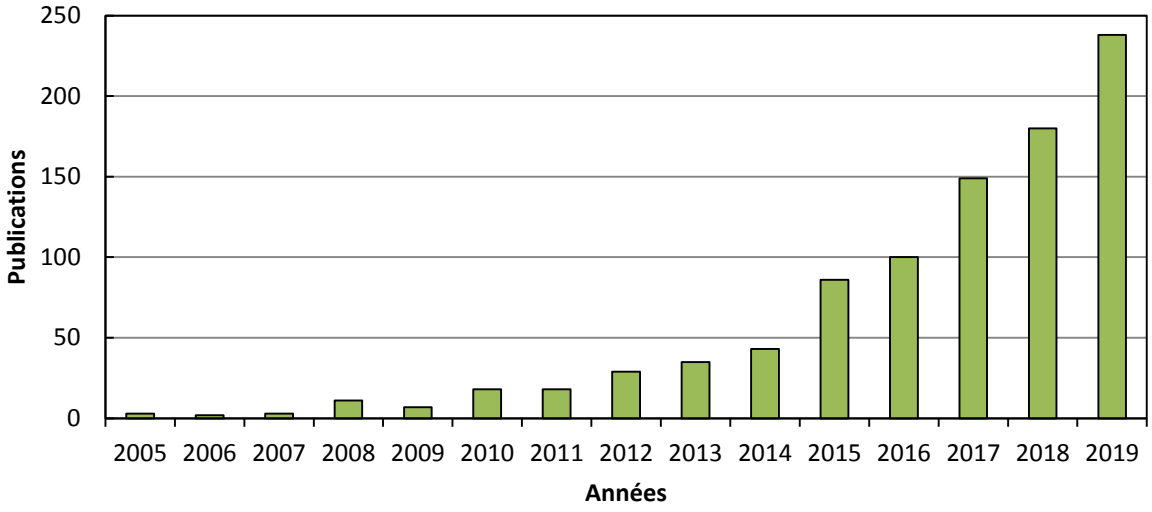

Figure 4.1. Publications avec les mots clés « food, encapsulation and nano* de 2005 à 2019 (Base de données Pubmed)

Comme mentionné dans le chapitre 3, certains composés bioactifs sont peu solubles dans l'eau, ce qui réduit leur efficacité et l'homogénéité de leur distribution dans l'aliment d'où l'intérêt de leur encapsulation dans une matrice plus commode à une application alimentaire.

Différentes matrices peuvent être utilisées. Les biopolymères, par exemple, ont été largement utilisés comme matériaux de paroi des microparticules pour la protection des composés bioactifs, comme le chitosane, l'alginate de calcium, l'amidon modifié, les protéines de lait, d'autres polysaccharides et les cyclodextrines (oligosaccharides cycliques). Une preuve de concept de la protection des agents bioactifs par micro/ nanoencapsulation a été fournie par différents auteurs, via des systèmes d'administration liposomale (c'est-à-dire des micelles de surfactants nanométriques) (Colas *et al.*, 2007, Matthew Taylor *et al.*, 2005), des microémulsions (Flanagan *et al.*, 2006, Ziani *et al.*, 2011), et des microcapsules biopolymères (Donsì *et al.*, 2011, Ong *et al.*, 2015), suggérant des mécanismes subcellulaires responsables de l'amélioration de la bioactivité.

4.2. PRINCIPALES FORMES D'ENCAPSULATION

4.2.1. Émulsions

4.2.1.1. Émulsion simple

Les émulsions sont des dispersions constituées de deux phases liquides non miscibles qui sont mélangées en utilisant une force mécanique et un surfactant (Kale *et al.*, 2017). Le choix du surfactant sur la base de la balance hydrophile-lipophile (HLB) ou le paramètre d'emballage critique (CPP) aide à développer l'émulsion souhaitée. Les surfactants avec de faibles valeurs de HLB 3-8 sont utiles pour former une émulsion eau dans huile (w / o) et celle avec des valeurs HLB élevées 8-18 sont utilisés pour former une émulsion o /w comme le montre la **Figure 4.2** (Kale *et al.*, 2017).

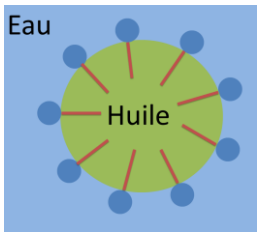

Figure 4.2. Schéma d'une émulsion simple o/w.

En se basant sur la taille des particules, David Julian McClements et al. (2011) et Kale *et al.* (2017) classifient les émulsions en:

- émulsion conventionnelle 100 µm < Rayon des particules < 100 nm produite par une homogénéisation mécanique vigoureuse ou à l'aide d'appareil de dispersion (tel que l'Ultra-turrax).

- microémulsion lorsque 10 nm < Rayon des particules < 100 nm et
- nanoémulsion lorsque 2 nm < Rayon des particules < 100 nm

Les émulsions o /w ont l'avantage d'améliorer la solubilité des composés hydrophobes ou solubles dans l'huile et de masquer le goût et l'odeur désagréables des composés actifs. Cependant, l'émulsion conventionnelle n'est pas assez stable, possède une courte durée de conservation et souvent déstabilisée par le crémage, le craquelage (rupture), la floculation et l'inversion de phase lors du stockage (Kale *et al.*, 2017).

Des études antérieures ont démontré que la réduction de la taille des particules de l'émulsion d'huile essentielle de lemongrass et d'alginate par microfluidisation permet d'améliorer son activité antimicrobienne contre *E. coli* (Salvia-Trujillo et al., 2014).

4.2.1.2. Émulsion double

Par émulsion double, on distingue l'émulsion (w/o/w) où une particule huileuse est piéger entre deux molécules d'eau et une émulsion (o/w/o) où une phase hydrophile est piégée entre deux phases huileuses et ceci grâce à la présence de tensioactifs. Cette technique a été développée pour la 1ère fois par Ogawa *et al.* (1988). Les émulsions doubles peuvent être utilisées à la fois pour la délivrance des composés peu solubles dans l'eau (exp : vitamines lipophiles) ou solubles dans l'eau, en raison de leur capacité à améliorer la solubilisation et la biodisponibilité de ces molécules. Elle peut être préparée spontanément ou en utilisant une énergie élevée qui génère des forces disruptives permettant de former de fines gouttelettes (valve à haute pression, homogénéisateur, microfluidisateur et sonicateur). La taille des gouttelettes de l'émulsion dépendra donc de l'intensité et de la durée de l'application de l'énergie, le type et la concentration des émulsifiants, la tension interfaciale et les viscosités relatives des phases continues et dispersées (Karthik *et al.*, 2015).

La préparation d'une émulsion double passe souvent par deux étapes, (1) émulsion w/o ou o/w, puis cette émulsion est ajoutée à une phase hydrophile ou hydrophobe pour former respectivement une émulsion w/o/w ou o/w/o. L'émulsion multiple est caractérisée par une grande efficacité d'encapsulation initiale. Des études antérieures ont montré aussi que l'émulsion double permet d'améliorer la stabilité et la biodisponibilité des composés bioactifs que ce soit hydrophiles ou lipophiles. Encapsuler la catéchine (hydrophile) et le curcumine (hydrophobe) en émulsion double w/o/w a permis d'augmenter leur biodisponibilité jusqu'à 4 fois et retarder leur libération avec un grand rendement d'encapsulation (88 à 97%) (Aditya *et al.*, 2015). Cependant, les difficultés relatives à sa préparation, sa dégradation facile pendant le stockage et la fuite rapide des composés bioactifs au cours du temps la laisse peu populaire pour une application à grande échelle (McClements, 2015).

4.2.1.3. Émulsion à multicouches

Une émulsion à multicouche se définit comme une émulsion dans laquelle les gouttelettes sont entourées de deux couches ou plus. L'émulsion à multicouche permet d'augmenter l'efficacité d'encapsulation et ainsi retarder la libération des molécules bioactives encapsulées hydrophobes et hydrophiles. Elle est généralement préparée par une technique couche par couche (LBL) (Mao *et al.*, 2017). Pour former une émulsion à multicouches, une succession d'ajout d'émulsifiant et de polymère (1), polymère (2),…. polymère (n) est réalisée en jouant sur leur différence de charge pour créer des forces d'attraction entre les charges opposées et ainsi maintenir les molécules bioactives à l'intérieur des couches formées (**Figure 4.3**Figure). Cependant augmenter le nombre de couches peut être défavorable à la libération des molécules bioactives. Le pH, par contre est très important dans ce genre de système, car à un certain pH (pH isoélectrique), le poly électrolyte peut se décharger et ainsi perdre sa charge.

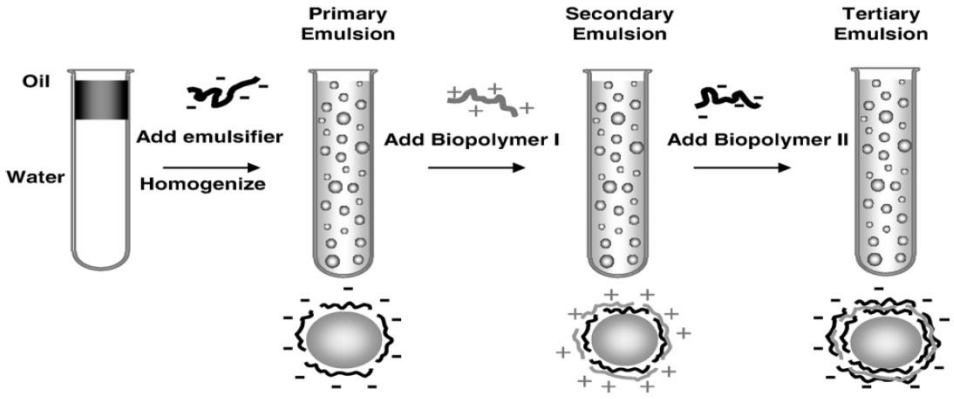

Figure 4.3. Principe de préparation de l'émulsion à multicouches (Guzey et al., 2006)

Cette méthode d'encapsulation a montré une efficacité à protéger les oméga 3 contre la peroxydation des lipides lorsqu'ils sont encapsulés dans le chitosane (Shaw *et al.*, 2007). Cependant, elle est peu appliquée en industrie à cause de sa mise au point difficile et du nombre élevé de paramètres à contrôler.

4.2.1.4. Encapsulation dans un polymère

Les polymères naturels sont des exsudats et des extraits végétaux. Bien qu'ils soient généralement non toxiques, biodégradables et abondants, leur composition peut varier.

Selon McClements (2015), on peut distinguer deux applications des polymères à des fins d'encapsulation :

- La formation de particules basée sur le principe de gélation de certains polymères en présence des ions, chauffage, refroidissement, enzyme. Le polymère présente un intérieur dense et composé principalement de molécules de biopolymères avec une quantité limitée d'eau piégée à l'intérieur.

- particules d'hydrogel tel que l'émulsion gélifiée qui ont un intérieur plus ouvert contenant un réseau de gel de biopolymère qui emprisonne les grandes quantités d'eau (**Figure 4.4**).

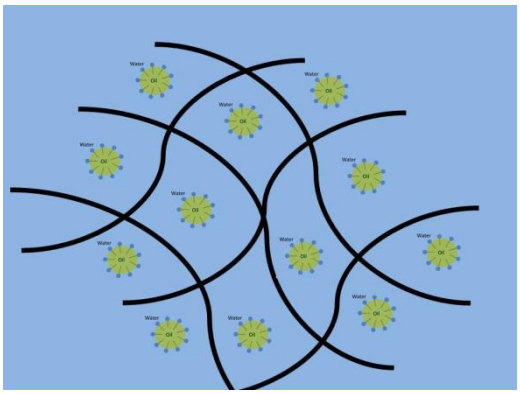

Figure 4.4. Schéma d'une émulsion gélifiée

Il est également nécessaire de considérer les interactions des molécules bioactives avec le biopolymère puisque ces interactions pourraient retarder leur libération, telles que les interactions électrostatiques ou hydrophobes (McClements, 2015). Cette technique a été utilisée dans l'encapsulation des probiotiques et a permis de réduire le risque de la contamination croisée en utilisant l'alginate et l'amidon libre ou complexé au calcium (Kailasapathy, 2006).

Le choix du polymère est important et doit être de grade alimentaire, biodégradable, et stable dans les systèmes alimentaires pendant le traitement, le stockage et la consommation. Les polymères les plus souvent utilisés sont les cyclodextrines et la zéine. D'autres polymères ont aussi été utilisés pour encapsuler l'huile essentielle du romarin tels que la gomme arabique, l'amidon, le maltodextrine et l'inuline (de Barros Fernandes *et al.*, 2014). Selon Lakkis (2008), on trouve :

- **Cires et les lipides** : ie. cire d'abeille, glycérol distearate.
- **Protéines** comme les protéines de soja et du lactosérum en forme native ou modifiée : capable d'encapsuler des composés hydrophiles et lipophiles. Le gel est produit par le déroulement des chaînes polypeptidiques avec exposition simultanée des résidus d'acides aminés hydrophobes (initialement cachés) et autoagrégation ultérieure des molécules de protéines dans un réseau tridimensionnel qui piègent l'eau par capillarité. Ces gels sont généralement appliqués pour l'encapsulation des composés thermorésistants (Lingyun Chen *et al.*, 2006). La préparation du gel nécessite généralement un traitement thermique. Ceci implique des interactions covalentes telles que les liaisons disulfures entre les chaînes polypeptidiques ce qui augmente le poids moléculaire et donc fait augmenter la longueur de la chaîne (Totosaus et al., 2002). Il est aussi possible de former un gel à température ambiante en utilisant des ions calcium ou des sels ferreux capables de réticuler avec les groupes carboxyles des protéines du

lactosérum préalablement dénaturés pour encapsuler des composés thermolabiles (Roff et al., 1996).

Carbohydrates : constitués par des monosaccharides, des oligosaccharides et des polysaccharides. Leur capacité d'encapsuler est due à leur structure moléculaire massive. Ils sont classés en fonction de leurs origines: végétale (amidon, cellulose, pectine et la gomme de guar, inuline); animale (chitosane); algale (alginate et le carraghénane), et microbienne (xanthane, dextrane et cyclodextrines). Contrairement aux supports lipidiques, les carbohydrates peuvent interagir avec une large gamme de composés bioactifs par l'intermédiaire de leurs groupes fonctionnels, ce qui fait d'eux des supports souples pour lier et piéger une variété d'ingrédients alimentaires bioactifs hydrophiles et hydrophobes. D'autre part, ils sont résistants à des températures élevées (Fathi *et al.*, 2014). Diego T. Santos *et al.* (2013) ont démontré que l'encapsulation des anthocyanines de l'extrait de l'écorce de jaboticaba par « expansion rapide de solution supercritique » ou par piégeage dans des billes de calcium-alginate permet d'améliorer leur stabilité contre la lumière et la température par rapport à l'extrait libre. Ainsi, on peut assurer leur biodisponibilité et leur stabilité pendant le stockage contre l'oxydation et la dégradation de leur activité biologique (CC Liolios *et al.*, 2009a).

Il est toutefois possible de combiner deux matrices ou plus pour améliorer la stabilité et la bioactivité des molécules encapsulées. de Barros Fernandes *et al.* (2014) ont démontré que l'usage des carbohydrates combinés ayant une capacité émulsifiante élevée est efficace à retenir les composés volatils et présentent également des températures de transition vitreuse assez basses démontrant ainsi une meilleure stabilité au cours du processus et au cours du stockage.

Dans cette étude, les carbohydrates ont été selectionnés pour les travaux d'encapsulation et bien précisemment, la pectine de betterave à sucre à un haut degré de méthylation.

4.2.1.5. Stabilité

La stabilité des émulsions durant le stockage repose en majeure partie sur la nature et la concentration du surfactant utilisé. On trouve commercialement une diversité d'émulsifiants d'origines animale, végétale ou synthétique. Ces émulsifiants sont souvent classés en non-ionique, cationique, anionique ou zwitterionic. Cependant, pour usage alimentaire, les émulsifiants cationiques sont rarement utilisés à cause de leur toxicité. Le choix entre cette panoplie de produits est parfois difficile et certains critères sont souvent recherchés y compris l'aspect économique, facilité d'usage, la solubilité et surtout la stabilité au cours du temps (donne la plus faible taille des particules). Certains émulsifiants ioniques, lorsqu'ils sont libres, peuvent avoir une activité antifongique. Cette activité est réduite en présence de la phase huileuse à cause de la séparation des molécules des tensioactifs ioniques entre les gouttelettes d'huile et les surfaces microbiennes réduisant ainsi la concentration efficace de tensio-actifs disponibles pour agir en tant qu'agents antimicrobiens (Ziani *et al.*, 2011). Comme mentionné plus tôt, l'émulsion est

généralement instable au cours du stockage. Cette instabilité est observée principalement via les phénomènes suivants (**Figure 4.5**):

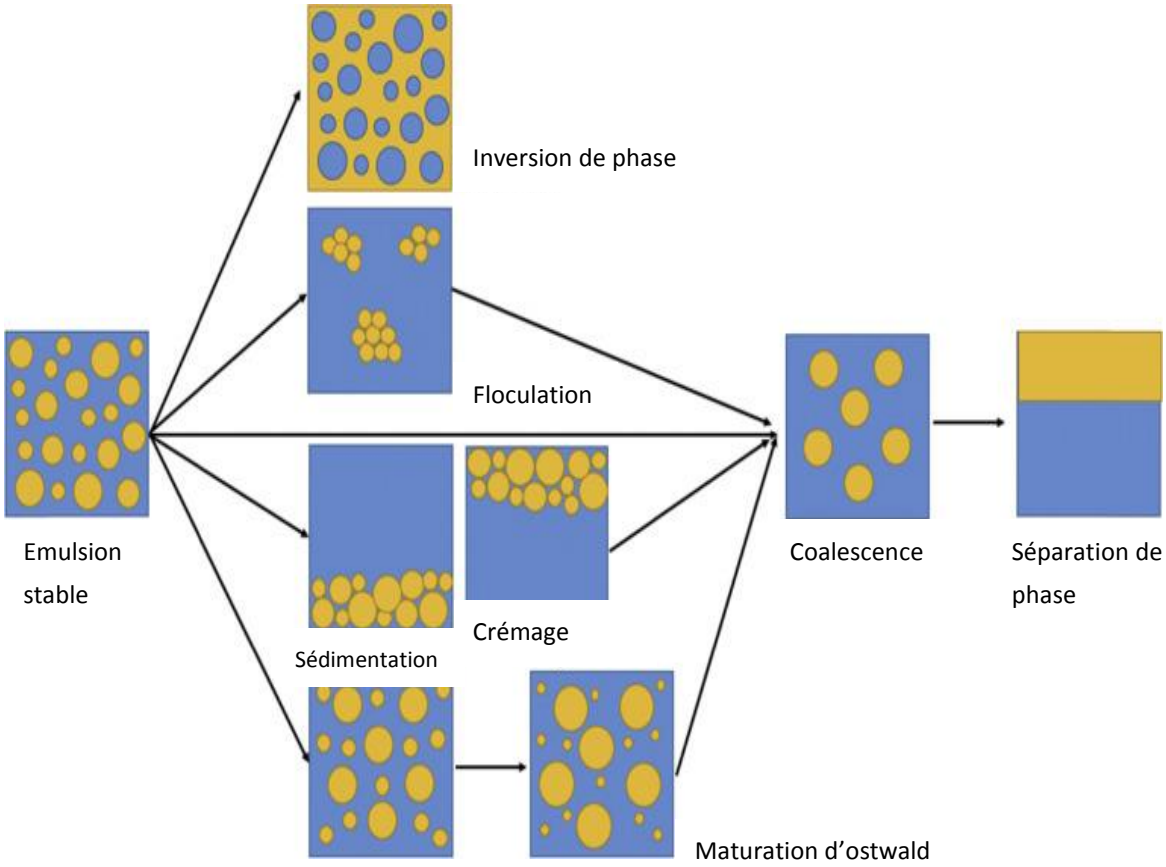

Figure 4.5. Représentation schématique des mécanismes d'instabilité de l'émulsion (Hu *et al.*, 2017).

- **La maturation d'Ostwald** : La maturation d'Ostwald consiste à la diffusion de petites particules graduellement vers des particules de taille plus grande donnant des particules de tailles supérieures. Ce phénomène se produit lorsque la phase dispersée (huile) a une certaine solubilité dans la phase continue (eau). La maturation d'Ostwald pourrait être réduite par l'incorporation dans l'émulsion d'autres huiles plus hydrophobes et ayant une faible solubilité dans l'eau (P. Taylor, 1998).

- **Coalescence :** c'est un indice de déstabilisation du système colloïdal et se manifeste par une augmentation de la taille des gouttelettes et une distribution fortement unimodale avec la possibilité d'une séparation de phase précoce à cause de la collision et la fusion au cours de temps (Karthik *et al.*, 2015). Plus la taille des particules est faible, plus le taux de coalescence est élevé à cause d'un comportement plus brownien. L'augmentation de la concentration de l'émulsifiant peut augmenter les forces d'attraction entre les gouttelettes en nanoémulsion. Il existe divers facteurs qui affectent le taux de

coalescence de la nanoémulsion tels que la concentration en sel, le pH, la composition, la formulation et les conditions de conservation.

- **Séparation gravitationnelle :** on distingue deux phénomènes : le crémage et la sédimentation. La séparation gravitationnelle est due principalement à une différence de densité. Le crémage se produit lorsque la phase dispersée (a une densité inférieure à la phase continue) se déplace vers le haut et forme une couche épaisse. Inversement, la sédimentation se produit si la phase dispersée a une densité plus élevée que la phase continue, entraînant le déplacement des gouttelettes vers le bas (sédiments) (Hu et al., 2017).
- **Floculation :** La floculation se manifeste durant le stockage à long terme. Elle est caractérisée par l'association de deux gouttelettes ou plus sans qu'elles fusionnent en une plus grosse gouttelette (Hu *et al.*, 2017).

L'évaluation de la stabilité des émulsions et des systèmes colloïdaux en général passe principalement par le suivi de différents paramètres tels que:

L'indice de polydispersité (PDI): donne une indication sur la distribution du poids moléculaire dans une dispersion. Plus le PDI est faible, plus la distribution des tailles des particules est homogène. En résumé, avec un PDI < 0.2, les échantillons ont un comportement de monomodes sphériques et étroites. Lorsque le PDI se rapproche de 1, l'émulsion contient plusieurs tailles des particules, les particules les plus larges ont plus tendance à flotter et ainsi engendrer le crémage. Ce phénomène peut être plus rapide dans le cas de collision avec les particules de petite taille (D Julian McClements, 2005). L'indice de polydispersité combiné au suivi de la taille des particules donne donc une indication sur la stabilité d'une émulsion. Il est également possible de prévoir le comportement des gouttelettes grâce à des simulations informatiques qui prennent en considération la polydispersité.

Le potentiel zêta : Ce paramètre reflète la charge à la surface des gouttelettes. L'amplitude et le signe de la charge des gouttelettes proviennent des propriétés de l'émulsifiant et du pH. Le potentiel zêta détermine souvent la nature des l'interaction entre les gouttelettes. Des forces de répulsion électrostatiques se produisent entre les gouttelettes avec le même signe de charge de surface, ce qui réduira le risque de floculation des particules et inhibera la propagation de la plupart des voies d'instabilité de l'émulsion décrites précédemment. En revanche, des forces d'attraction électrostatiques apparaissent lorsque des particules de charges opposées entrent en contact et, selon l'amplitude de la force d'attraction, le phénomène d'instabilité des gouttelettes peut résulter selon différents mécanismes. Par exemple, les gouttelettes ont tendance à se rassembler les unes près des autres mais ne fusionnent pas physiquement en une particule plus grande lorsque l'amplitude de la force d'attraction est faible. Cependant, la coalescence se produit lorsque les forces d'attraction sont fortes, conduisant à l'apparition d'une plus grosse gouttelette au détriment des plus petites (Hu *et al.*, 2017).

4.2.2. Liposome

4.2.2.1. Généralités

Les liposomes sont des vésicules sphériques composées d'une ou plusieurs couches lipidiques le plus souvent de nature phospholipidique. Ils sont capables d'encapsuler une molécule active hydrosoluble, liposoluble ou amphiphile, de la vectoriser et de maîtriser sa libération en fonction des conditions environnementales (**Figure 4.6**). Lorsque les molécules encapsulées sont des protéines, on les appelle protéoliposomes (Gulati *et al.*, 1998).

Les liposomes sont composés majoritairement de phospholipides et de cholestérol (permet d'améliorer la fluidité de la bicouche lipidique). La disposition des phospholipides est semblable à celle de la membrane cellulaire mais sous forme de vésicule renfermant un volume aqueux au centre ce qui permet de piéger des molécules hydrophiles (**Figure 4.6**). Ceci implique la facilité avec laquelle ces vésicules peuvent transporter et libérer des composés bioactifs dans les cellules humaines d'où le grand intérêt de leur usage dans la délivrance médicamenteuse.

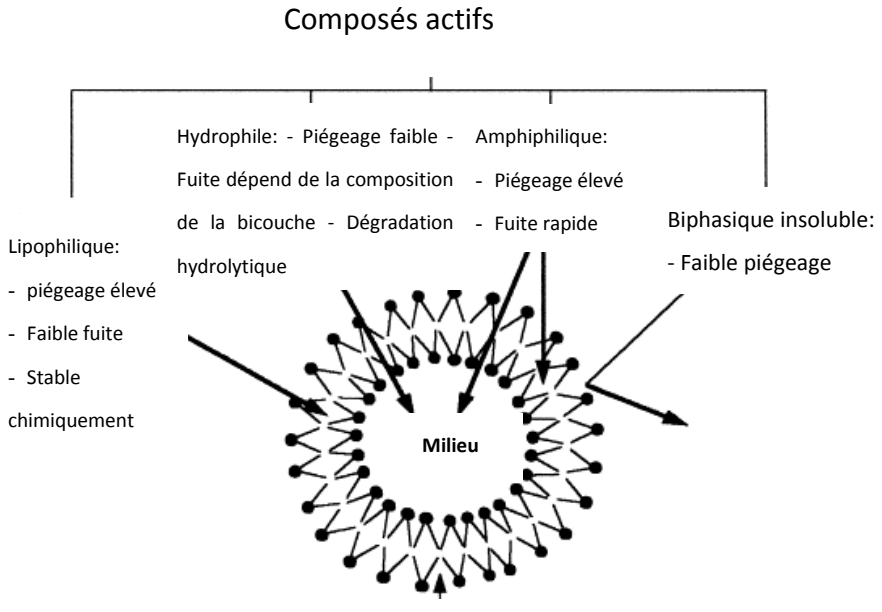

Figure 4.6. Encapsulation des composés bioactifs dans le liposome selon leur nature (Gulati *et al.*, 1998)

La queue hydrophobe permet d'assurer l'étanchéité des liposomes dans des milieux aqueux et aussi de piéger des composés lipophiles. Ce système est un support idéal pour des applications dans différents domaines, y compris l'alimentaire, les cosmétiques, l'agriculture et les produits pharmaceutiques. Cependant, malgré leurs propriétés intéressantes, les liposomes sont très peu utilisés à grande échelle en alimentaire en

raison de leur faible stabilité physique dans les conditions environnantes de nombreux produits alimentaires. De plus, ils ont généralement une faible efficacité d'encapsulation pour les substances bioactives hydrophiles, car une fraction assez grande reste en dehors des vésicules pendant le processus de formation (McClements, 2015). Aussi, l'interaction avec la matrice alimentaire au cours de la chaîne de vie du produit est peu connue. En effet, l'interaction avec les autres composés du milieu est aussi un paramètre important pour déterminer la stabilité du liposome dans la matrice alimentaire. Elle peut être prédite par la caractérisation des propriétés interfaciales (rhéologie, hydrophobicité et charge de surface).

La stabilité des liposomes dépend de la nature de (1) la molécule encapsulée, sa composition et sa concentration, (2) de la composition et la concentration en lipide et aussi (3) des interactions entre le composé actif et la bicouche lipidique. Le liposome peut être composé d'une ou plusieurs couches sphériques d'une épaisseur de 4 nm. Les liposomes sont classés selon la taille des vésicules et selon le nombre de bicouches (**Tableau 4.1**). Ces deux paramètres définissent l'efficacité d'encapsulation et la demi-vie des liposomes (Akbarzadeh *et al.*, 2013).

Tableau 4.1. Classification des liposomes (Akbarzadeh *et al.*, 2013, McClements, 2015)

Classes	**Caractéristiques**
Vésicule multi lamellaire MLV	ont une structure en oignon (> 400 nm)
Vésicule uni lamellaire	vésicules uni lamellaire large LUV (80 nm)
	petites vésicules uni lamellaire SUV (20 à 80 nm)
	liposomes unilamellaires géants GUV (> 1 µm)
Vésicules multi-vésiculaires (MVV)	liposomes à plusieurs vésicules non concentriques encapsulées dans de grandes vésicules uniques (1 µm)

Le passage de MVV ou MLV vers SUV et LUV nécessite l'application de l'énergie à ces structures phospholipidiques qui va leur permettre de se refermer sur elles-mêmes formant une vésicule (liposome) ayant moins de bicouches et de taille plus faible. Pendant ce processus, le piégeage des solutés présents dans le milieu aqueux survient (McClements, 2015).

4.2.2.2. Préparation

La préparation du liposome se fait généralement par dispersion par solvant où la phase lipidique est solubilisée dans un solvant organique. Le solvant est évaporé et un film lipidique est formé appelé pro-liposome. Les liposomes sont formés une fois ce film lipidique est réhydraté avec un solvant aqueux contenant les molécules à encapsuler (McClements, 2015). L'utilisation des solvants organiques dans cette méthode rend la sécurité de son application en agroalimentaire douteuse. Pour une application alimentaire, il est possible de faire la dispersion mécanique des phospholipides naturels tels que les lécithines qui sont capables de former des vésicules sans ayant recours aux solvants

organiques. A la différence de l'émulsion conventionnelle, pour préparer des liposomes le ratio lécithine : huile doit être supérieure à 1.

Les liposomes sont formés principalement par des phospholipides. La plupart des phospholipides naturels sont capables en milieu aqueux de former des liposomes. Selon le type de phospholipides utilisé les propriétés du liposome formé peuvent changer. En fait, les lipides peuvent modifier de conformation, de biodistribution, de charge de surface, de perméabilité et de libération des formulations liposomales. Le type des phospholipidiques influe également l'efficacité d'encapsulation, la toxicité et la stabilité des liposomes. On trouve 5 types de phospholipides : Phosphatidylcholine (PC), phosphatidyléthanolamine (PE), phosphatidylsérine (PS), phosphatidyleglycérol (PG) et l'acide phosphatidique (PA). Pour chaque type il existe plusieurs sous types dépendamment de la nature de la chaîne d'acide gras.

La plupart des phospholipides naturels ont des chaînes d'acides gras asymétriques. La présence de doubles liaisons dans les chaînes d'acide gras engendre un changement de conformation (**Figure 4.7**). Ce genre de conformation peut être intéressant dans le cas d'encapsulation de composés lipophiles ou ces derniers peuvent être piégés entre les chaînes d'acide gras.

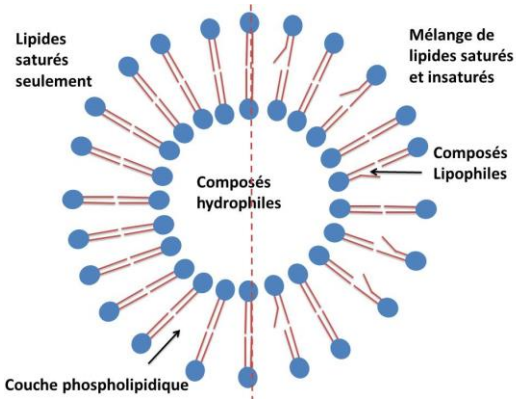

Figure 4.7. Bicouche de phospholipides selon le niveau de saturation des chaînes d'acides gras.

Comme mentionné précédemment, la lécithine est très riche en phospholipides et pourra être utilisée dans le domaine alimentaire. Cependant, la composition et les propriétés de la lécithine varient énormément selon son origine (Soja, maïs, tournesol, colza, œuf, cerveau bovin…). Par exemple, la lécithine du jaune d'œuf contient une grande quantité de PC et son niveau de saturation est supérieur à celui des lécithines de soja, d'où les liposomes préparés à base de cette lécithine ont une meilleure stabilité à l'oxydation (Nara *et al.*, 1997). Cependant, à cause du prix dispendieux de la lécithine des jaunes d'œuf, elle est très peu utilisée à l'échelle industrielle.

4.2.2.3. Application dans les aliments

L'une des premières applications des liposomes dans les produits alimentaires était dans la fabrication des fromages, en ajoutant les protéinases encapsulées dans les liposomes aux mélanges de fromages. Ceci a permis d'améliorer la stabilité, l'activité et la libération des enzymes, d'améliorer la saveur du fromage et réduire le coût de production (Magee *et al.*, 1981, Thompson, 2003). Dong-Hoon Lee *et al.* (2000) ont encapsulé l'enzyme bromélaïne (utilisée comme attendrisseur de viande) dans les liposomes et ils ont également constaté une meilleure stabilité et biodisponibilité de l'enzyme. Matsuzaki *et al.* (1989) et Rao *et al.* (1994) ont utilisé la β-galactosidase piégée dans les liposomes afin de faciliter la digestion des produits laitiers par les personnes intolérantes au lactose ce qui permis de stabiliser l'enzyme pendant le stockage.

Les vitamines ont également été encapsulées dans des liposomes pour améliorer leur rétention telle que vitamines A et E, et les carotènes ce qui a permis de prolonger la demi-vie des antioxydants piégés et de faciliter leur absorption intracellulaire (Tesoriere *et al.*, 1996). Les liposomes ont également été utilisés pour augmenter la qualité nutritionnelle des produits laitiers en piégeant la vitamine D dans la crème et le fromage ce qui a permis de protéger les vitamines de la dégradation (Banville *et al.*, 2000).

Une autre application intéressante est l'encapsulation des conservateurs alimentaires dans des liposomes afin de contrôler les altérations du fromage. Le lysozyme, par exemple, encapsulé dans des liposomes a été utilisé dans les fromages pour remplacer les nitrites (Thompson, 2003). Un autre groupe de chercheurs a encapsulé les antimicrobiens pediocine et nisine Z dans les liposomes et a constaté que l'encapsulation dans des liposomes permet de réduire ou éliminer les interactions indésirables dans l'aliment et d'augmenter sa durée de conservation (Benech *et al.*, 2003, Degnan *et al.*, 1992, Verheul *et al.*, 1997).

4.2.3. Particules solides

Il est possible aussi d'encapsuler les composés hydrophiles en les piégeant à l'intérieur des particules lipidiques solidifiées et sont donc protégés de la phase aqueuse environnante. Elles sont généralement préparées à partir de triglycérides et les cires à haut point de fusion. Deux méthodes peuvent être utilisées: (1) les composés bioactifs hydrophiles peuvent être dispersés dans la phase lipidique sous forme de fines particules solides (exp: en mélangeant les molécules bioactives en poudre avec la phase huileuse liquide avant solidification). (2) les composés bioactifs hydrophiles peuvent être dissous dans l'eau en premier lieu, puis transformé en une émulsion w/o, qui est ensuite utilisée pour former des particules solides (McClements, 2015). La formation de particules solides s'est avéré efficace à maintenir l'intégrité et la bioactivité des protéines encapsulées dans le poly (lactic-co-glycolic acid) PLGA (Xie *et al.*, 2008). D'autres études ont confirmé cette observation et ont démontré aussi que la formation des particules solides permet d'éviter

la dégradation protéolytique et une libération prolongée des molécules incorporées (António J. Almeida *et al.*, 2007).

4.2.4. Complexes moléculaires

Les complexes moléculaires consistent à former des complexes entre les molécules bioactives et d'autres types de molécules, comme les protéines, les glucides ou les lipides. Par exemple, les polyphénols peuvent former des complexes avec diverses protéines et carbohydrates. Les cyclodextrines sont souvent utilisées pour former des complexes avec des molécules bioactives relativement hydrophobes (tels que les vitamines solubles dans l'huile, des caroténoïdes et des acides gras), mais ils peuvent également être utilisés pour d'autres molécules bioactives dispersibles dans l'eau (tels que les polyphénols). Ceci augmentera la viscosité et améliorera la stabilité thermique et le pouvoir antioxydant des extraits encapsulés (Kalogeropoulos *et al.*, 2010). Les cyclodextrines ont la capacité de protéger les composés actifs contre l'oxydation, les réactions induites par la lumière, la décomposition thermique, la perte due à la volatilité, la sublimation, etc. Ils peuvent éliminer ou réduire le goût indésirable / odeurs, contaminations microbiologiques, fibres / autres composants indésirables et hygroscopicité (Szente *et al.*, 2004).

La formation de complexes cyclodextrine-bioactifs dépend des caractéristiques de solubilité de la molécule biologiquement active à encapsuler. Les composants bioactifs dispersibles dans l'eau (tels que des polyphénols) peuvent être simplement dissous dans de l'eau et ensuite mélangés avec une solution de cyclodextrine.

4.2.5. Nanoparticules solides lipides

Elles consistent en des nanoémulsions cristallisées avec la phase dispersée étant constituée d'un mélange de support lipidique sous forme solide et d'ingrédients bioactifs (**Figure 4.8**)(Weiss *et al.*, 2008). Trois principales techniques de préparation sont possibles : (1) Homogénéisation du mélange en chauffant (fonte), (2) Microémulsification du mélange en chauffant (fonte) et (3) Homogénéisation du mélange à froid.

Cette méthode d'encapsulation a l'avantage d'avoir une grande efficacité d'encapsulation et une facilité de production à grande échelle. Cependant, il y a un risque de recristallisation et possibilité d'explosion. Il est important de noter que la libération pour les particules solides est plus longue en raison de l'augmentation du temps de dégradation de la matrice solide qui permet une plus grande protection contre les réactions chimiques telles que l'oxydation (Fathi *et al.*, 2012b).

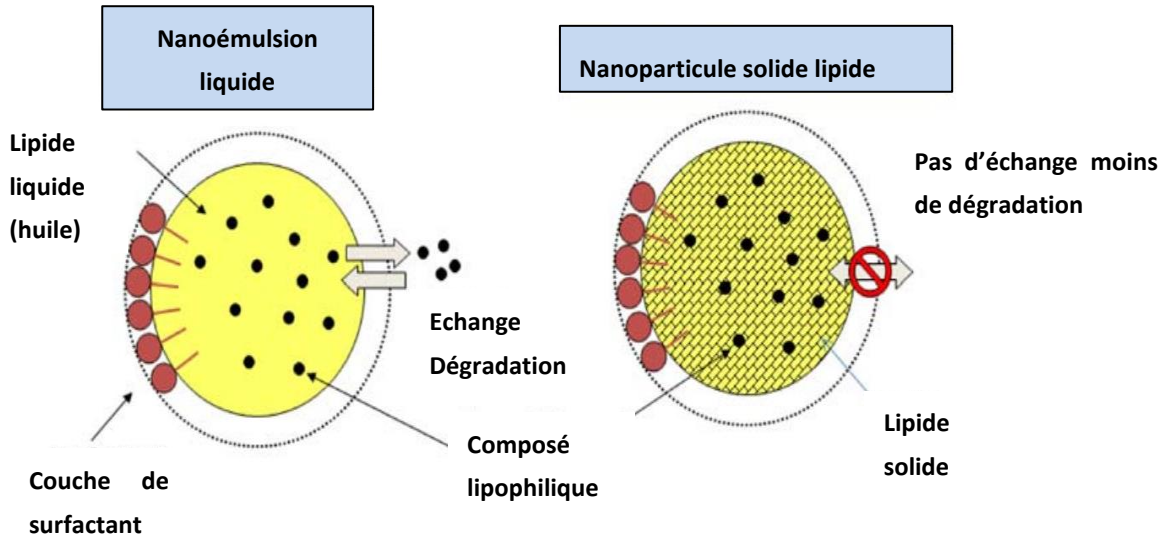

Figure 4.8. Différence entre nano émulsion liquide et solide (Weiss *et al.*, 2008)

4.3. LIBÉRATION CONTROLÉE

L'encapsulation permet également une libération plus contrôlée des composés bioactifs. Selon (Fathi *et al.*, 2012b), il existe deux types de libération : (1) une libération prolongée qui est plus intéressante pour libérer, par exemple, les antimicrobiens d'une façon stable pendant un grand laps de temps ce qui permet d'augmenter la durée de conservation des aliments et (2) une libération retardée qui est utilisée souvent pour les aliments nutraceutiques et dans le domaine pharmaceutique. Selon Pothakamury *et al.* (1995), l'avantage de contrôler le taux de libération des composés actifs sur une période prolongée, permet d'éviter la perte des vitamines et des minéraux au cours du traitement et de la cuisson et séparer les composés incompatibles et réactifs. McClements (2015) distingue la libération des composés hydrophiles de ceux lipophiles. Le choix de la forme d'encapsulation (poudre, gel…) est important et il dépend aussi de la nature des composes bioactifs à encapsuler plus précisément leur nature hydrophile ou lipophile.

4.3.1. Libération des composés hydrophiles

La cinétique de libération des composés hydrophiles dépend de la forme des sphères formées : homogènes, poreuses et enrobées (**Figure 4.9**).

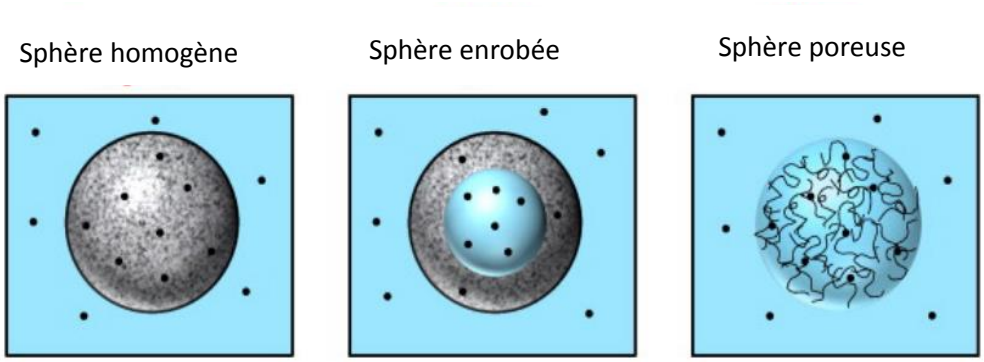

Figure 4.9 : Libération de composants bioactifs de différents types de particules colloïdales (McClements, 2015)

Lorsque les sphères sont homogènes, la libération dépend de la taille des molécules bioactives et de leur coefficient de diffusion. Plus la taille des particules est faible, plus le coefficient de diffusion est élevé et la libération est accélérée (McClements, 2015).

Pour retarder la libération des composés bioactifs, il est possible d'enrober les sphères avec un matériel imperméable comme un polymère hydrophobe ou avec du gras solide. Ceci engendre la diminution de la perméabilité de la coquille (exp : baisse du coefficient de diffusion de la coquille, diminution du coefficient de partage de la coquille-noyau, ou augmentation de l'épaisseur de la coquille) (McClements, 2015).

Lorsque les sphères polymériques sont poreuses, la libération des molécules bioactives est plus lente avec la diminution de la taille des pores et l'augmentation de la taille de la molécule bioactive. Ainsi, pour piéger des agents bioactifs hydrophiles à l'intérieur des particules poreuses, il est important de veiller à ce que la taille des pores soit inférieure à la taille des molécules bioactives (McClements, 2015). Les particules poreuses peuvent être conçues pour gonfler ou rétrécir en réponse à un déclencheur environnemental (comme le pH, la force ionique, ou de la température), ce qui conduira à des changements dans la taille des pores et donc la vitesse de libération. Ce phénomène peut être utilisé pour concevoir des systèmes de délivrance qui libèrent un composant hydrophile en réponse à un déclencheur environnemental spécifique.

4.3.2. Libération des composés lipophiles

La libération des composés lipidiques se fait le plus souvent par diffusion selon la loi de Fick. Deux phases caractérisent la diffusion des composés lipophiles: (1) éclatement initial suivi (2) d'une libération plus lente ultérieure (Hosseini *et al.*, 2013, Luo *et al.*, 2011).

Luo *et al.* (2011) dans leur étude sur la libération du tocophérol à partir du complexe zéine-chitosane, ont observé que la libération du tocophérol est réduite avec la concentration en zéine. Aussi, la libération du tocophérol du complexe zéine- chitosane est plus lente que celle de la zéine seule. D'autres travaux sur la libération des huiles essentielles ont démontré que la libération de l'huile essentielle de l'origan à travers le chitosane dépend fortement du ratio chitosane : huile essentielle (Hosseini *et al.*, 2013).

4.4. TECHNOLOGIES D'ENCAPSULATION APPLIQUÉES

La plupart des formes d'encapsulation décrites dans la section 4.2 nécessite l'application d'une énergie. Plusieurs techniques ont été développées pour répondre à ce besoin et diverses approches ont été développées y compris : sublimation, électrospray, fluide supercritique, émulsification / évaporation du solvant, ultrasonication et homogénéisation à haute pression (**Tableau 4.2**).

Le choix des techniques dépend de (a) la capacité de chacune à contrôler la taille des particules et la morphologie; b) les propriétés des composés à encapsuler (thermosensibilité); c) l'efficacité d'encapsulation et d) le rendement.

Tableau 4.2. Comparaison entre les différentes technologies d'encapsulation

	Forme	**Avantages**	**Inconvénients**	**Exemple d'utilisation**	**Référence**
Spray drying	Poudre	Possibilité de récupération d'une émulsion ou suspension encapsulant les composés bioactifs	consommation d'énergie Rendement faible à cause des pertes	Encapsulation d'huiles et d'arômes	(Martín *et al.*, 2010)
Sonication	liquide	nettoyage plus facile Taille de 150–700 nm	contamination par les métaux de pointe de la sonde et le titane Perte d'activité	Préparation de nanoémulsions	(Jafari *et al.*, 2006) (Salvia-Trujillo *et al.*, 2014)
Microfluidisation	Liquide	Taille de 30–700 nm PDI faible	coût élevé (énergie) Nettoyage difficile	Préparation de nanoémulsions	(Jafari *et al.*, 2006) (Salvia-Trujillo *et al.*, 2014)
CO_2 supercritique	liposome	diamètre de 150 nm	Installation complexe	Préparation de liposomes	(Fages *et al.*, 2003, Zhao *et al.*, 2015)

4.4.1. Spray drying

Le spray drying ou le séchage par atomisation est une technique développée dans les années 1950 et à partir des années 1990 elle a été utilisée pour objectif d'encapsulation. Cette technique permet d'encapsuler les molécules bioactives sous forme de poudre. Elle utilise de l'air chauffé qui percute les particules de liquide finement pulvérisées. L'évaporation de l'eau libre donne à la sortie une poudre fine encapsulant les molécules d'intérêt. Cette technique a été utilsée dans l'encapsulation d'huile essentielle de romarin et l'huile de lin (Carneiro *et al.*, 2013, de Barros Fernandes *et al.*, 2014). Pour, les

molécules hydrophobes, une émulsion est préparée avant son passage à l'appareil. Cette technique a l'inconvénient de perte élevée de poudre dans le flux d'air sortant et aussi une consommation élevée d'énergie lors du procédé (Mujumdar, 2014).

4.4.2. Microfluidisation

Le microfluidisateur est composé principalement de 3 compartiments : le réservoir, la chambre d'interaction et la chambre de refroidissement. Brièvement, le fluide à traiter passe du réservoir vers la chambre d'interaction, sous une haute pression à des vitesses élevées à travers des microcanaux produisant des grandes forces de cisaillement. Cette force appliquée génère des forces disruptives permettant de former de fines gouttelettes. A cause de la haute pression (allant jusqu'à 25,000 psi), le fluide traité est chauffé. Donc, il traversera un circuit de refroidissement. Cette technique a les avantages de (1) produire une plus petite taille de particule avec une distribution plus étroite, (2) facile à extrapoler à grande échelle (3) reproductible. Cependant, le microfluidisateur est plus difficile à nettoyer et il consomme beaucoup d'énergie. Des études antérieures ont également montré que la préparation des nanoémulsions d'huiles essentielles de lemongrass par microfluidisation améliore l'activité anti-microbienne proportionnellement au nombre de cycles (Hossain *et al.*, 2019, Mujumdar, 2014, Salvia-Trujillo *et al.*, 2014).

4.4.3. Sonication

La sonication est la technologie la plus utilisée dans la préparation des liposomes nanométriques (entre 20 et 80 nm). Elle peut être sous forme de sonde ou de bain. L'usage de la sonde utilise une énergie élevée permettant de chauffer la solution. Un traitement de sonication avec la sonde pendant 1 h permet d'estérifier plus de 5 % des lipides (Swain *et al.*, 2016). Un bain d'eau ou de glace est alors suggéré. Par contre, avec la sonication en bain c'est plus facile de contrôler la température et aussi de protéger les différents compartiments de la sonde.

Le principe de fonctionnement du sonicateur consiste à la transformation de l'énergie électrique en vibration mécanique. La vibration est amplifiée et transmise sur toute la longueur de la sonde où la pointe se dilate et se contracte longitudinalement. La distance du déplacement de la pointe dépend de l'amplitude sélectionnée par l'utilisateur. En augmentant l'amplitude, l'intensité de la sonication augmente dans l'échantillon. En milieu liquide, la vibration rapide de la pointe provoque la cavitation, la formation et l'effondrement violent de bulles microscopiques. L'effondrement de milliers de bulles de cavitation libère une énergie considérable dans le domaine de la cavitation. L'effet de l'érosion et le choc de l'effondrement de la bulle de cavitation est le principal mécanisme de traitement de fluide (Sivakumar *et al.*, 2014). Le diamètre de la pointe de la sonde devrait être choisi en fonction de la quantité d'échantillons. Une pointe à petit diamètre offre une sonication de haute intensité, mais l'énergie se concentre dans une petite zone concentrée. Cependant, à des diamètres de pointe plus grands, de plus grands volumes

peuvent être traités, mais avec une intensité plus faible. Le choix de la sonde dépend aussi de la viscosité de l'échantillon. La puissance de sonication est mesurée en watts. L'amplitude est une mesure de l'excursion de la pointe de la sonde. Il est préférable d'utiliser un récipient étroit. L'énergie ultrasonore est générée à partir de la pointe et est dirigée vers le bas. Quand un échantillon est traité, le liquide est poussé vers le bas et dans toutes les directions. Si le récipient est trop large, il ne sera pas mélangé efficacement et une partie de l'échantillon demeurera non traitée à la périphérie (Sivakumar *et al.*, 2014).

Plusieurs inconvénients sont reliés à cette technique: le très faible volume interne des vésicules du liposome ce qui affecte l'efficacité d'encapsulation, la possible dégradation des phospholipides et des composés à encapsuler, l'élimination des grosses molécules, la contamination par les métaux de pointe de la sonde et le titane (Akbarzadeh *et al.*, 2013). De plus, la sonication engendre une perte plus importante en terme d'activité antimicrobienne contre *E. coli* dépendamment de l'amplitude appliquée. Un traitement à 100 µm pendant 3 minutes engendre une perte totale d'activité bactéricide de l'huile essentielle de lemongrass contrairement à la microfluidisation où l'activité antimicrobienne est maintenue (Salvia-Trujillo *et al.*, 2014).

CHAPITRE 5 : OBJECTIFS, HYPOTHÈSES, ET MÉTHODOLOGIE DU PROJET

5.1. OBJECTIFS GÉNÉRAUX

L'objectif de ce projet de thèse est de développer des formulations antimicrobiennes à base de produits naturels essentiellement fournis par Biosecur lab en combinaison avec d'autres extraits naturels agissant en synergie et efficaces à réduire les pathogènes des légumes prêts-à-manger et du pain.

Afin d'assurer une meilleure biodisponibilité et d'améliorer l'activité antimicrobienne, le second objectif de cette thèse est de développer des méthodes de stabilisation des formulations antimicrobiennes (nanoémulsion, nanoliposomes et immobilisation dans un polymère) et de caractériser leur efficacité lorsqu'ils sont appliqués *in situ* sur la matrice alimentaire.

5.2. HYPOTHÈSES

- Les antimicrobiens naturels peuvent avoir une efficacité antimicrobienne similaire à celle des antimicrobiens chimiques et peuvent ainsi être utilisés comme conservateurs des produits alimentaires.
- Il y a une amélioration de l'activité antimicrobienne et un meilleur contrôle de la libération des antimicrobiens naturels lorsqu'ils sont stabilisés sous la forme de nanoémulsion/ nanoliposomes ou émulsion gélifiée.
- Les antimicrobiens naturels encapsulés dans des capsules n'ont pas d'impact négatif sur les propriétés sensorielles et physicochimiques de l'aliment.

5.3. OBJECTIFS SPÉCIFIQUES

- Cribler les antimicrobiens naturels selon leur pouvoir antimicrobien contre les microorganismes susceptibles de contaminer le pain et les carottes précoupées et évaluer une potentielle synergie lorsqu'ils sont utilisés en combinaison.
- Caractériser les antimicrobiens efficaces à contrôler les microorganismes sélectionnés.
- Développer une nanoémulsion stable en sélectionnant les émulsifiants et leurs concentrations et en optimisant les paramètres de sonication et de microfluidisation.
- Développer une émulsion gélifiée en sélectionnant un biopolymère capable de protéger l'activité antimicrobienne et de contrôler la libération des composés actifs.
- Développer des nanoliposomes avec une grande efficacité d'encapsulation et une bonne stabilité.
- Caractériser les différentes capsules développées *in vitro* pour leur stabilité et efficacité.

- Évaluer l'efficacité des différentes formes d'encapsulation *in situ* sur les modèles alimentaires et vérifier l'impact de leur application sur les propriétés sensorielles et physicochimiques de l'aliment (texture, couleur, etc.).

5.4. MÉTHODOLOGIE

- Le développement de la formulation antimicrobienne sera effectué par un criblage de différents antimicrobiens principalement d'origine végétale en se basant sur la détermination du diamètre d'inhibition, la concentration minimale inhibitrice, bactéricide et fongicide contre les pathogènes cibles et l'évaluation d'une potentielle synergie lorsqu'ils sont utilisés en combinaison selon la méthode de Yosra Ben-Fadhel *et al.* (2017).
- La caractérisation des composantes des antimicrobiens les plus efficaces (polyphénols totaux, flavonoïdes totaux, pouvoir anti radicalaire et antioxydant) sera effectuée selon les méthodes de Dewanto *et al.* (2002) et Fattouch *et al.* (2007).
- La stabilisation de la nanoémulsion sera effectuée en sélectionnant le ratio hydrophile/lipophile requis (HLB), le ratio huile : émulsifiant et les paramètres optimaux de la sonication et microfluidisation et ceci selon la méthode de Maherani *et al.* (2018).
- La stabilisation de la formulation antimicrobienne dans un biopolymère et la caractérisation des capsules formées sera réalisée selon la méthode Ong *et al.* (2015) et Severino *et al.* (2014b).
- L'optimisation des paramètres de la préparation du nanoliposome sera réalisée selon la méthode de Rafiee *et al.* (2017). La caractérisation des nanoliposomes sera réalisée selon la méthode de Cury *et al.* (2015).
- La caractérisation des microcapsules / nanoliposomes sera basée sur la mesure de la taille des particules et la stabilité selon la méthode de Maherani *et al.* (2018). L'évaluation des propriétés thermiques, structurales et antimicrobiennes et antioxydantes sera réalisée selon la méthode de Cury *et al.* (2015), S. Salmieri *et al.* (2015) et Maherani *et al.* (2018) respectivement. La libération contrôlée sera réalisée selon la méthode de Paula *et al.* (2010) et Sessa *et al.* (2014).
- L'évaluation de la capacité antimicrobienne *in situ* des microcapsules et nanoliposomes sera réalisée selon la méthode de Yosra Ben-Fadhel *et al.* (2017).

CHAPITRE 6: ANTIMICROBIAL PROPERTIES OF ENCAPSULATED ANTIMICROBIAL NATURAL PLANT PRODUCTS FOR READY-TO-EAT CARROTS

Propriétés antimicrobiennes d'antimicrobiens naturels d'origine végétale encapsulés pour application sur des carottes prêtes-à-manger

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Le nombre de figures et de tableaux et le style des références ont été présentés selon le guide des auteurs du journal.

CONTRIBUTIONS DES AUTEURS

Ce travail de recherche a été réalisé sous la supervision du Pr. Monique Lacroix. Toutes les expériences ont été réalisées en laboratoire par Yosra Ben Fadhel avec l'aide de Mélinda Aragones et les discussions sur les résultats et les protocoles ont été assistés par Stéphane Salmieri. L'article a été écrit par Yosra Ben Fadhel, tandis que les corrections et révisions ont été effectuées par Stéphane Salmieri, Dr Behnoush Maherani, et Pr. Monique Lacroix.

RÉSUMÉ

L'activité antimicrobienne des antimicrobiens naturels (extraits de fruits, huiles essentielles et dérivés) a été évaluée contre six espèces bactériennes (*E. coli* O157: H7, *L. monocytogenes*, *S*. Typhimurium, *B. subtilis*, *E. faecium* et *S. aureus*), deux moisissures (*A. flavus* et *P. chrysogenum*) et une levure (*C. albicans*) par la méthode de diffusion sur disque. Ensuite, les composés antimicrobiens ayant une grande capacité inhibitrice ont été évalués pour déterminer leur concentration minimale inhibitrice, bactéricide et fongicide (CMI, CMB et CMF respectivement). La teneur en phénols totaux et en flavonoïdes, l'activité antiradicalaire et le pouvoir antioxydant des composés sélectionnés ont également été évalués. Sur la base d'essais *in vitro*, cinq composés antimicrobiens ont été sélectionnés pour leur concentration efficace la plus faible. Les résultats ont montré que la plupart de ces composés antimicrobiens présentaient une concentration élevée en phénols totaux et en flavonoïdes et une bonne activité anti-oxydante et anti-radicalaire. Une étude *in situ* a montré que le mélange d'antimicrobiens naturels, appliqué à la surface de la carotte coupée, réduisait de manière significative le dénombrement de la flore mésophile aérobie totale (FMT) initiale, des moisissures et de levures et permettait de prolonger de deux jours la durée de conservation des carottes par rapport au contrôle. Cependant, le traitement chimique (mélange d'acide peroxyacétique et de peroxyde d'hydrogène) a révélé une activité antifongique et une légère réduction de la FMT.

ABSTRACT

The antimicrobial activity of natural antimicrobials (fruit extracts, essential oils and derivates), was assessed against six bacteria species (*E. coli* O157:H7, *L. monocytogenes*, *S*. Typhimurium, *B. subtilis*, *E. faecium* and *S. aureus*), two molds (*A. flavus* and *P. chrysogenum*) and a yeast (*C. albicans*) using disk diffusion method. Then, the antimicrobial compounds having high inhibitory capacity were evaluated for the determination of their minimum inhibitory, bactericidal and fungicidal concentration (MIC, MBC and MFC respectively). Total phenols and flavonoids content, radical scavenging activity and ferric reducing antioxidant power of selected compounds were also evaluated. Based on *in vitro* assays, five antimicrobial compounds were selected for their lowest effective concentration. Results showed that, most of these antimicrobial compounds had a high concentration of total phenols and flavonoids and a good anti-oxidant and anti-radical activity. *In situ* study showed that natural antimicrobials mix, applied on the carrot surface, reduced significantly the count of the initial mesophilic total flora (TMF), molds and yeasts and allowed an extension of the shelf-life of carrots by two days as compared to the control. However, the chemical treatment (mix of peroxyacetic acid and hydrogen peroxide) showed antifungal activity and a slight reduction of TMF.

Keywords: natural antimicrobials; encapsulation; shelf-life; microbiological quality

1. Screening of natural antimicrobial extracts

Disk diffusion method : selection of 5 extracts

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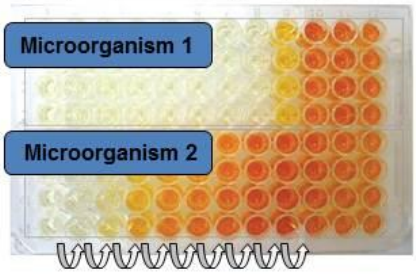


Serial dilutions

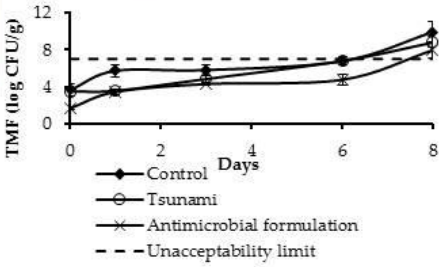
Determination of MIC/MBC/MFC

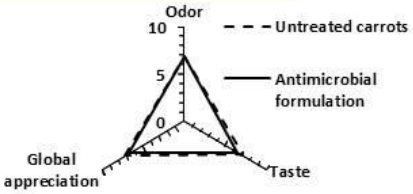
2. Characterization

	Total phenols (mg gallic acid/ g of AM)	Total flavonoids (mg catechin /g of AM)
Biosecur F440D	4.38 ± 0.16 [a]	1.26 ± 0.06 [a]
Pan tropical EO	34.62 ± 3.68 [b]	17.63 ± 1.40 [b]
Mediterranean EO	220.57± 17.67 [c]	34.75 ± 2.4 [c]
Asian EO	1.41 ± 0.18 [a]	0.56 ± 0.07 [a]
Citrus EO	1.51 ± 0.03 [a]	0.06 ± 0.03 [a]

	FRAP Eq µM of ascorbic acid/g of AM	Radical Scavenging Activity mM trolox
Biosecur F440D	0.30±0.04 [ab]	0.28 ±0.05 [d]
Pan tropical EO	0.43±0.02 [b]	0.15 ±0.02 [c]
Mediterranean EO	0.76±0.03 [c]	0.18 ±0.03 [c]
Asian EO	0.04±0.00 [a]	0.02 ±0.00 [a]
Citrus EO	0.03±0.00 [a]	0.07±0.01 [b]

3. *In situ* application on pre-cut carrots





Sensory evaluation

1. Introduction

Plants, spices, fruits and vegetable extracts have been exploited since antiquity for their aromas, coloring ability, antioxidant and antimicrobial properties (Mahendra Rai, 2011). However, at the beginning of the 19th century, a rapid rise of the use of chemical additives has been observed. Among the chemical additives used in food, nitrites, sulfide dioxide, sulfites, parabens, peroxyacetic acid and hydrogen peroxide are the best known. However, these additives are controversial as many have shown potential health risks, mainly carcinogenic effects, irritation and the appearance of resistant strains (Gagnaire *et al.*, 2002, Mahendra Rai, 2011). There is, therefore, a growing interest in identifying natural antimicrobial extracts which have the advantage of being effective with much less toxic and less allergenic effects. Natural antimicrobial extracts have demonstrated various antiviral, antifungal, antibacterial, anti-parasitic, antioxidant, and even insecticidal activities (Hashemi *et al.*, 2017, Nollet *et al.*, 2017). For example, it was demonstrated that garlic juice and tea extract could inhibit bacteria even those resistant to antibiotics, such as ciprofloxacin, methicillin and vancomycin (Yee-Lean Lee *et al.*, 2003b). In addition to their antimicrobial properties, natural antimicrobials often have functional properties already used as anticancer, radioprotective and hypoglycemic (Mahendra Rai, 2011). For example, it was observed that lime juice extract can inhibit the growth of pancreatic cancer cells (Jaiprakash R Patil *et al.*, 2009). Antioxidant properties have also been reported for certain plant extracts like garlic and onion. Antioxidant properties can help in the prevention of meat discoloration, the preservation of vitamin content (B_1 and B_2) and the prevention of lipid oxidation (Yosra Ben-Fadhel *et al.*, 2016). Some of the active compounds present in plants, herbs, spices, fruits and vegetables are known as secondary metabolites. The main groups of compounds responsible for the antimicrobial activity of plants extracts include phenols (phenolic acids, flavonoids: i.e., flavonols, tannins), quinones, saponins, coumarins, terpenoids and alkaloids (Cowan, 1999). Natural extracts under the form of essential oils are rich in flavonoids, terpenes, terpenoids and aromatic and aliphatic constituents and could be obtained by hydro or steam distillation, solvent extraction, ultrasound, microwave, ohmic heating, supercritical CO_2 extraction or pulsed electric field (Hashemi *et al.*, 2017). Most of their active compounds are found in leaf extract (i.e., rosemary, sage), flowers and flower buds (i.e., cloves), bulbs (i.e., garlic, onion), rhizomes (i.e., asafetida) and fruits (i.e., pepper)(Tiwari *et al.*, 2009). Depending on plant type and bacterial strain, essential oil derivatives could have a high antibacterial activity. Bertoli *et al.* (2011) reported that 60 % of plant essential oils have antifungal activity. Their mode of action on microorganisms has been the object of several studies and demonstrated that essential oils, due to their hydrophobic nature, are able to react with the lipid layer of the bacterial cell membrane, thereby increasing the permeability of membranes inducing leakage of ions and cell contents, lysis and death of bacteria (Samia Ayari *et al.*, 2009). Their efficiency against several bacteria, molds and yeasts made of the essential oils a good candidate for food industry to insure food safety. Unfortunately, their use in food industry is restricted by a low dose due to their strong sensorial impact and toxicity (Baser *et al.*, 2015, Tisserand *et al.*, 2013). On the other hand, the hydrophobic nature of essential oils affects their homogeneity and bioavailability on the food surface. Their encapsulation in a more suitable matrix could help to avoid this inconvenient and can prevent volatilization and oxidation of their active compounds. Moreover, encapsulation could mask the strong aroma and prevent the degradation of the active compounds (MIS Santos *et al.*, 2017).

Carrots have been implicated in several outbreaks in England and Wales during 1992–2005, in the United States during 1973–1997 (Jalava *et al.*, 2006, Sivapalasingam *et al.*, 2004) and in 2004 (Gaynor *et al.*, 2009). The most frequent pathogens involved in these outbreaks are *E. coli* O6 (strain that produced the heat-stable and heat labile toxins (O6: NM LT ST), VTEC, *Yersinia pseudotuberculosis* which caused gastrointestinal illness and

erythema nodosum among schoolchildren in Finland and *Shigella sonnei* (Da Silva Felício *et al.*, 2015, Sivapalasingam *et al.*, 2004). Others studies demonstrated the possibility of growth of *Salmonella spp*. and *Listeria monocytogenes* on carrots (Ruiz-Cruz *et al.*, 2007). The fungal strains of *Alternaria, Rhizopus, Aspergillus, Stemphylium* and *Botrytis* were also found to contaminate carrots (Banwart, 2012, Tournas, 2005b). The mechanism of contamination of carrots remains not well known. Monaghan *et al.* (2016) reported inadequate hand hygiene in the field can transfer bacterial contamination to hand-harvested carrots. Direct contact with wildlife feces during storage and cross-contamination of the equipment during washing and peeling could also be contributing factors (Jalava *et al.*, 2006).

The main objective of this study was to assess the antimicrobial activities of 17 antimicrobial agents against nine different microorganisms (Gram negative, Gram positive, molds and yeast) that could affect food products in order to select the most efficient antimicrobial extracts. The total phenols and flavonoids content, the anti-radical and antioxidant activity were assessed for each selected extract. In this study, a strategy was developed in order to reduce the efficient dose of natural antimicrobial extracts by the development of formulation containing a mixture of natural extracts encapsulated in o/w emulsion which could act in synergy. Then, the antimicrobial efficiency of the antimicrobial-loaded emulsion was tested *in situ* onto pre-cut carrots. Finally, sensorial evaluation was done on the treated carrots.

2. Materials and Methods

2.1. Antimicrobial Extracts

Biosecur F440D (33–39%) was provided by Biosecur Lab, Inc (Mont St-Hilaire, Québec, Canada). Citral was provided from BSA, Inc. (BSA Ingredients s.e.c/l.p., Montreal, QC, Canada). Cranberry juice (*Vaccinium macrocarpon*) was provided by Atoka Cranberries, Inc. (Manseau, QC, Canada) and was stored at −80 °C until used. Fourteen essential oils from spices, fruits and plants were bought from Biolonreco, Inc. (Dorval, QC, Canada) and their main constituents are presented in Table 6.1. Biosecur F440D, citral and essential oils were stored at 4 °C.

Table 6.1. List of organic essential oils (EO) and their composition.

Common Name	Botanic Name	Part	Compositions (%) *
Bergamote EO	*Citrus bergamia*	Zest	Limonene (36.2), Linalyle acetate (29.7), linalool (13.2), γ-terpinene (6.8), β-pinene (5.4)
Pan tropical EO	*Cinnamomum verrum*	Peel	E-cinnamaldehyde (55.1), cinnamyl acetate (9.6), β-caryophyllene (4.0)
Citrus EO	*Cymbopogon winterianus*	Aerial part	Citronellal (35.4), geraniol (20.1), Citronellol (12.2), elemol (4.6), Limonene (3.0), citronellyl acetate (2.9), germacrene D (2.7), geranyl acetate de (2.5), linalool (0.6)
Ginger EO	*Zingiber officinalis*	Rhizome	α-zingiberene (25.4), β-sesquiphellandrene + α-curcumene (13.9), Camphene (10.5), β-phellandrene + 1, 8-cineole (8.3), β-bisabolene + β-selinene (7.7), E,E-α-farnesene (4.2), α-pinene (3.3)
Asian EO	*Cymbopogon flexuosus*	Herb	Geranial (39.1), neral (31.6), geraniol (6.7), geranyl acetate (3.7)

Marjolaine shells EO	*Origanum majorana*	Flower top	Terpinene-4-ol (28.0), γ-terpinene (15.5), α-terpinene (9.5), Cis-thuyanol (7.3), α-terpineol (3.7)
Peppermint EO	*Mentha x piperita*	Aerial part	Menthol (30.6), menthone (29.3), 1,8-cineole + β-phellandrene (5.2), menthyl acetate (4.5), neomenthol (3.1), Isomenthone (4.4), menthofurane (4.2), Limonene (2.4)
Myrte cineole EO	*Myrtus communis*	leaf	α-pinene (51.5), 1,8-cineole (23.9), Limonene (10.4), Linalool (3.0)
Sweet orange EO	*Citrus sinensis*	Zest	Limonene (94.8)
Tea tree EO	*Melaleuca alternifolia*	Leaf	Terpinene-4-ol (37.6), γ-terpinene (21.1), α-terpinene (10.1), Terpinolene (4.8), 1,8-cineole + β-phellandrene (4.2), α-pinene (2.6), α-terpineol (2.5)
Mediterranean EO	*Origanum compactum*	Flower top	Carvacrol (46.1), thymol (17.6), γ-terpinene+ trans-β-ocimene (14.8), p-cymene (8.5)
Thyme leaf savory EO	*Thymus satureioides*	Flower top	Borneol (27.0), α-terpineol (11.9), camphene (10.5), α-pinene + α-thuyene (6.5), β-caryophyllene (5.5), Carvacrol (5.3), p-cymene (3.9), Linalol (3.7), Terpinene-4-ol + methyl carvacrol ether (2.9), 1,8-cineole + β-phellandrene (2.9), Thymol (2.8)
Cloves EO	*Eugenia caryophyllus*	Floral button	Eugenol (81.8), Eugenyl acetate (12.9), β-caryophyllene (3.4)
Thyme thymol EO	*Thymus vulgaris CT6*	Flower top	Thymol (46.6), p-cymene (16.9), γ-terpinene (9.3), Linalool (4.1), Carvacrol (3.5)

* Composition was provided by Biolonreco, Inc. and was determined by CPG-SM Hewlett Packard /CPG- FID; Column: HP Innowax 60-0.5 -0.25; Carrier gas Helium: 22 psi.

2.2. Preparation of Bacterial Cultures

Six bacterial strains, four Gram positive: *Listeria monocytogenes* HPB 2812 (Health Canada, Health Products and Food Branch, Ottawa, Canada), *Staphylococcus aureus* ATCC 29213 (American Type Culture Collection, Rockville, MD, USA), *Enterococcus faecium* ATCC 19434 (American Type Culture Collection, Rockville, MD, USA) and *Bacillus subtilis* ATCC 23857 (INRS-Institut Armand-Frappier, Laval, QC, Canada), and two Gram negative: *Escherichia coli* O157:H7 (EDL 933, provided by Pr. Charles Dozois) and *Salmonella* Typhimurium SL 1344 (INRS-Institut Armand-Frappier, Laval, QC, Canada) were used as target bacteria in antimicrobial tests. *Aspergillus flavus* (INRS-Institut Armand-Frappier, Laval, QC, Canada) and *Penicillium chrysogenum* (INRS-Institut Armand-Frappier, Laval, QC, Canada) were used as fungal strains and *Candida albicans* ATCC10231 (INRS-Institut Armand-Frappier, Laval, QC, Canada) as yeast. All the bacteria were stored at −80 °C in Tryptic Soy Broth medium (TSB; BD, Franklin Lakes, NJ, USA) containing glycerol (20% *v*/*v*). Before each experiment, bacterial stock cultures were propagated through two consecutives 24 h growth cycles in TSB at 37 °C to reach the concentration of approximately 10^9 CFU/mL. The grown cultures were then diluted in sterile peptone water 0.1% (Alpha Biosciences, Inc., Baltimore, MD, USA) to obtain a working culture of approximately 10^6 CFU/mL.

For fungal evaluation, *A. flavus and P. chrysogenum* were propagated through 72 h growth cycle on potato dextrose agar (PDA, Difco, Becton Dickinson) at 28 °C ± 2 °C. Colonies were isolated from the agar media using sterile platinum loop, suspended in sterile peptone water, and filtrated through sterile cell strainer (Fisher scientific, Ottawa, ON, Canada). *C. albicans* was inoculated in potato dextrose broth (PDB, Difco, Becton Dickinson) for 24 h at 28 °C. The filtrate was adjusted to 10^6 CFU/mL using a microscope before dilution to reach approximately 10^6 CFU/mL for the disk diffusion agar and the minimum inhibitory, bactericidal and fungicidal concentration (MIC, MBC and MFC, respectively) determination (Yosra Ben-Fadhel *et al.*, 2017).

2.3. Preliminary Study

First, 100 µL of the tested microorganisms 10^6 CFU/mL were seeded on sterile Petri dishes containing Muller Hinton Agar (MHA, BD, Franklin Lakes, NJ, USA). Then, 5 µL of each pure antimicrobial compounds were deposited on the surface of a sterile 6-mm filter disk. A negative control was used by deposing 5 µL of sterile water on the surface of the disk. All plates were sealed with parafilm to avoid evaporation and incubated for 24 h at 37 °C for bacteria and for 48 h to 72 h at 28 °C for molds and yeasts followed by the measurement of the diameter zone of the inhibition expressed in mm. On the basis of the disk diffusion results, the most efficient antimicrobial compounds have been selected to determine their MIC, MBC and MFC, their total phenols and flavonoids content and their antioxidant and anti-radical properties and to evaluate the *in situ* antimicrobial efficiency of the mixture on pre-cut carrot surface.

2.4. Antimicrobial Efficiency

The minimum inhibitory concentration (MIC) and the minimum bactericidal and fungicidal concentration (MBC and MFC) were determined on the emulsion as an encapsulation form composed of essential oils 2.5% (*w*/*w*), tween 80 2.5% (*w*/*w*) and 95% (*w*/*w*) distilled water. The mixture was homogenized by vortex for 1 min and by Ultra-Turrax (IKA T25 digital Ultra-Turrax disperser, IKA Works Inc., Wilmington, NC, USA) for 1 min at 15,000 rpm. Because of its water solubility, Biosecur F440D was prepared at 0.4% (*w*/*w*) in distilled water. All the prepared solutions were then filtered through 0.2 µm syringe filter.
The MIC value of each antimicrobial compound was determined in sterilized flat-bottomed 96-well microplate according to the serial microdilution method (Yosra Ben-Fadhel *et al.*, 2017). Briefly, serial dilutions (200:100 µL) of the antimicrobial compounds were made in Mueller Hinton Broth (MHB, Difco, Becton Dickinson) for bacteria and in Potato Dextrose Broth (PDB, Difco, Becton Dickinson) for molds and yeast and dispensed into 96-well microplates to obtain a dilutions range of 2000–15 ppm for Biosecur F440D and 12,500–145 ppm for essential oils. Then, a volume of 15 µL of bacteria, molds and yeast suspension (10^6 CFU/mL) was added. Two control samples were evaluated; the 1st was to control the growth of the evaluated microorganisms where a volume of 100 µL of MHB/PDB was mixed to 15 µL of the selected microorganism. The 2nd control was the blank where a volume of 15 µL of distilled water was added to 100 µL of each antimicrobial dilution. The MIC of tween 80 at 2.5% was also evaluated. The final volume in all the wells was 115 µL. Microplates were sealed with acetate foil to avoid evaporation and then incubated on a shaker (Forma Scientific. Inc., Marietta, OH, USA) at 80 rpm at 37 °C for 24 h and 28 °C for 48 h respectively for bacteria and molds/yeasts to insure a better homogenization. The absorbance was then measured at 595 nm in an absorbance microplate reader (BioTek ELx800®, BioTek Instruments Inc., Winooski, VT, USA). The MIC is considered to be the lowest concentration of the antimicrobial compounds that completely inhibits bacterial and fungal strain growth by showing equal absorbance as blank. Afterwards, to assess the MBC and the MFC, 5 µL of each well were taken from the microplate and were deposit on a Petri dish containing Tryptic Soy Agar (TSA) for bacteria

and PDA for molds and yeasts. Finally, Petri dishes were incubated for 24 h at 37 °C for bacteria or 48–72 h at 28 °C for molds and yeasts respectively. The MBC and the MFC were respectively determined as the concentration where no colony was detected.

2.5. Total Phenol Determination

The total phenol content was carried out using a Folin–Ciocalteu colorimetric method according to Dewanto *et al.* (2002). Pure essential oils and Biosecur F440D were diluted in anhydrous ethanol and water respectively to obtain suitable dilution within the standard curve ranges of 0–200 µg of gallic acid/mL. Measurements were done at 760 nm versus the blank prepared similarly with water or ethanol. All values were expressed as mean (milligrams of gallic acid equivalents per g of antimicrobial compounds).

2.6. Radical Scavenging Activity (DPPH)

The antioxidant activity of the antimicrobial compounds was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a free radical (Fattouch *et al.*, 2007). The reaction for scavenging DPPH radicals was performed in polypropylene tubes at room temperature. One milliliter of a 40 µM of methanolic solution of DPPH was added to 25 µL of diluted antimicrobial compounds. The mixture was shaken vigorously and left for 90 min. The absorbance of the resulting solution was measured at 517 nm. Anhydrous methanol was used as a blank solution, and DPPH solution without any sample served as control. The Trolox equivalent antioxidant capacity (TEAC) values were calculated from the equation determined from linear regression after plotting known solutions of Trolox or ascorbic acid with different concentrations (0–1 mM). The DPPH inhibition percentage was calculated using Equation (1) and the antiradical activity was expressed as mM of Trolox or ascorbic acid.

$$\text{Radical scavenging activity (\%)} = (\text{Control OD} - \text{Sample OD}) \times 100/\text{Control OD} \quad (1)$$

2.7. Ferric-Reducing Antioxidant Power (FRAP)

Total antioxidant activity was estimated by FRAP assays (Benzie *et al.*, 1996). Three aqueous stock solutions containing 0.1 M acetate buffer (pH 3.6), 10 mM TPTZ [2,4,6-tris(2-pyridyl)-1,3,5-triazine] in 40 mM hydrochloric acid solution, and 20 mM ferric chloride were prepared and stored under dark conditions at 4 °C. Stock solutions were combined (10:1:1, *v*/*v*/*v*) to form the FRAP reagent just prior to analysis. FRAP reagent was heated in a water bath for 30 min at 37–40 °C. For each assay, 2.8 mL of FRAP reagent and 200 µL of diluted sample were mixed. After 10 min, the absorbance of the reaction mixture was determined at 593 nm. The standard curve was prepared with ascorbic acid (0–2 mM). Results were expressed as equivalent µM of ascorbic acid per gram of antimicrobial.

2.8. Determination of Total Flavonoids Content

Total flavonoids content was determined by using a colorimetric method (Dewanto *et al.*, 2002). Briefly, 0.25 mL of diluted antimicrobial compounds or (+) catechin standard solution was mixed with 1.25 mL of distilled water followed by the addition of 75 µL of a 5% $NaNO_2$ solution. After 6 min, 150 µL of a 10% $AlCl_3$ $6H_2O$ solution was added and allowed to stand for 5 min at room temperature before 0.5 mL of 1 M NaOH was added. The mixture was brought to 2.5 mL with distilled water and mixed well. The absorbance was measured immediately against the blank at 510 nm in comparison with the standards prepared similarly with known (+)-catechin concentrations. The results were expressed as mean (micrograms of catechin equivalents per gram of antimicrobial).

2.9. In Situ Test on Pre-Cut Carrots

2.9.1. Antimicrobial Loaded Emulsion

To encapsulate the natural antimicrobial compounds, an emulsion was prepared by mixing Biosecur F440D® to citrus, Asian, Mediterranean and pan tropically essential oils composed mainly with lemongrass, oregano and cinnamon essential oils respectively (Takala *et al.*, 2013). Sunflower lecithin (HLB 7) and sucrose monopalmitate (HLB 18) were used as emulsifiers (180 ppm) to obtain a stable emulsion with a HLB = 12 and an oil phase: emulsifier's ratio of 1:1. The emulsion was magnetically homogenized then mixed with Ultra-Turrax at 10,000 rpm for 1 min.

2.9.2. Samples Preparation

Freeze pre-cut carrots were provided by Bonduelle, Inc (Sainte-Martine, Canada). Carrot was washed with water then divided into 3 groups: untreated carrots (control), treated carrots with antimicrobial formulation-loaded emulsion (containing a mixture of Biosecur F440D extract and Asian, Mediterranean, citrus and pan tropical essential oils) and treated carrots with commercial chemical antimicrobial (0.03% of Tsunami: a mix of 15.2% of peroxyacetic acid and 11.2% of hydrogen peroxide). For treated samples, carrots were dipped in the antimicrobial solution for 30 s, kept drying under laminar flow hood for 15 min to discard the exceeding solution. Samples were then stored in Whirl-Pak™ Sterile Filter Bags (Nasco, Whilpack®, Fort Atkinson, WI, USA) at 4 °C for 8 days (20 g per bag). Emulsifiers were considered too low to not affect the antimicrobial activity of the emulsion.

2.9.3. Shelf-life Estimation

The total mesophilic bacterial count (TMF) was evaluated during 8 days of storage at 4 °C. The TMF was selected based on previous studies, as TMF contains a complex mix of different autochthonous microorganisms including *Candida* spp. (Babic *et al.*, 1992), *Entrobacter* spp., *Salmonella* spp. and *S. aureus* (Itohan *et al.*, 2011). To estimate the initial count of TMF, a bacterial analysis was carried out for the control on day 0. During storage, all treatments and control were evaluated on day 1, 3, 6 and 8. On each day of analysis, 60 g of 0.1% (*w*/*v*) peptone water (Alpha Biosciences Inc., Baltimore, MD, USA) were added to filter bag containing 20 g of carrots previously prepared. The carrot samples were mixed during 2 min at high speed (260 rpm) in a Lab-blender 400 stomacher (Laboratory Equipment, London, UK), then 100 µL were seeded on TSA for TMF evaluation and on PDA with chloramphenicol for molds and yeasts evaluation. Plates were incubated at 37 °C and 28 °C during 48–72 h for TMF and molds and yeast respectively. Results were expressed as bacterial count and fungal count (log CFU/g) during storage at 4 °C.

Shelf-life limit was considered at the limit of unacceptability, when TMF count and the total molds and yeasts reached the current authorities regulation level of 10^7 CFU/g and 10^4 CFU/g, respectively (MAPAQ, 2009a) . Equation (2) was used to describe the growth of bacteria (**Y**) over time during the exponential phase.

$$Y = X\exp(\mu t) \quad (2)$$

where X is the initial population, μ the growth rate of TMF (Ln CFU/g/day) and t the number of storage days.

2.10. Sensory Evaluation

In order to evaluate the effect of the developed antimicrobial formulation on the sensory properties of carrots, the sensory evaluation, was carried out by comparing the control to

treated carrots with the developed antimicrobial formulation. The sensorial evaluation of treated and untreated carrots was done using a hedonic test (da Silva *et al.*, 2017). The level of appreciation was determined using nine points (1 = dislike extremely; 5 = neither like nor dislike; 9 = like extremely). Samples were treated with the antimicrobial formulation-loaded emulsion (containing a mixture of Biosecur F440D and Asian, Mediterranean, citrus and pan tropical essential oils) and kept to dry. The sensorial evaluation was done by a panel of 24 untrained people after 1 day of the treatment application. For each panelist, 3 pieces of carrots were served to evaluate the flavor, the odor and the global appreciation. Treated samples consisted of carrot samples coated with the antimicrobial formulation.

2.11. Statistical Analysis

Each experiment was done in triplicate (n = 3). For each replicate 2 samples were analyzed. Analysis of variance (ANOVA), Duncan's multiple range tests for equal variances and Tamhane's test for unequal variances were performed for statistical analysis using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Differences between means were considered significant when the confidence interval was lower than 5% ($p \leq 0.05$).

3. Results

3.1. Preliminary Study

Results of the disk diffusion method (**Table 6.2**) showed that from 17 evaluated antimicrobial compounds, five antimicrobial agents that showed high inhibitory diameter against all the tested microorganisms were identified. Based on their bioactivity, these antimicrobial compounds could be also grouped into four distinctive groups: Group 1 contains pan tropical, Mediterranean and thyme essential oils which have a large spectral activity against bacteria, yeast and molds with an inhibitory diameter ≥23.7 mm. Their effectiveness was higher against yeast and molds with an inhibitory diameter between 38.3 and 80 mm for *C. albicans*, *P. chrysogenum*, and *A. flavus* as compared to an inhibitory diameter between 23.7 and 44.3 mm for *S*. Typhimurium, *L. monocytogenes*, *B. subtilis*, *E. coli*, *S. aureus* and *E. faecium*. Group 2 contains Asian, cloves, citrus and thyme savory leaves essential oils and citral and was very efficient to inhibit molds and yeasts. Asian essential oil and citral showed an average antibacterial activity against six bacterial strains with an inhibitory diameter ≤22.5 mm and an antifungal activity with an inhibitory diameter between 23.0 mm and 80.0 mm. Citrus and cloves essential oils were efficient to reduce *B. subtilis*, *S. aureus, C. albicans, A. flavus* and *P. chrysogenum* showing an inhibitory diameter between 22.0 and 68.7 mm. Otherwise, they showed above-average efficiency against the other microorganisms. Group 3 contains Biosecur F440D which possesses a good antimicrobial activity against all the microorganisms. The inhibitory diameter of Biosecur F440D varied from 12.3 mm to 25.4 mm for *E. faecium* and *S. aureus*, respectively, showing a medium antimicrobial activity whether against bacteria molds or yeast. Biosecur F440D was more efficient to inhibit bacteria, molds and yeasts than cranberry juice. Group 4 contains bergamot, marjoram, peppermint, sweet orange, tea tree, myrtle and ginger essential oils and cranberry juice, and showed a very low antimicrobial activity. Pepper mint essential oil was efficient only to inhibit the growth of *C. albicans* showing an inhibitory diameter of 31.3 mm. Results showed that essential oils of bergamot, sweet marjoram, sweet orange, myrtle and ginger with an inhibitory diameter ≤18.3 mm showed a very low antimicrobial activity against bacteria, molds and yeasts.
Based on these results, five antimicrobial extracts were selected to characterize their MIC, MBC, MFC and to determine their total phenols and flavonoids composition and their antiradical and antioxidant properties: citrus and Asian essential oils for their antifungal

activity, pan tropical and Mediterranean essential oils for their large spectral activity and Biosecur F440D for its good activity and its hydrophilic properties.

Table 6.2. Inhibitory diameter of antimicrobials extracts against tested microorganisms (*n* = 3).

		Inhibition Diameter: Mean ± std.dev (mm)								
		Gram Positive				Gram Negative		Yeast	Molds	
		L. monocytogenes	*B. subtilis*	*E. faecium*	*S. aureus*	*S.* Typhimurium	*E. coli*	*C. albicans*	*A. flavus*	*P. chrysogenum*
1	**Biosecur F440D**	16.6 ± 1.9	18.9 ± 1.0	12.3 ± 0.7	25.4 ± 2.4	12.5 ± 1.1	13.7 ± 0.9	22.8 ± 1.6	14.1 ± 0.7	13.6 ± 3.0
2	**Cranberry juice**	8.7 ± 0.9	9.3 ± 1.6	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	7.0 ± 1.2	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0
3	**Bergamote EO**	6.0 ± 0.0	13.7 ± 1.2	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	10.7 ± 0.4	6.0 ± 0.0	8.5 ± 0.3
4	**Citrus EO** *	8.4 ± 0.6	68.7 ± 4.9	13.9 ± 0.4	35.6 ± 2.8	13.8 ± 1.0	14.8 ± 2.0	36.2 ± 5.5	22.4 ± 5.2	45.7 ± 4.0
5	**Cloves EO**	14.8 ± 1.2	24.9 ± 3.4	19.0 ± 1.9	22.0 ± 2.5	20.0 ± 0.8	20.1 ± 3.3	27.2 ± 0.7	38.6 ± 1.2	41.7 ± 1.2
6	**Marjoram EO**	13.9 ± 0.9	17.4 ± 3.5	16.1 ± 0.7	17.1 ± 0.8	17.3 ± 1.1	19.1 ± 2.4	13.0 ± 0.2	6.0 ± 0.0	11.2 ± 0.4
7	**Pepper menthe EO**	7.8 ± 0.4	19.1 ± 3.4	15.5 ± 0.9	18.9 ± 3.3	13.3 ± 0.4	14.4 ± 1.9	31.3 ± 1.8	6.0 ± 0.0	9.9 ± 1.1
8	**Sweet orange EO**	6.0 ± 0.0	14.0 ± 1.5	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	11.1 ± 0.9	6.0 ± 0.0	8.5 ± 0.1
9	**Mediterranean EO**	23.8 ± 0.5	44.3 ± 4.1	33.9 ± 4.4	42.7 ± 4.0	28.5 ± 3.6	27.2 ± 2.5	52.0 ± 1.6	59.0 ± 2.6	80.0 ± 0.0
10	**Tea tree EO**	12.2 ± 0.4	17.2 ± 1.6	16.5 ± 0.8	18.3 ± 3.9	16.7 ± 2.2	17.3 ± 1.7	12.3 ± 1.3	6.0 ± 0.0	9.5 ± 0.4
11	**Thyme savory leaves EO**	11.3 ± 0.3	21.3 ± 3.7	16.0 ± 0.9	27.6 ± 2.6	17.7 ± 1.5	19.3±3.6	30.6 ± 1.9	20.5 ± 1.9	33.4 ± 0.5
12	**Myrte EO**	8.6 ± 0.4	11.0 ± 1.7	6.8 ± 0.9	9.3 ± 0.9	10.1 ± 2.4	8.7 ± 0.6	12.8 ± 1.2	6.0 ± 0.0	11.3 ± 1.3
13	**Ginger EO**	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	7.8 ± 2.9	6.0 ± 0.0	7.1 ± 1.3	12.4 ± 0.6	16.3 ± 1.4	11.4 ± 0.4
14	**Pan tropical EO**	31.1 ± 3.4	30.6 ± 2.1	23.7 ± 0.3	25.4 ± 2.1	32.0 ± 6.4	29.2 ± 1.3	61.0 ± 5.8	70.3 ± 3.4	63.0 ± 0.2

15	**Citral EO**	12.5 ± 1.4	10.2 ± 1.3	11.8 ± 1.6	18.4 ± 0.8	11.5 ± 1.4	10.4 ± 0.6	80.0 ± 0.0	23.0 ± 3.0	80.0 ± 0.0
16	**Asian EO**	8.8 ± 0.3	10.3 ± 2.6	9.2 ± 0.6	22.5 ± 1.2	9.6 ± 1.0	10.2 ± 0.7	42.7 ± 1.6	62.6 ± 6.1	80.0 ± 0.0
17	**Thyme thymol EO**	32.1 ± 2.2	41.4 ± 4.0	26.9 ± 3.4	31.3 ± 4.0	27.2 ± 3.7	30.5 ± 4.0	53.9 ± 2.6	38.3 ± 2.3	44.2 ± 5.3

* EO: Essential oil.

3.2. Determination of MIC, MBC and MFC

The results of MIC, MBC and MFC of the selected antimicrobial compounds are presented in **Table 6.3**. Results showed that Biosecur F440D was the most efficient in inhibiting the bacterial growth, showing a MIC and a MBC between 17 and 171 ppm against all evaluated bacterial strains. Pan tropical essential oil was also more efficient in inhibiting the growth of molds and *C. albicans* showing a fungicidal activity against *A. flavus* and *P. chrysogenum* with a MFC between 155 and 621 ppm. Pan tropical and Mediterranean essential oils showed the highest antimicrobial activity against almost all microorganisms tested showing a bactericidal and fungicidal activity. They inhibited the growth of all evaluated microorganisms at a concentration ≤1241 ppm for pan tropical essential oil and ≤2474 ppm for Mediterranean essential oil. These results indicate that these two essential oils have an interesting antimicrobial potential. Asian essential oil showed a high activity in inhibiting the growth of molds and yeast and showed a MFC of 311, 622 and 4979 ppm for *C. albicans*, *A. flavus* and *P. chrysogenum,* respectively. On the other hand, Biosecur F440D had a bactericidal activity against all the evaluated bacterial strains as compared to essential oils which have fungicidal activity.

Table 6.3. Minimum inhibitory, bactericidal and fungicidal concentrations (MIC, MBC and MFC) of the selected antimicrobial compounds.

		MIC, MBC and MFC Expressed in parts-per-million, PPM (Mean Value ± SD, n =3)					
		Biosecur F440D	**Pan Tropical EO**	**Mediterranean EO**	**Asian EO**	**Citrus EO**	**Tween 80 2.5%**
L. monocytogenes	MIC	171 ± 5	621 ± 3	619 ± 2	4974 ± 0	4974 ± 0	> 12500
	MBC	171 ± 0	1241 ± 5	1237 ± 3	4974 ± 0	4974 ± 0	-
B. subtilis	MIC	33 ± 1	1241 ± 6	1237 ± 3	2487 ± 0	4974 ± 0	> 12500
	MBC	33 ± 0	1241 ± 0	2470 ± 0	4974 ± 0	4974 ± 0	-
E. faecium	MIC	142 ± 33	1241 ± 6	2474 ± 7	4979 ± 0	4974 ± 0	> 12500
	MBC	142 ± 28	2488 ± 8	4947 ± 10	4979 ± 7	4974 ± 0	-
S. aureus	MIC	17 ± 0	1050 ± 0	1049 ± 1	1056 ± 0	2474 ± 0	> 12500
	MBC	17 ± 0	2227 ± 0	2224 ± 0	2239 ± 0	2474 ± 0	-
S. Typhimurium	MIC	171 ± 5	621 ± 3	309 ± 1	1245 ± 2	4974 ± 0	> 12500
	MBC	171 ± 4	621 ± 2	619 ± 1	1245 ± 1	4974 ± 0	-
E. coli	MIC	114 ± 3	621 ± 3	619 ± 2	1245 ± 2	2474 ± 0	> 12500
	MBC	114 ± 2	1243 ± 5	619 ± 1	1245 ± 0	2474 ± 0	-
C. albicans	MIC	427 ± 12	155 ± 1	155 ± 0	311 ± 0	1245 ± 0	> 12500

	MFC	628 ± 0	621 ± 3	618 ± 1	311 ± 0	1245 ± 0	-
A. flavus	MIC	836 ± 23	621 ± 3	2474 ± 7	4979 ± 0	4979 ± 0	> 12500
	MFC	1261 ± 26	621 ± 1	4958 ± 5	4979 ± 7	4979 ± 0	-
P. chrysogenum	MIC	552 ± 11	155 ± 1	1237 ± 3	622 ± 1	1245 ± 0	> 12500
	MFC	609 ± 76	155 ± 1	2477 ± 5	622 ± 0	1245 ± 0	-

3.3. Total Phenols and Flavonoids

Results of total phenols and total flavonoids content (**Table 6.4**) showed that Mediterranean and pan tropical essential oils were highly concentrated in total phenols (respectively 220.57 and 34.62 mg gallic acid equivalent/ g of antimicrobial) and total flavonoids (respectively 34.62 and 17.63 mg catechin equivalent/g of antimicrobial). Biosecur F440D showed a concentration of 4.38 mg gallic acid equivalent/g of antimicrobial for total phenols content and 1.26 mg catechin equivalent/g of antimicrobial for total flavonoids. Citrus and Asian essential oils showed the least concentration of total phenol and flavonoid content with, respectively, 1.51 and 1.41 mg gallic acid equivalent/g of antimicrobial and 0.06 and 0.56 mg catechin equivalent/g of antimicrobial.

Table 6.4. Total phenols and total flavonoids content of the antimicrobial extracts.

Natural antimicrobial products	Total Phenols (mg gallic acid/g of AM) *	Total Flavonoids (mg catechin/g of AM) *
Biosecur F440D	4.38 ± 0.16 [a]	1.26 ± 0.06 [a]
Pan tropical EO	34.62 ± 3.68 [b]	17.63 ± 1.40 [b]
Mediterranean EO	220.57 ± 17.67 [c]	34.75 ± 2.4 [c]
Asian EO	1.41 ± 0.18 [a]	0.56 ± 0.07 [a]
Citrus EO	1.51 ± 0.03 [a]	0.06 ± 0.03 [a]

* Within each column, means with the same letter are not significantly different ($p > 0.05$); AM: Antimicrobial.

3.4. Radical Scavenging Activity and FRAP

Biosecur F440D and Mediterranean, Asian, pan tropical and citrus essential oils were tested for their ability to scavenge radicals by the DPPH method. Biosecur F440D has the highest radical scavenging activity above all the other compounds with 0.28 mM of Trolox (**Table 6.5**). The radical scavenging of Biosecur F440D was two times higher than Mediterranean essential oil (0.18 mM equivalent), three times higher than citrus essential oil (0.07 mM equivalent) and 10 times higher than Asian essential oil (0.02 mM of Trolox equivalent).

The antioxidant activity measured with the ferric reducing power assay revealed similar results to those obtained with the DPPH technique (**Table 6.5**). The highest antioxidant activities were obtained with Mediterranean essential oil (0.76 Eq µM of ascorbic acid equivalent/g of extract), followed by pan tropical essential oil and Biosecur F440D (0.43 and 0.30 Eq µM of ascorbic acid

equivalent/g of antimicrobial respectively). Asian and citrus essential oils have the lowest values (below 0.04 Eq µM of ascorbic acid equivalent/g of antimicrobial).

Table 6.5. Ferric reducing antioxidant power (FRAP) and Radical Scavenging Activity of the antimicrobial compounds.

	FRAP *	Radical Scavenging Activity *	
Natural antimicrobial products	**Eq µM of Ascorbic acid/g of AM**	**mM Trolox**	**mM AA**
Biosecur F440D	0.30 ± 0.04 [ab]	0.28 ± 0.05 [d]	0.29 ± 0.05 [d]
Pan tropical EO	0.43 ± 0.02 [b]	0.15 ± 0.02 [c]	0.15 ± 0.02 [c]
Mediterranean EO	0.76 ± 0.03 [c]	0.18 ± 0.03 [c]	0.19 ± 0.03 [c]
Asian EO	0.04 ± 0.00 [a]	0.02 ± 0.00 [a]	0.02 ± 0.00 [a]
Citrus EO	0.03 ± 0.00 [a]	0.07 ± 0.01 [b]	0.07 ± 0.01 [b]

* Within each column, means with the same letter are not significantly different ($p > 0.05$).

3.5. In Situ Analysis

Results of the growth of TMF, molds and yeasts (**Figure 6.1**) showed that on Day 0, the encapsulation of the antimicrobial formulation in o/w emulsion (containing a mixture of Biosecur F440D and Asian, Mediterranean, citrus and pan tropical essential oils), applied on the surface of carrots, allowed 2 log reductions for TMF and 1 log reduction for molds and yeasts as compared to the control ($p \leq 0.05$). The mix of selected antimicrobial ingredients-loaded emulsion was more effective than the commercial mix (Tsunami 100). A significant reduction of TMF, molds and yeasts counts was also observed during the whole storage period showing a 1 log reduction of TMF on carrots treated with the antimicrobial ingredients-loaded emulsion as compared to the control which signifies a better control of the microbiological growth of TMF on pre-cut carrots. The antimicrobial activity of the commercial mix of peroxyacetic acid and hydrogen peroxide against TMF was also lower than the antimicrobial ingredients-loaded emulsion during the whole storage. The shelf-life of pre-cut carrots was reached on Day 6 for untreated carrots, treated carrots with the commercial chemical preservatives and on Day 8 for treated carrots with the developed antimicrobial-loaded emulsion (**Figure 6.1a**). By considering Days 1, 3 and 6, the growth rate was also lower in treated carrot with the antimicrobial formulation and with Tsunami samples showing a growth rate of 0.1291 and 0.1852 Ln CFU/g/day respectively as compared to 0.2193 Ln CFU/g/day for untreated samples (**Table 6.6**).

Table 6.6. Growth rate of total mesophilic flora (TMF) in refrigerated pre-cut carrots.

Sample	Growth Rate of TMF (Ln CFU/g/day)
Control	0.2193
Tsunami	0.1852
Antimicrobial formulation	0.1291

By considering the results of total molds and yeasts (**Figure 6.1b**), the shelf-life of pre-cut carrots was reached on Day 1 for untreated carrots and on Day 3 for both treated carrots with the antimicrobial-loaded emulsion and treated carrots with the chemical preservative (Tsunami). The obtained *in situ* results indicated that the antimicrobial formulation was effective against TMF and molds and yeasts**,** not only immediately after treatment but also during a mid-term storage.

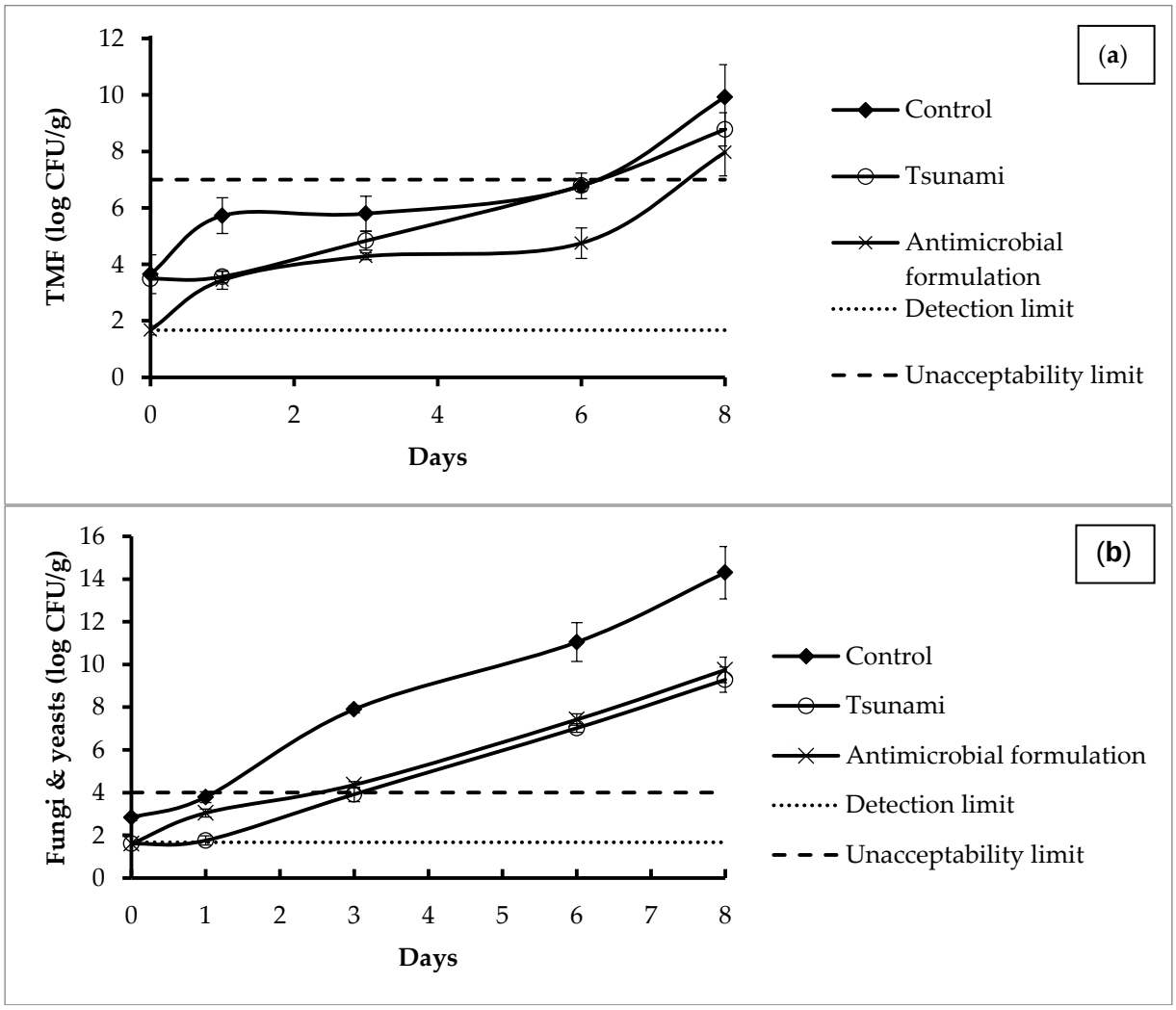


Figure 6.1. Total mesophilic flora (**a**) and total molds and yeasts (**b**) growth on pre-cut carrots.

3.6. Sensory Evaluation

Sensory analysis of pre-cut carrots treated or not with the antimicrobial formulation-loaded emulsion, was done by evaluating its odor, taste and global appreciation, using a nine-point hedonic scale and a panel of 24 untrained people and results are presented in **Figure 6.2**. Results showed that the antimicrobial treatment did not have any detrimental effect on the sensorial quality of the coated carrots. The values of the odor, the taste and the global appreciation were 6.8, 6.6 and 6.6 for the carrots treated with the antimicrobial formulation as compared to 6.8, 7.1 and 7.2 for the control samples. The odor was not affected by the applied treatment and a slight reduction on the attributed note was observed on the taste and the global appreciation. Overall, no significant negative effect ($P > 0.05$) was observed.

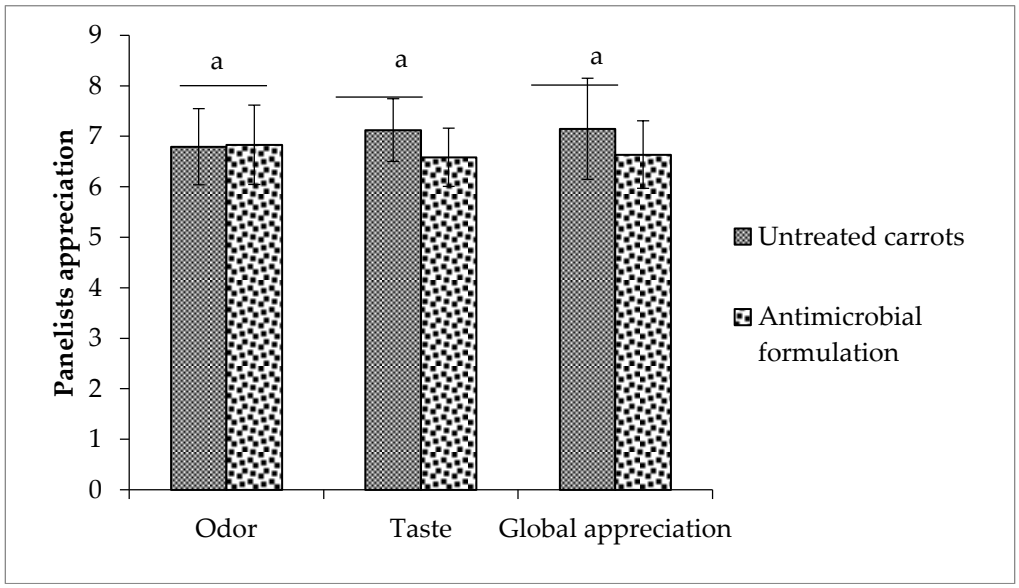


Figure 6.2. Effect of antimicrobial treatment on sensorial properties of pre-cut carrots.

4. Discussion

Valorization of natural antimicrobials has been extensively investigated during the last decades. In the present study, it was demonstrated that natural antimicrobials have a good antioxidant and antimicrobial activity against a wide range of food pathogens and spoilage microorganisms, and that their combination allows a better control of the microbiological quality of pre-cut carrots without altering their sensory properties.

Using the disk diffusion method, we have identified five antimicrobial compounds that showed a high inhibitory diameter against the tested microorganisms: Biosecur F440D and citrus, Asian, Mediterranean and pan tropical essential oils. Similar results for inhibitory diameter obtained by disk diffusion were also reported by Baser *et al.* (2015) for cinnamon and citronella against *L. monocytogenes* and *S*. Typhimurium. Despite the medium inhibitory diameter (12.5–25.4 mm) of Biosecur F440D as compared to essential oils, its MIC and MBC was the lowest against all the evaluated bacteria. According to Ghabraie *et al.* (2016) and Lopez *et al.* (2005), the antimicrobial activity of essential oils is due to both solid and vapor-phase fractions. The antimicrobial activity of the vapor-phase could be observed only when essential oils are seeded on surface which was the case with the disk diffusion method. With the MIC method, the antimicrobial evaluation was done in liquid medium which reduces significantly the antimicrobial effect of the vapor fraction. However, Biosecur F440D, because of its water solubility, has a

bactericidal activity when employed in liquid media and the obtained MIC was similar to the MBC (Table 3). Results obtained with disk diffusion agar confirmed previous observations and showed a higher or similar sensitivity of Gram positive bacteria to essential oils than Gram negative (Baser *et al.*, 2015, Dussault *et al.*, 2014). On the other hand, results obtained with MIC and MBC of essential oils showed that overall, essential oils were more efficient to inhibit Gram-negative bacteria than Gram positive as well showing a lowest MIC and MBC. These results suggest that volatile compounds in essential oils (MW < 300) could have a higher efficiency against Gram negative probably due to its various chemical compounds: alcohols, ethers or oxides, aldehydes, ketones, esters, amines, amides, phenols, heterocycles, and mainly the terpenes. It is known that the composition has an impact on the antimicrobial efficiency (Dhifi *et al.*, 2016).

The antimicrobial behavior observed in the *in vitro* study of each antimicrobial compound differs mainly due to the difference in their chemical composition and nature. The Mediterranean and the pan tropical essential oils are highly effective antimicrobial compounds, leads to a significant inhibition against almost all evaluated microorganisms.

The Mediterranean essential oil, for example, is rich in total phenols and total flavonoids (Table 4). Similar results were observed by Wogiatzi *et al.* (2009) where several oregano origins were evaluated. Wogiatzi *et al.* (2009) demonstrated that the total phenol content is also intimately related to the plant area of cultivation (foot/middle mountain). The hydroxyl group (-OH) of the phenolic compounds could interact with the membrane cell of bacteria and reduce the pH gradient through the cytoplasmic membrane which disrupts its structure and causes the loss of intracellular ATP and cell death (Gyawali *et al.*, 2014). The -OH group can also bind to the active site of enzymes (i.e., ATPase, histidine carboxylase), thereby altering the cellular metabolism of microorganisms (Gyawali *et al.*, 2014, Nazzaro *et al.*, 2013). The presence of phenolic compounds is also responsible for the good antioxidant activity of the Mediterranean essential oil observed, which act as free radical terminators (Shahidi *et al.*, 1992). Mediterranean essential oil is thus able to reduce the redox potential of the culture medium and to reduce the growth of microorganisms.

The antimicrobial activity of pan tropical essential oil is related to its high concentration on cinnamaldehyde. Cinnamaldehyde is capable of modifying the lipid profile of the microbial cell membrane probably due to its high antioxidant activity (Schmidt *et al.*, 2006) which allows it to oxidase lipids on the bacterial membrane. Cinnamaldehyde can also inhibit the respiratory tract in certain bacteria by disrupting K^+ and pH homeostasis (Nazzaro *et al.*, 2013). In this study, pan tropical essential oil was also characterized by a great antifungal activity probably due to its ability to inhibit b-(1,3)-glucan and chitin synthesis in yeasts and molds which are the major structural compounds of the fungal cell walls (Bang *et al.*, 2000).

Asian essential oil is highly concentrated on geranial and neral. These two isomers are the main compounds of the monoterpene citral which its antimicrobial activity is well known against several bacteria and molds (Dorman *et al.*, 2000, Cristiane de Bona da Silva *et al.*, 2008). Despite the antifungal effectiveness of Asian and citrus essential oils with disk diffusion method, the effectiveness in broth media was lower due probably to the ability of some microorganisms to transform citronellal and citral and other of their components to the sole carbon and energy source (Baser *et al.*, 2015). The antifungal activity of citral and cinnamaldehyde is the result of perturbation in ergosterol biosynthesis which causes a damage to the intracellular structure, loss of intracellular substance and membrane damage (Wang *et al.*, 2018).

Citrus essential oil is highly concentrated with citronellol and geraniol, and showed a lower antimicrobial activity when compared to the other antimicrobials mainly due to the presence of only one double bond on its main compounds (Gyawali *et al.*, 2014). Nakahara *et al.* (2013) showed that citronellal and linalool has antifungal activity at a dose of 112 ppm. The antifungal

activity of components found in citrus essential oil (i.e. mono-terpenes) was previously reported to the interference of such compounds with enzymatic reaction of wall, i.e., structure (Aguiar *et al.*, 2014, Neeta Sharma *et al.*, 2008). This allows a lack of cytoplasm, damage of integrity and finally the mycelial death (Pereira *et al.*, 2011). Simic *et al.* (2008) showed also that the antimicrobial activity of citronella essential oil is intimately related to the association of citronella and citronellol due probably to a synergistic effect of their combination.

Biosecur F440D was efficient to inhibit the growth of Gram positive and Gram-negative bacteria showing a bactericidal activity. According to Álvarez-Ordóñez *et al.* (2013), citrus extracts at higher concentrations than the MIC, pore formation in the cell membrane is observed inducing leakage of nucleic acids. According to the same authors, to achieve a significant bacterial reduction, the exposure time or the antimicrobial concentration used should be two to four times higher than the MIC. Citrus extract mainly acts on the membrane. It causes conformational damage and/or compositional in some or all components of the cell membrane. It mainly affects the carboxyl groups of membrane fatty acids and thus impairs the macromolecular structure of the bacterial membrane. Several studies have tried to identify the components that are involved in the antimicrobial activity of citrus extract. It possesses strong antioxidant and antimicrobial properties, pleasant aromas and flavors, especially due to the presence of flavonoids. Citrus flavanones include naringenin, hesperidin, hesperitin and prunine and have a broad spectrum of action against many Gram-negative bacteria.

Citrus flavonoids have also a direct role in scavenging reactive oxygen species (ROS) as confirmed by the obtained results of antiradical activity (Calabro *et al.*, 2004). This suggests that the ROS could be involved in the bactericidal activity observed on citrus extracts. Inoue *et al.* (2002) supported this suggestion and showed that ROS act in conjunction to induce the strong bactericidal activity. The antiradical activity is also due to the presence of vitamin C at a high concentration in citrus extract which is a natural free radical scavenger.

The obtained results of *in vitro* study showed a very good antimicrobial and antioxidant properties of the selected natural antimicrobials. As their mode of action against bacteria fungi and yeasts differs, the mix of natural antimicrobial-loaded emulsion applied on carrots as a food model, presented a large spectral activity against targeted microorganisms.

The application of this developed formulation encapsulated in o/w emulsion at a concentration that did not affect the sensory properties of carrots (Figure 2) was efficient to reduce TMF, molds and yeasts growth during storage at 4 °C. The developed formulation was also more effective than the chemical antimicrobial (mix of peroxyacetic acid and hydrogen peroxide) to control TMF and had similar efficiency to control molds and yeasts. Based on previous studies, the developed formulation seems to be also more effective than other chemical methods such as HOCl, 4% H_2O_2 which showed less than 2 log reduction of TMF of carrots (Amanatidou *et al.*, 2000). *In situ* efficiency is mainly due to combined activity of different compounds. The use of such combination could help to better control spoilage of fruits and vegetables. According to Bassolé *et al.* (2012), combining cinnamon and oregano yielded in most cases, in a synergistic activity against *E. coli* and *S*. Typhimurium. Monoterpene hydrocarbon (α-pinene) when mixed with limonene or linalool also showed additive and synergistic effects (Bassolé *et al.*, 2012). The obtained results present a new antimicrobial formulation based on natural plant extracts that allowed a better control of initial microflora that could replace the methods presently used in industries such as blanching and ozonized water.

5. Conclusions

This study showed that natural antimicrobial extracts are rich on antioxidant and antiradical compounds. Biosecur F440D has the highest radical scavenging activity and has a bactericidal activity against all evaluated bacteria. Pan tropical essential oil has particularly an antifungal

activity. Mediterranean essential oil was highly rich on total phenol and has the highest antioxidant activity**.** The mixture of natural antibacterial extracts when encapsulated in o/w emulsion and applied on carrot surface showed a better antimicrobial effectiveness than commercial chemical treatment widely used to treat vegetables. The mixture could be used as food treatment to extend the shelf-life of pre-cut carrots by two days without affecting their sensory properties. Finally, this user-friendly antimicrobial formulation-loaded emulsion could be applied in the food industry as a way to fulfill federal regulation requirements.

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CHAPITRE 7: PHYSICOCHEMICAL AND MICROBIOLOGICAL CHARACTERIZATION OF PECTIN-BASED GELLED EMULSIONS COATING APPLIED ON PRE-CUT CARROTS

Caractérisation physicochimique et microbiologique d'un enrobage d'émulsions gélifiée à base de pectine appliqué sur des carottes précoupées

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CONTRIBUTIONS DES AUTEURS

Ce travail de recherche a été réalisé sous la supervision du Pr. Monique Lacroix. Toutes les expériences ont été réalisées en laboratoire par Yosra Ben Fadhel avec l'aide de Johanne Manus et les discussions sur les résultats et les protocoles ont été assistés par Stéphane Salmieri. L'article a été écrit par Yosra Ben Fadhel, tandis que les corrections et révisions ont été effectuées par Stéphane Salmieri, Dr Behnoush Maherani, et Pr. Monique Lacroix.

RÉSUMÉ

Deux formulations antimicrobiennes composées de Biosecur F440D (extrait d'agrumes) et d'un mélange de quatre huiles essentielles (HE) piégées dans une matrice à base de pectine ont été caractérisées par spectroscopie infrarouge à transformée de Fourier (FTIR). L'effet de la matrice à base de pectine sur la libération des composés actifs et leur stabilité pendant le stockage ont également été évalués. De plus, la durée de conservation des carottes précoupées enrobées d'émulsions gélifiées et son activité antimicrobienne contre *Listeria monocytogenes* et *Penicillium chrysogenum* ont été évaluées. Sur la base de l'analyse FTIR, le piégeage de Biosecur F440D dans la matrice de pectine a été obtenu en particulier par liaison hydrogène et d'étirement CH. Un fort étirement asymétrique $–CH_3$ dans les groupes alkyles a également été observé pour le piégeage du mélange d'HE dans la matrice de pectine. Le piégeage des ingrédients bioactifs dans la matrice de pectine a ralenti la libération des deux formulations et a donné une meilleure stabilité de la teneur en phénols totaux et de la couleur, en particulier dans l'émulsion à base de Biosecur F440D. L'application d'une émulsion gélifiée sur des carottes précoupées a améliorée la durée de conservation d'un jour par rapport à la carotte non traitée pour une émulsion gélifiée à base de Biosecur et de 2 jours pour une émulsion gélifiée à base d'HE. Une amélioration de l'activité antimicrobienne contre *L. monocytogenes* et *P. chrysogenum* a également été observée pour les deux émulsions et leurs émulsions gélifiées respectives.

ABSTRACT

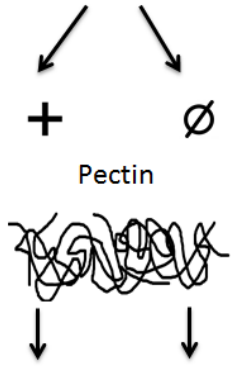
Two antimicrobial formulations composed of Biosecur F440D (citrus extract) and a mixture of four essential oils (EOs) entrapped in pectin-based matrix were characterized by Fourier-transform infrared spectroscopy (FTIR). The effect of pectin-based matrix on active compounds' release and their stability during storage time were also evaluated. Furthermore, shelf-life of pre-cut carrots coated with gelled emulsions and its antimicrobial activity against *Listeria monocytogenes* and *Penicillium chrysogenum* were assessed. Based on FTIR analysis, entrapment of Biosecur F440D in pectin matrix was obtained especially by hydrogen bonding and CH stretching. A strong asymmetric $–CH_3$ stretching in alkyl groups were also observed for the entrapment of mixture of EOs in pectin matrix. The entrapment of bioactive ingredients in pectin matrix slowed down the release behavior for both formulations and gave a better stability of total phenols content and color especially in Biosecur F440D based emulsion. Application of gelled emulsion on pre-cut carrots improved the shelf-life by one day as compared to untreated carrot for Biosecur-based gelled emulsion containing EOs and by 2 days for EOs-based gelled emulsion containing Biosecur F440D. An improvement in antimicrobial activity against *L. monocytogenes* and *P. chrysogenum* was also observed for both emulsions and their respective gelled emulsions.

Preparation of 2 formulations

Essential oils (EOs)

+

Biosecur F440D
Citrus extracts



PF_1/ PF_2 F_1 / F_2

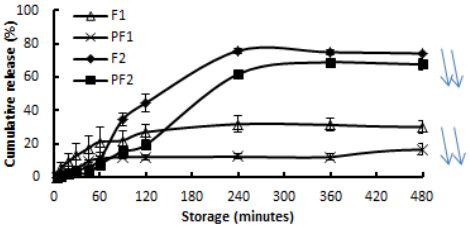
Physicochemical Characterization

FTIR

- **Pectin + F440D** : hydrogen bonding and CH stretching

- **Pectin + EOs:** asymmetric - CH_3 stretching in alkyl groups

Release of bioactive ingredients



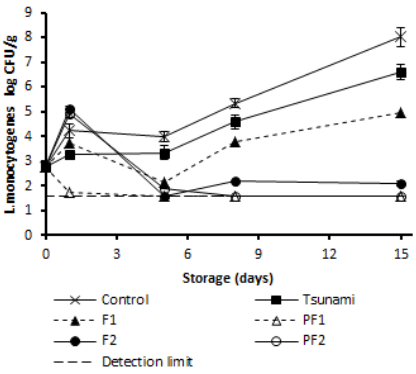
Stability

Better stability of total phenols content and color

***In situ* characterization**

Shelf-life extension by 2 days of carrots

Better control of *L. monocytogenes* and *P. chrysogenum* on carrots



1. Introduction

Preprocessed food is increasingly growing in food market. This phenomenon is principally due to the new lifestyle of consumers that has generated an increase of the demand of ready-to eat foods (Senauer, 2001). These preprocessed foods are more likely to be cross-contaminated during the process (preparation, handling or packaging). *E. coli, Listeria, Salmonella, Alternaria*, *Rhizopus*, *Aspergillus* and *Penicillium* could contaminate preprocessed food and could have serious economically impacts on the companies with recalls and on the health system with foodborne diseases (Ignatius Uche *et al.*, 2015, Kora *et al.*, 2005, Samuel *et al.*, 2016) .

To skirt this issue, food manufacturers add preservatives – mainly chemical additives – to the food during processing to avoid spoilage with foodborne pathogens and to extend the shelf-life. However, consumers increasingly want foods that are not only safe, but that also positively promote good health and have a long shelf-life. This encourages the development of antimicrobial formulations based on natural compounds and chemical additives-free food (Senauer, 2001). Thus, it becomes important to identify natural antimicrobials that have a similar or better efficacy to those of chemical additives with much less toxic and allergenic effects.

Using natural antimicrobial compounds as preservatives replacing chemical products is highly sought-after due to their safety. They can act as bio-preservatives, reducing or eliminating pathogenic bacteria, yeasts and molds (Y/M) and increasing the overall quality of food products. They have the advantages of eliminating antibiotic-resistant bacteria (*i.e* methicillin) and biofilms and suppress antibiotic resistance (KW Lee *et al.*, 2003a, Naveed *et al.*, 2013). When applied on meat surface, natural antimicrobials can also prevent discoloration of meat and lipid oxidation and could maintain the vitamin content (Ben Fadhel *et al.*, 2016). Natural antimicrobials also often possess antioxidant, nutraceutical and functional properties *i.e*. anti-cancer and radioprotective properties (Jaiprakash R Patil *et al.*, 2009, Manoj P Rai *et al.*, 2011). Phenolic and anthocyanins, highly produced when vegetable tissue is stressed, are produced to protect plant against environmental pathogens by rendering the tissue unpalatable and poisonous. This gave them both antioxidant and antimicrobial effects and could have a good potential for food application to avoid food spoilage (Friedman, 1997, Stevenson and Hurst, 2007).

Despite their use advantages, marketing of food products incorporating natural antimicrobials is restricted due to the hydrophobicity, instability and the volatility of some natural antimicrobials which reduces their efficiency and homogeneity in foods. Indeed, a high concentration of fat and/or protein in foods allows the antimicrobial activity loss of natural antimicrobials such as EOs during storage, since the latter come into direct contact with the fats and/or proteins of the food which oxidize, resulting in rapid loss of antimicrobial activity (Huq *et al.*, 2015). This means that once applied *in situ*, the activity of natural antimicrobials does not cover the entire shelf-life of foods. In addition, the concentration needed to control bacteria and molds often affects the sensory properties of foods by acidic taste or herbal and chemical odors (Gutierrez *et al.*, 2008).

To recover this issue, some strategies were developed including the encapsulation of antimicrobial compounds and combined treatments. In fact, combining natural antimicrobials at low doses together or with other methods such as heat or physical treatment could reduce the impact on the nutritional value and the sensory quality of food. The encapsulation of bioactive compounds could protect their bioactivity against temperature, humidity, lipid peroxidation or other components of the food system. Encapsulation was described to assure a controlled release over time, improve the targeting of encapsulated ingredients and thus increase food shelf-life (Đorđević *et al.*, 2015, Fathi *et al.*, 2012a, Huq *et al.*, 2015, C. C. Liolios *et al.*, 2009b, McClements, 2015, Stephane Salmieri *et al.*, 2014a, Salvia-Trujillo *et al.*, 2014). For example,

the use of biopolymers was reported to improve the physical stability of natural antimicrobials and to minimize their impact on the flavor and taste of food products (Severino *et al.*, 2015).

Encapsulation in a convenient matrix helps sensitive ingredients to disperse homogenously in food products and to improve their stability and efficiency during process and storage time. Currently, this technology is increasingly used in food industry to encapsulate the flavorings, antioxidants, colorants, sweeteners, antimicrobial agents, bioactive and nutraceutical molecules, probiotics and enzymes (Kashappa Goud H Desai *et al.*, 2005b, Donsì *et al.*, 2011). The main objective of this study was to characterize different techniques of encapsulation such as emulsions and pectin-based gelled emulsions to entrap two natural antimicrobial formulations based on Biosecur F440D and EOs. The effect of their application on the shelf-life and on specific spoilage microorganisms in pre-processed carrots was also assessed.

2. Materials and methods

2.1. Material

Biosecur F440D (33-39% of citrus fruits extract) was provided by Biosecur Lab Inc. (Mont St-Hilaire, QC, Canada). EOs were provided by Bio Lonreco Inc. (Dorval, QC, Canada) and their main constituents are presented in **Table 7.1**. High methylester pectin (Genu Beta, degree of esterification: DE > 50) was kindly provided by CP Kelco US Inc. (Atlanta, GA, USA). Sunflower lecithin and sucrose monopalmitate were kindly provided by Compass Foods (Singapore).

Table 7.1. List of organic EOs and their main composition.

	Botanic name	Part	Compositions (%)
1	*Cinnamomum verrum*	Peel	E-cinnamaldehyde (55.1), cinnamyl acetate (9.6), β-caryophyllene (4.0)
2	*Cymbopogon winterianus*	Aerial part	Citronellal (35.4), geraniol (20.1), citronellol (12.2), elemol (4.6), Limonene (3.0), citronellyl acetate (2.9), germacrene D (2.7), geranyl acetate de (2.5), linalool (0.6)
3	*Cymbopogon flexuosus*	Herb	Geranial (39.1), neral (31.6), geraniol (6.7), geranyl acetate (3.7)
4	*Origanum compactum*	Flower top	Carvacrol (46.1), thymol (17.6), γ-terpinene+ trans-β-ocimene (14.8), p-cymene (8.5)

*Composition was provided by Bio Lonreco Inc and was determined by CPG-SM HP; Column: HP Innowax 60–0.5-0.25; Carrier gas Helium: 22 psi.

2.2. Preparation of emulsions and gelled emulsions

An oil-in-water (o/w) emulsion was prepared by mixing Biosecur F440D® with the EOs mixture (Asian and Italian EOs) (Takala *et al.*, 2013). Two emulsions were prepared: F_1 as a Biosecur F440D-based emulsion (60,000 ppm of Biosecur F440D + 720 ppm of EOs mixture) developed by Yosra Ben-Fadhel *et al.* (2019a) and F_2 as EOs-based emulsion (28,000 ppm of EOs mixture + 4,000 ppm of Biosecur F440D) developed based on preliminary checker board method (supplementary data 1). Sunflower lecithin (HLB 7) and sucrose monopalmitate (HLB 18) were used as emulsifiers and added to the emulsion with a ratio of oil phase: emulsifiers 1:1 (w/w). Emulsions were stabilized by using a Ultra-Turrax T25 high-shear homogenizer (IKA Works Inc., Wilmington, NC, USA) at 10,000 rpm for 1 min. Emulsions were diluted 1/4 in MilliQ (MQ-water) or in 27,000 ppm of high methyl ester pectin to obtain a final concentration of 15,000 ppm of Biosecur F440D and 180 ppm of EOs for F_1 and PF1 and 7,000 ppm of EOs and 1,000 pm of Biosecur F440D for F_2 and PF2. The emulsions and gelled emulsions were magnetically stirred at room temperature and then degassed.

2.3. Characterization of gelled emulsions

2.3.1. FTIR analysis

The interactions between pectin and antimicrobial ingredients was evaluated by FTIR spectroscopy. Dried pectin-based films were prepared with and without antimicrobial ingredients. Pectin solutions were degassed and 15 mL per Petri dish was poured and allowed to dry for 72 h. The FTIR spectra of the films were recorded using a Spectrum One spectrophotometer (Perkin-Elmer, Woodbridge, ON, Canada) equipped with an attenuated total reflectance (ATR) device for solids analysis and a high-linearity lithium tantalate detector. Spectra were analyzed using Spectrum 6.3.5 software. Films were then placed onto a zinc selenide crystal, and the analysis was performed within the spectral region of 650–4000 cm^{-1} with 64 scans recorded at a 4 cm^{-1} resolution. After attenuation of total reflectance and baseline correction, spectra were normalized with a limit ordinate of 1.5 absorbance units (Stephane Salmieri *et al.*, 2014b).

2.3.2. Release of bioactive compounds

The release of antimicrobial compounds was evaluated *in vitro* according to Paula *et al.* (2010) and Sessa *et al.* (2014) by measuring total phenol content using UV-Vis spectroscopy. Five mL of samples (emulsions F_1 and F_2 and their respective gelled emulsions PF_1 and PF_2) were placed in a dialysis bag (Mw cut off: 14 kDa) and were kept in a beaker containing 200 mL of 10 % ethanol as a simulated medium for preprocessed vegetables and fruits according to the European Commission (2011) No 10/2011 standard method (2011), since vegetables are composed from 90 % of water and 10 % of organic phase. The release of both emulsions and gelled emulsions from dialysis membrane was studied at room temperature under mild stirring conditions. At certain time intervals, 1.0 mL of release medium was withdrawn and replaced with an equal volume of the corresponding fresh medium to maintain a constant volume. The total phenols content of test solutions was determined by using Folin-Ciocalteu's reagent (Dewanto *et al.*, 2002).

The cumulative release was determined following the equation (1):

$$\text{Cumulative percentage release } (\%) = \frac{\text{Volume of sample withdraw (mL)}}{\text{Bath volume}} * P(t-1) + Pt \quad \text{(Eq 1)}$$

Where:

P_t: Percentage release (w/v) at time t

$P_{(t-1)}$: Percentage release (w/v) at time previous to 't'.

In vitro release data was examined through Korsmeyer-Peppas model to describe the release kinetics. Equation 2 is a simple relationship which described bioactive ingredients release from a polymeric system to find out the mechanism of bioactive ingredients release. The first 60% of bioactive ingredients release data was fitted in Korsmeyer-Peppas model:

$$Mt / M\infty = k_{kp}\, t^n \quad \text{(Eq 2)}$$

where:

n: the release exponent

k_{kp}: constant of apparent release.

Based on this model, the magnitude of the release exponent "n" indicates the release mechanism. An n value ≤ 0.45 corresponds to Fickian diffusion. Values of n between 0.45 and 0.89 are regarded as an indicator of both phenomena (drug diffusion in hydrated matrix and polymer relaxation) commonly called anomalous transport. An n value = 0.89 corresponds to a

zero-order release mainly occurring by swelling process. An n value > 0.89 indicates a non-Fickian transport mechanism, controlled by relaxation of polymer chains. Non-Fickian release occurs mainly by two mechanisms, diffusion and relaxation of polymer chain which characterize the deterioration of the microcapsules (Huanbutta *et al.*, 2018).

2.3.3. Total phenols content determination

The total phenols (TP) content of F_1, F_2, PF_1 and PF_2 was determined using a Folin-Ciocalteu colorimetric method according to Dewanto *et al.* (2002) with a standard curve ranges of 0-200 µg of gallic acid mL^{-1}. A quantity of 125 µL of sample (F_1, F_2, PF_1 or PF_2) was mixed with 0.5 mL of distilled water in a test tube followed by addition of 125 µL of Folin-Ciocalteu's reagent. Samples were mixed with vortex at room temperature. After 6 minutes of reaction, 1.25 mL of a 7% sodium carbonate aqueous solution was added to the mix and the final volume was adjusted to 3 mL by adding water. Samples were allowed to stand for 90 min at room temperature before measurement at 760 nm versus a blank prepared similarly with water. All values were expressed as mean µg of gallic acid equivalents (GAE)/mL. The method was preliminary verified for its repeatability and showed a low standard deviation (≤ 10%).

2.3.4. Stability of emulsions

Total phenol content and color evaluation of emulsions (F_1, F_2) and their respective gelled counterparts (PF_1, PF_2) were carried out during 90 days of storage at room temperature under normal indoor light condition and 40-50% of RH (Dewanto *et al.*, 2002). Color evaluation was conducted using a Konica Minolta CR10 Plus Color reader (Folio Instruments Inc., Montreal, QC, Canada). L* (lightness, from black = 0 to white = 100), a* (green-red from -300 to +299), and b* (blue-yellow from -300 to +299), the hue (H° from yellow = 90° to green = 180°), the chroma C* (0 = duller to 200 = brighter) and the total change of color (ΔE*) were quantified on each sample. The hue angle was measured from the positive a* axis according to the following equations:

$$h° = \tan^{-1}(b^*/a^*) \text{ if } a^* > 0 \quad \text{(Eq 3)}$$

$$h° = \tan^{-1}(b^*/a^*) + 180° \text{ if } a^* < 0 \quad \text{(Eq 4)}$$

The chroma was calculated according to the following equation:

$$C = \sqrt{(a^{*2} + b^{*2})} \quad \text{(Eq 5)}$$

The total change of color ΔE* was quantified on each sample following Equation 6.

$$\Delta E = \sqrt{[(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]} \quad \text{(Eq 6)}$$

2.3.5. Evaluation of the antimicrobial activity of the coating on pre-cut carrots

Effect on the shelf-life of pre-cut carrots

Frozen pre-cut carrots were provided by Bonduelle Inc. (Sainte-Martine, QC, Canada). Carrot samples were divided into 6 groups: untreated (control), treated with F_1, PF_1, F_2, PF_2 and a commercial antimicrobial (0.03% of Tsunami 100), corresponding to a mix of 15.2% (v/v) of peracetic acid and 11.2% (v/v) of hydrogen peroxide. For treated samples, carrots were dipped in the antimicrobial solution for 30 s, and dried under a laminar flow hood for 15 min. Samples were divided in Nasco Whirl-Pak™ sterile filter bags (Fisher Scientific, Ottawa, ON, Canada) (20 g per bag) then stored at 4 °C until reaching the limit of microbial acceptance (LOA).

On each day of analysis, 60 g of 0.1% (w/v) peptone water (Alpha Biosciences Inc., Baltimore, MD, USA) was added to filter bags containing 20 g of carrots. The carrot samples were mixed during 2 min at high speed (260 rpm) in a Lab-blender 400 Stomacher (Laboratory Equipment,

London, UK), then seeded on Tryptic Soy Agar (TSA, BD Difco, Mississauga, ON, Canada) for total mesophilic flora (TMF) evaluation and on Potato Dextrose Agar with chloramphenicol (PDA-Chl, BD Difco) for Y/M evaluation. Plates were incubated at 37 °C during 48 h for TMF and at 28 °C during 72 h for Y/M, respectively. Results were expressed as bacterial counts (log CFU g^{-1}) and fungal counts (log conidia g^{-1}) during storage at 4 °C.

Effect on L. monocytogenes and P. chrysogenum

L. monocytogenes was stored at -80 °C in Tryptic Soy Broth (TSB) medium (TSB; BD Difco) containing glycerol (10% v/v). Before experiment, bacterial stock cultures were propagated through 2 consecutive 24 h-growth cycles in TSB at 37 °C to reach the concentration of approximately 10^9 CFU mL^{-1} and at the same day of experiment, the grown cultures were diluted in sterile peptone water to obtain a working culture of approximately 10^4 CFUmL^{-1}.

For fungal evaluation, *P. chrysogenum* was propagated through 72 h-growth cycle on PDA-Chl medium at 28 °C ± 2 °C. Conidia were isolated from the agar media using sterile platinum loop, suspended in sterile peptone water, and filtered through sterile cell strainer 40 µm (Fisher scientific, Ottawa, ON, Canada). The filtrate was adjusted to 10^4 conidia mL^{-1}.

Frozen pre-cut carrots were irradiated at the Canadian Irradiation Center at 10 kGy in a UC-15A irradiator (Nordion Inc., Kanata, ON, Canada) equipped with a ^{60}Co source at a dose rate of 10.492 kGy h^{-1} at room temperature to sterilize them before inoculation with selected microorganisms and then stored at 4 °C. Carrot samples were divided into 6 groups as for shelf-life study: untreated carrots (control), treated carrots with F_1, F_2, PF_1, PF_2 or with Tsunami 100 (0.03%). Samples were treated with antimicrobials as described previously, then inoculated with 1 mL of *L. monocytogenes* or *P. chrysogenum* at 10^4 CFU mL^{-1} in order to obtain a final concentration of 10^2 CFU g^{-1}. Samples were then stored at 4 °C during 15 days of storage.
On each day of analysis, 60 g of 0.1% (w/v) peptone water (Alpha Biosciences Inc.) were added to filter bags containing carrots. The carrot samples were mixed during 2 min at high speed (260 rpm) in a Lab-blender 400 Stomacher (Laboratory Equipment), then seeded on PALCAM for *L. monocytogenes* and on PDA-Chl for *P. chrysogenum*. Plates were incubated at 37 °C during 48 h for *Listeria* evaluation and at 28 °C during 72 h for *P chrysogenum*. Results were expressed as bacterial counts (log CFU g^{-1}) and fungal count (log conidia g^{-1}) during storage at 4 °C.

2.4. Statistical analysis

The experiment was done in triplicate and for each repetition a duplicate was analyzed. For each sample, triplicates were analyzed. Analysis of variance (ANOVA), Duncan's multiple range tests for equal variances and Tamhane's test for unequal variances were performed for statistical analysis using SPSS 18.0 software (SPSS Inc, Chicago, IL, USA). Differences between means were considered significant when the confidence interval was lower than 5 % ($P \leq 0.05$).

3. Results and discussion

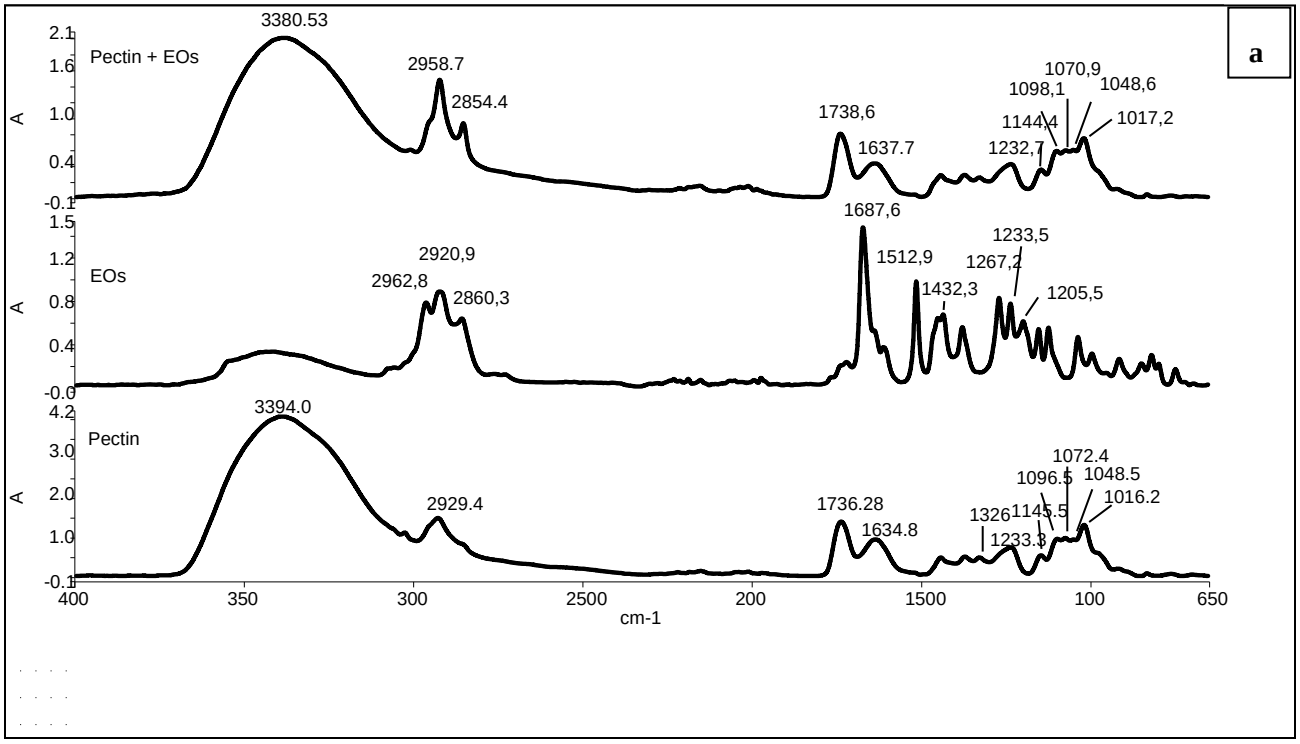
3.3. Characterization of the gelled emulsions

3.3.1. FTIR analysis

FTIR spectrum of pectin

The FTIR spectra presented the interaction of EOs (**Fig. 7.1a**) and Biosecur F440D (**Fig. 7.1b**) with pectin. Regarding the pectin spectrum, OH stretching band covers 3600-3100 cm^{-1}, CH stretching is observed at 3000-2800 cm^{-1}. The fingerprint region is ranged in the region *ca.* 2000-1000 cm^{-1} and can be decomposed into several vibrations bands. In the 1st sub-region 1800-1200 cm^{-1} (state of carboxyl groups), 2 bands correspond to major functional groups

presented in pectin such as bands at 1736 cm^{-1} attributable to C=O stretching of methyl ester (typically used to probe the DE of pectins), 1633 cm^{-1} and 1370 cm^{-1} respectively related to asymmetric (asym) and symmetric (sym) C=O stretching due to the COO^{-} group of polygalacturonic acid, 1441 cm^{-1} to asym CH_3 bending of methyl groups, 1326 cm^{-1} to CH bending and 1232 cm^{-1} to OH bending of carboxylic groups, accordingly to some authors (Acikgoz, 2011, Guo *et al.*, 2014, Kyomugasho *et al.*, 2015). In the 2nd sub-region 1200-1000 cm^{-1} (ring breathing resonance mode of pyranose cycle), characteristic peaks corresponding to the typical profile of carbohydrates include one band at 1144 cm^{-1} attributable to asym C–O–C stretching vibration in glycosidic ring whereas the bands in the region 1100-1000 mostly imply C–C and C–O stretching modes: 1096 cm^{-1} related to C–C stretching of the ring structure, 1072 cm^{-1} to C–O stretching of skeletal vibrations, 1048 cm^{-1} to C–OH stretching of secondary alcohols, 1016 cm^{-1} to C–H–OH stretching in cyclic compounds (and side group vibrations of C–C, C–OH and C–H ring). Such vibrations were confirmed by other authors (Fissore *et al.*, 2013, Marry *et al.*, 2000, Synytsya *et al.*, 2003).



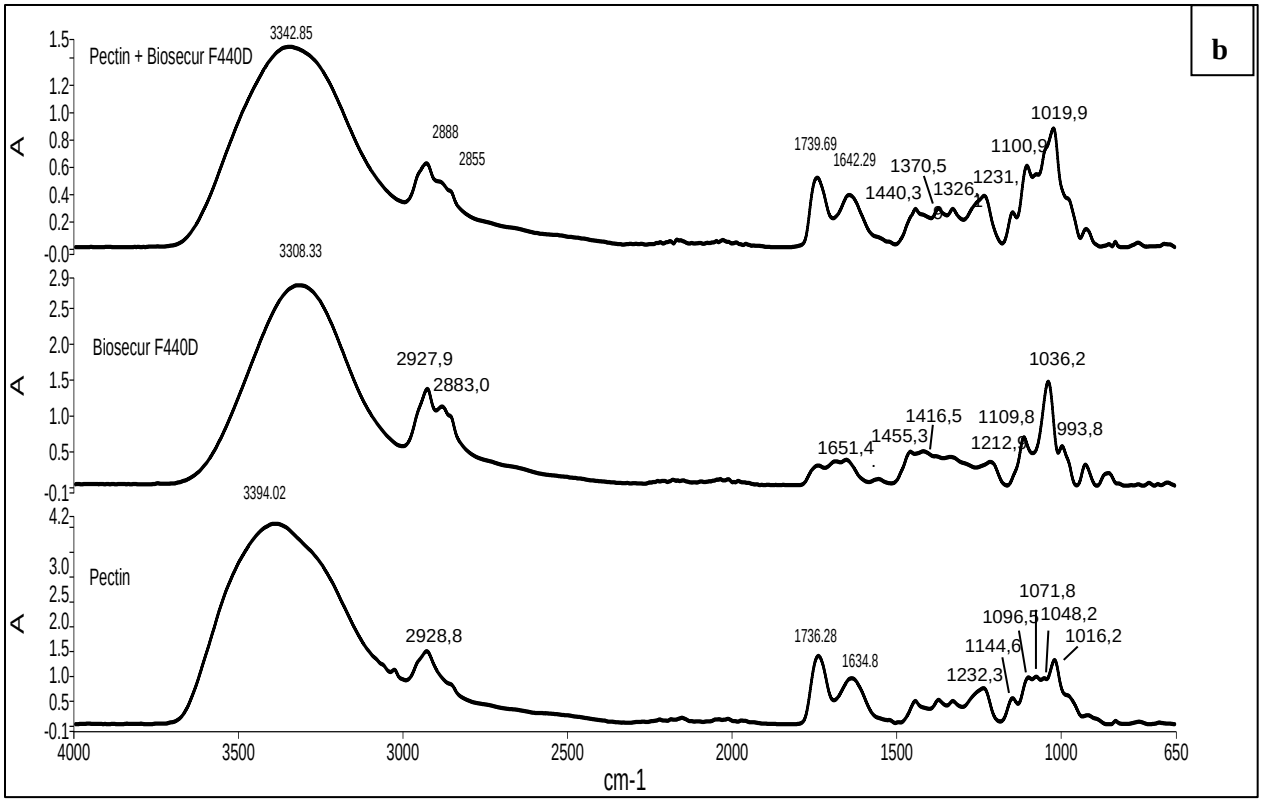


Figure 7.1. FTIR spectra of pectin and pectin incorporating (**a**) EOs and (**b**) Biosecur F440D.

Effect of antimicrobial ingredients on molecular interactions with pectin.

Effect of Biosecur F440D – (pectin + F440D) spectrum. When compared to the spectrum of pectin, a shift of OH stretching band from 3394 cm^{-1} to a lower frequency at 3343 cm^{-1} was observed in presence of Biosecur F440D incorporated in pectin matrix. This feature suggests that pectin-based coating could have an effect on the stabilization of Biosecur F440D by hydrogen bonding with respect to that observed for EOs for which no shift of OH stretching was observed or probably it is due to Biosecur F440D which the OH peak was observed at much lower wavenumber (3308 cm^{-1}). In the range of 2980-2850 cm^{-1} which is related to CH stretching of alkyl groups, an increase of the signal was observed with the appearance of two shoulders at 2888 and 2857 cm^{-1}, related to CH asym and sym stretching of CH_2 in aliphatic compounds and mainly due to glycerol used as an excipient in Biosecur F440D formulation. The characteristic band of pectin at 1635 cm^{-1} (carbonyl stretching of COO^- group in polygalacturonic structure) was shifted towards 1642 cm^{-1} in the complex (Pectin + Biosecur F440D) spectrum, suggesting a possibly interaction made by the pectin hydrophobic pockets (highly methylated carboxylates) with the hydrophobic motif of EOs: the aromatic ring.

Similar results were observed by Majumdar *et al.* (2009) with the entrapment of hesperidin (a flavonoid glycoside) by inclusion complex and by Bosio *et al.* (2012) with the encapsulation of doxorubicin in pectin hydrogels.

Effect of EOs – (Pectin + EOs) spectrum. The incorporation of EOs in pectin matrix allowed the apparition of 2 new bands at 2959 cm^{-1} and 2854 cm^{-1} respectively related to the presence of EOs, which could be observed in IR spectrum of EOs. According to Lv *et al.* (2014), the

incorporation of oil to polysaccharide (i.e. gelatin-gym Arabic) would induce hydrophobic interactions and hydrogen bond.

On the basis of these FTIR results, it can be concluded that the incorporation of Biosecur F440D in pectin matrix may be conducted by hydrogen bonding and CH stretching. These interactions are supposed to enhance the stability of the bioactive compounds. The incorporation of EOs in pectin matrix did not show specific interactions.

3.3.2. Release profile

The release profiles of phenolic compounds from both emulsions (F_1 and F_2) and from their respective gelled emulsions (PF_1 and PF_2) from dialysis membrane in ethanol 10 % at room temperature are shown in **Fig. 7.2**. Results showed that the release of phenolic compounds was slow during the early stage of 60 min and showed 7.7%, 10.7%, 10.7% and 21.3% of cumulative release from PF_2, PF_1, F_2 and F_1, respectively. Afterwards, the release increased and reached 12.2%, 19.5%, 27.1% and 44.6% after 120 min for PF_1, PF_2, F_1 and F_2 respectively. A stabilization was observed after 120 min for F_1 and PF_1 showing a respective release of 27.1 and 12.2 %. After 240 min, a release of 75.8% and 61.9 % was observed respectively for F_2 and PF_2. The release of phenolic compounds was also faster from F_2 than F_1 showing 74.3% at 480 min as compared to 30.2 % for F_1. Similar results were also observed with gelled emulsions where the release of phenolic compounds of PF_2 was 67.7 % compared to 16.6 % for PF_1. It is interesting to note that phenolic compounds release from gelled emulsion PF_1 and PF_2 was slower than their non-gelled counterparts (F_1 and F_2 respectively). A cumulative release of 19.5 % of phenolic compounds from PF_2 was observed after 120 min as compared to 44.6% for phenolic compounds release from F_2 and 16.6% for PF_1 as compared to 30.2 % for F_1 at 480 min. These results suggest that the release of bioactive ingredients from formulated antimicrobials to simulated media got delayed in formulations entrapped in pectin matrix when compared to emulsions, allowing a slower release during storage time.

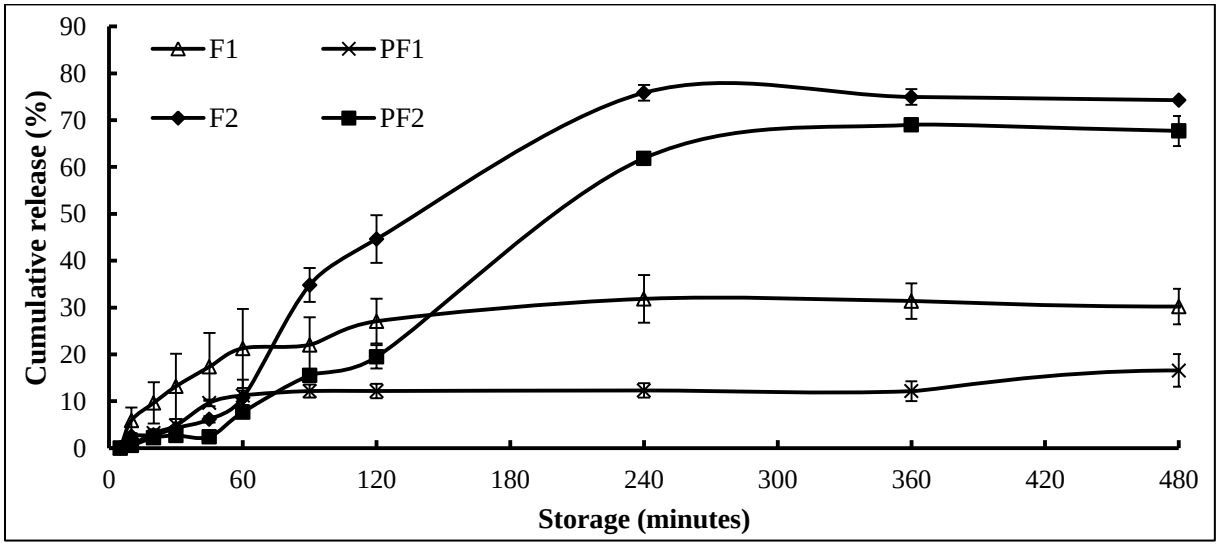


Figure 7.2. Effect of the entrapment of two antimicrobial formulations in pectin matrix on their release profile.

To analyze kinetics and the mechanism of release, the parameters k, n and R^2 values were determined for emulsion and gelled emulsion (**Table 7.2**). The R^2 value for all samples was > 0.8 demonstrating that the Korsmeyer-Peppas model fitted the release of phenolic compounds

from antimicrobial ingredients. The values of '*n*' and '*k*' were found to vary with the entrapment in pectin. The release exponent value of $n < 0.45$ for both F_1 and PF_1 was obtained, which indicates that the Biosecur F440D release was controlled by a Fickian diffusion which is characterized by the retention of an intact polymer during release. However, for F_2 and PF_2, $n > 0.89$ corresponding to a sigmoidal release pattern and ascribed to a Super Case II transport, in which EOs release seemed to be controlled by the polymer relaxation (Wei *et al.*, 2006). This type of release is principally due to the polymer degradation and erosion that often associated with stresses and state-transition in hydrophilic glassy polymers which swell in water or biological fluids (Peppas *et al.*, 1989). The obtained EOs-release behavior is similar to that observed by Paula *et al.* (2010) and Kulkarni *et al.* (2000) where EOs and natural pesticide were released from angico gum/chitosan nanoparticles or from alginate beads crosslinked with glutaraldehyde respectively, showing a non-Fickian behavior (case II transport). The observed release behavior was due to an increase in the crosslinker density in beads made from guar gum and polyacrylamide.

Table 7.2. Kinetic release parameters of natural antimicrobials from emulsions and gelled emulsions.

	n	**k**	**R^2**
F_2	1.4152	0.10	0.9405
PF_2	1,0313	0,09	0.9573
F_1	0.3516	0.25	0.8073
PF_1	0.3385	0.22	0.8769

3.3.3. Total phenols content stability

The stability of TP content in the developed emulsions is presented in **Fig. 7.3**. The objective of this study was to evaluate the effect of pectin incorporation on the relative phenol stability of formulations. For each antimicrobial formulation, results showed a higher TP content into gelled emulsions. This could be related to the presence of some phenolic compounds present in sugar-beet pectin 124.65 ± 7.77 GAE µg/mL. In agreement with these results, Sun *et al.* (1998) have stated that phenolic compounds were also determined in sugar-beet pectin. It is effectively known that beet is rich in different types of phenolic compounds such as esterified ferulic acid and other phenolic monomers, principally vanillin and p-hydroxybenzoic acid (Obata *et al.*, 1963, Sun *et al.*, 1998). Although the contribution on TP was not the same for both formulations, this contribution remains stable during the whole storage, which allows measuring relative stability throughout storage. This different contribution could be due to an interference with Folin-Ciocalteu (FC)'s reagent of some chemical groups in F_1 formulation such as ascorbic acid, organic acids and sugars interacting with pectin as described above in FTIR spectra **(Fig.7.1b)**. Moreover, Ghafar *et al.* (2010) and Sánchez-Rangel *et al.* (2013) have reported that these chemical groups can react with FC, which supports our statements of the different contributions of TP in formulations. It is interesting to note that the incorporation of EOs and Biosecur F440D into pectin allowed a protection of the bioactive compounds and a higher stability of the TP content in the emulsions during storage at room temperature. TP contents in the pectin gel after 90 days of storage amounted to 364 GAE µg/mL in PF_1 and 1534 GAE

µg/mL in PF_2, corresponding respectively to 34 % and 44 % of loss from day 0 as compared to 52 % and 54 % of loss for their respective emulsions F_1 and F_2.

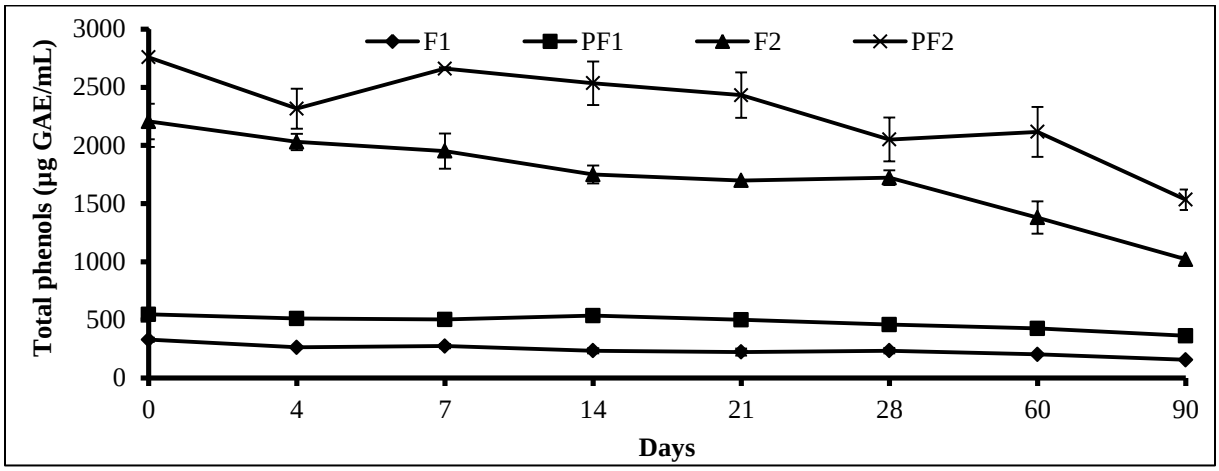


Figure 7.3. Total phenols content of emulsions and their respective gelled emulsions during storage at room temperature.

The determination of the TP content during storage clearly demonstrated that pectin matrix led to TP stabilization during time, suggesting that bioactive ingredients of natural antimicrobials were protected against oxidation over time. Maier *et al.* (2009) also demonstrated that polyphenol-enriched pectin and gelatin gels are valuable sources of phenolic compounds with high antioxidant activity for over a storage period of up to 24 weeks. This result was supported by the obtained results of FTIR showing a shifting of OH band to lower frequency when Biosecur F440D was incorporated in pectin matrix, the appearance of 2 peaks related to aliphatic C–H and strong asym $–CH_3$ stretching vibrations when EOs were incorporated in pectin. .

3.3.4. Color stability

Fig. 7.4 shows the variation of ΔE (4.a), a* (4.b), b* (4.c), L* (4.d), C* (4.e) and H° (4.f) for the emulsions (F_1 and F_2) and their gelled counterparts (PF_1 and PF_2) during 90 days of storage at room temperature. Results show that on day 0, the emulsions displayed lower b*and a* values and higher L* and H° values than their gelled counterparts and no change was observed in the chroma value, indicating that gelled emulsions are darker (lower L* value) and have a yellow color (higher b* value). On the other hand, the total color change of both emulsions and gelled emulsions increased during storage from 1.5 to 13.2 for F_1, from 1.3 to 2.1 for PF_1, from 0.47 to 3.6 for F_2 and from 0.6 to 3.4 for PF_2 on day 90. These results suggest that F_1 was highly affected by color change as compared to F_2, and this correlated with an increase in a*, b* and C* value and a decrease of L* and H° value. According to David Julian McClements (2002), changes in emulsion color result from alterations in their composition or microstructure mainly due to an increase of the droplet size in o/w emulsions over time related to coalescence, Ostwald ripening, or flocculation.

On the other hand, as Biosecur F4440D is a citrus extracts concentrate, consequently the color of F_1 could be changed because of enzymatic oxidation *i.e.* polyphenol oxidases (PPO) activity in presence of oxygen (Coseteng *et al.*, 1987, Chang Y Lee *et al.*, 1990, Rocha *et al.*, 2002). Quinones are the end-products of oxidation reaction and can be polymerized with other quinones or phenolics termed as melanin producing brown pigments (Holderbaum *et al.*, 2010). To control PPO/ peroxidase (POD) in food products, various anti-browning technologies such as high pressure processing (HPP) or heat treatments are used in food industry. The addition of

organic acids or antioxidant agents allows controlling the atmospheres in order to reduce the oxygen availability in foodstuff (MEM Almeida *et al.*, 1995, Kaushik *et al.*, 2016).

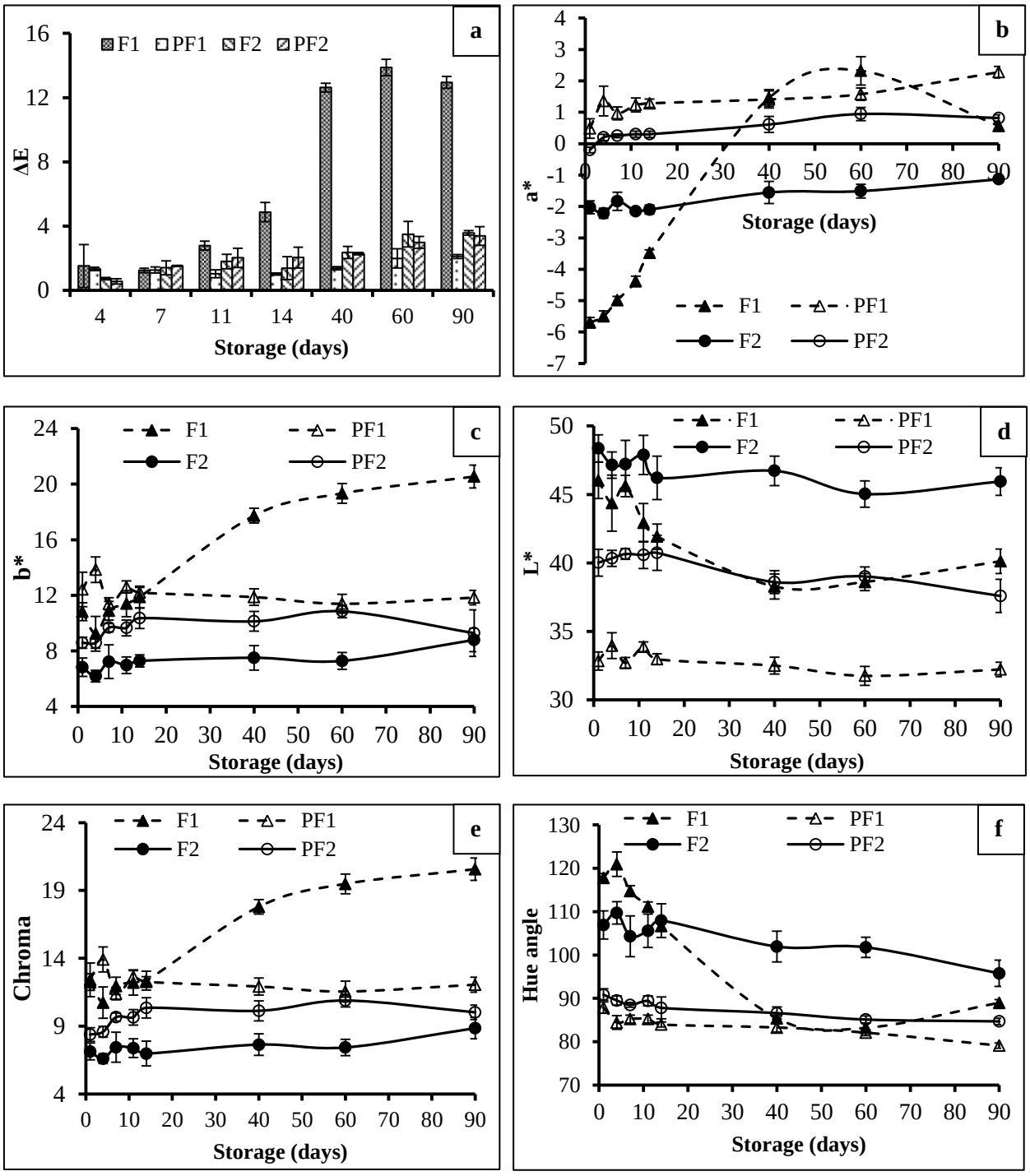


Figure 7.4. Color attributes of emulsion and gelled emulsion (**a**) ΔE, (**b**) a*, (**c**) b*, (**d**) L*, (**e**) Chroma and (**f**) Hue angle during storage time at 25 °C.

In current study, pectin-based matrix was able to play the role of an anti-browning coating as ΔE was significantly lower ($P \leq 0.05$) for gelled emulsion of PF_1 (≈ 2.1) on day 90 as compared to 13.2 for F_1. These observations suggest that the color of PF_1 was well maintained during storage at room temperature as compared to F_1. This phenomenon could be due to the stabilization of the TP content present in Biosecur F440D by the pectin matrix against oxidation over time.

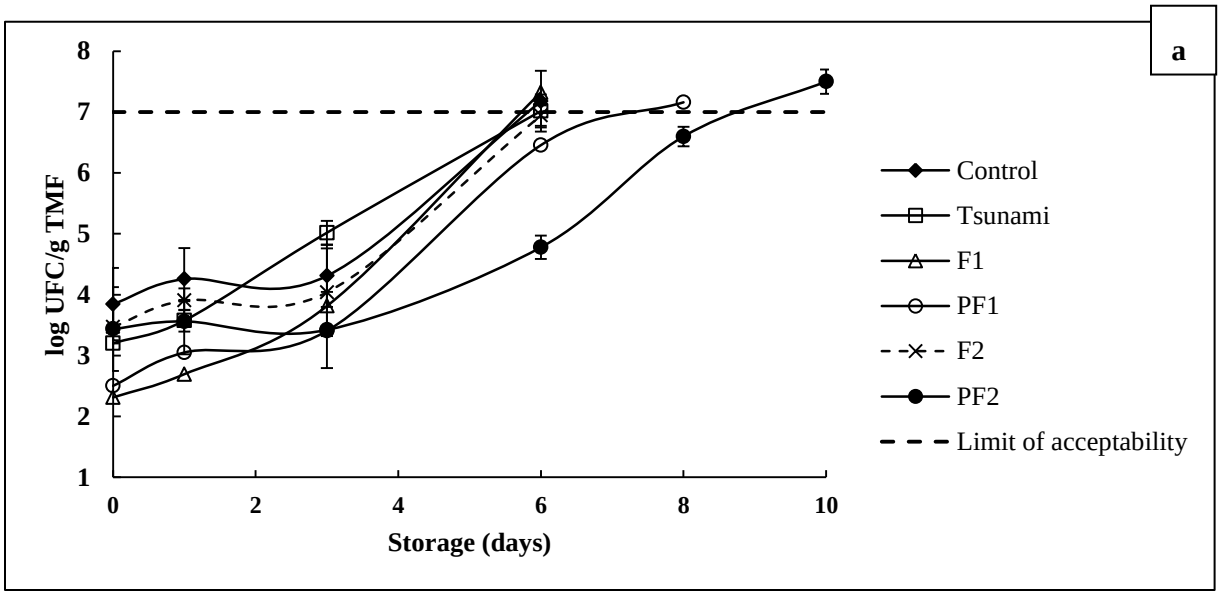
3.1. Effect of gelled emulsion on the antimicrobial activity

Shelf-life is defined as the LOA when the TMF and Y/M counts reach a level of 10^7 $CFUg^{-1}$ and 10^4 conidia g^{-1} respectively, according to current authorities regulation (MAPAQ, 2009b). The results of TMF and Y/M counts of carrot samples are presented in **Fig. 7.5**. Results show that on day 0, the antimicrobial formulations F_2, PF_2 and the Tsunami treatment were not efficient in reducing the count of TMF. However, F_1 and PF_1 were highly effective in reducing TMF on the surface of carrots and showed 1.59 and 1.3 log reductions at day 0 compared to the control.

Based on TMF count (**Fig. 7.5a**), shelf-life of the control and carrots treated with F_1, F_2 and Tsunami was reached after 6 days. On the other hand, both PF_1 and PF_2 allowed reaching the LOA at day 7 and 9 respectively showing a respective shelf-life extension of 1 and 2 days.

By considering the Y/M counts (**Fig. 7.5b**), no significant effect ($P > 0.05$) of treatments was observed on day 0 and the Y/M counts were ≈ 1.7 log conidia/g. Thereafter, control showed a shelf-life of 2 days whereas F_1, F_2, PF_1 and Tsunami showed a shelf-life of 3 days, and PF_2 showed a shelf-life of 4 days, therefore leading to a shelf-life extension of 2 days.

Based on these results, the entrapment of natural antimicrobials in pectin matrix allowed a shelf-life improvement of pre-cut carrots. This could be related to the stabilization of EOs in pectin matrix by maintaining TP content and by slowing down the release of bioactive ingredients as observed in sections 3.3.2 and 3.3.3.



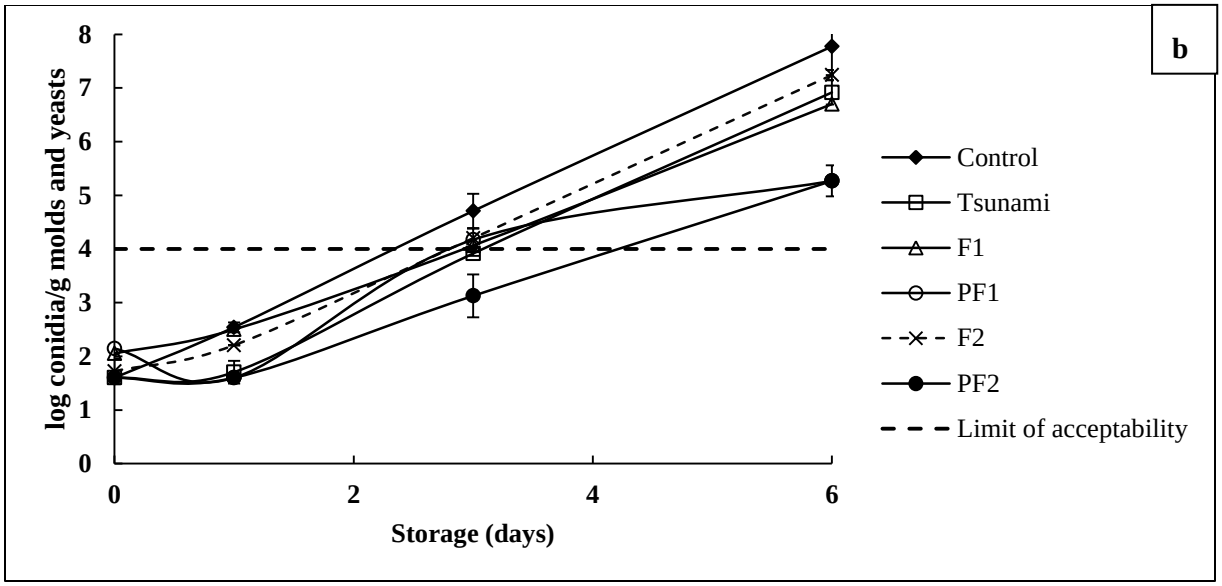


Figure 7.5. Effect of the antimicrobial coating on the count of (**a**) TMF and **(b)** Y/M in carrots during storage at 4 °C.

The growth of *L. monocytogenes* on carrot samples during storage at 4 °C is shown in **Fig. 7.6a**. The obtained results showed that for untreated carrots (control), *L. monocytogene*s count increased from 2.6 log CFU/g on day 0 to 8.0 log CFU/g on day 15 during storage at 4 °C. The application of the chemical preservative (Tsunami) on carrot surface allowed a 1.4-log reduction on day 15. For carrots treated with both F_2 and PF_2, a significant reduction ($p \leq 0.05$) was observed on day 5 as compared to untreated carrots with 2.4 and 2.1-log reductions for F_2 and PF_2, respectively. On day 8, PF_2 completely reduced the count of *L. monocytogenes* under the limit of detection (1.59 log CFU/g) as compared to F_2 where the *L. monocytogenes* level was 2.2 and 2.1 log CFU/g on day 8 and 15, respectively. Similar results were observed with F_1 where 4.9 log CFU/g were observed on day 15 compared to a complete elimination of *L. monocytogenes* with PF_1 (< 1.59 log CFU/g).

The growth of *P. chrysogenum* on carrot samples during storage at 4 °C is shown in **Fig. 7.6b**. Results show that no significant difference ($P > 0.05$) was observed between treatments during the first week of storage and a decrement in *P. chrysogenum* count for all samples was observed. In fact, *Penicillium* is a strain that has difficulty to adapt to cold storage (Plaza *et al.*, 2003). The optimal conditions are storage at room temperature. After 8 days, a quick increase of *P. chrysogenum* population was observed to reach 3.1 and 3.3 log conidia/g on day 15 for carrots treated with Tsunami and untreated carrots respectively. However, after treatment with F_1 and F_2 and their gelled counterparts PF_1 and PF_2, the count of *P. chrysogenum* reached 1.75 log conidia/g on day 15 for carrots treated with F_1 as compared to a total inhibition (< 1.59 log conidia/g) in samples treated with F_2, PF_1 and PF_2.

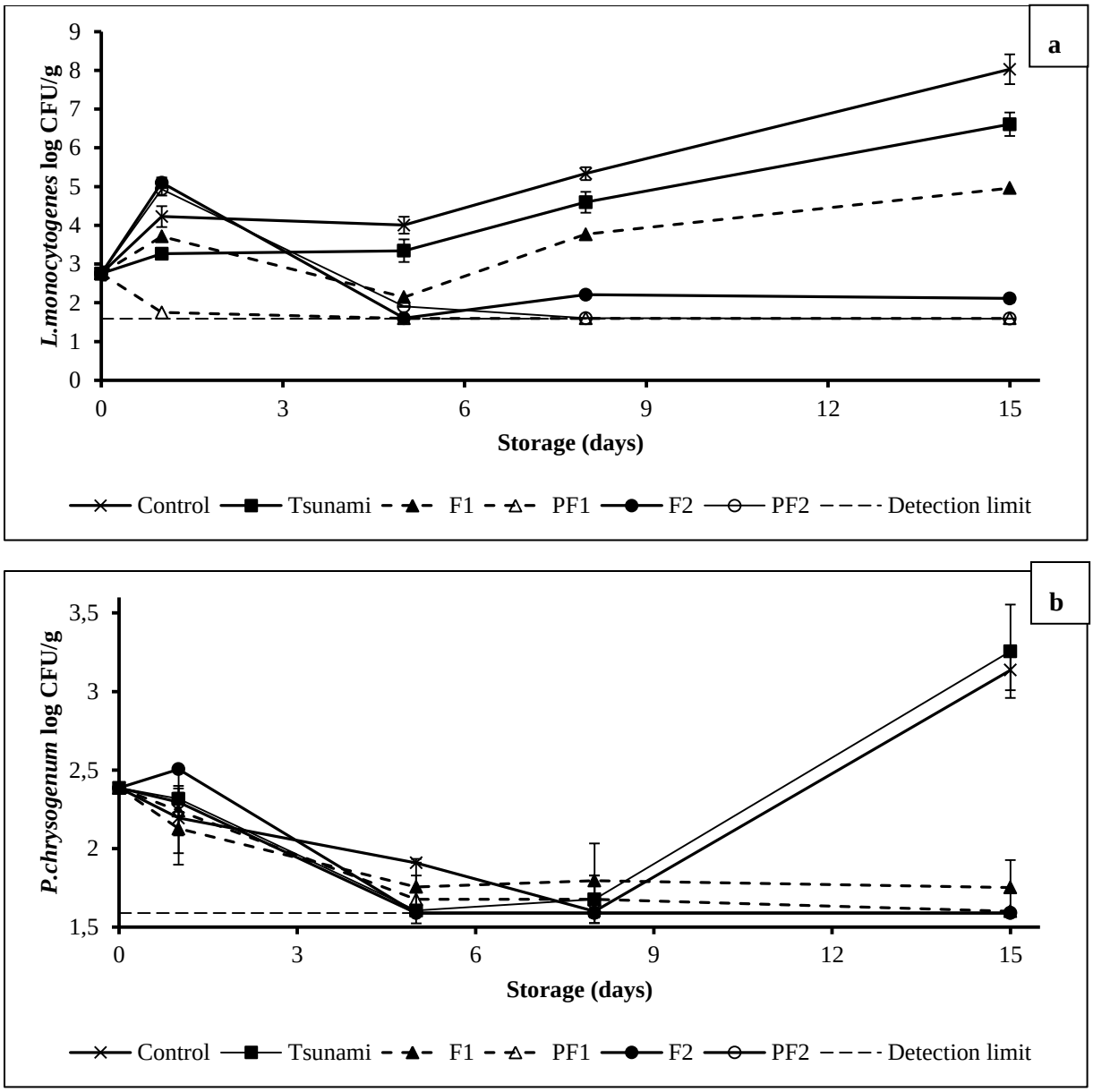


Figure 7.6. Effect of the antimicrobial coating on the population of (**a**) *L. monocytogenes* and **(b)** *P. chrysogenum* in carrots during storage at 4 °C.

Based on these microbiological results, it is obvious that the addition of pectin to the emulsions have permitted to improve their antimicrobial activity to a significant higher inhibiting effect of *L. monocytogenes* and *P. chrysogenum* in pre-cut carrots. It is to be noted that the efficiency of pectin-based formulations developed in this study were higher than the chemical preservative (*i.e.* Tsunami) widely used for vegetables treatment.

Similar results were observed in some previous studies with the encapsulation of the poorly water-soluble antifungal agent “clotrimazole” in hydroxypropyl methylcellulose based matrix against *C. albicans*. This was related to a better solubility of clotrimazole in an emulgel (Shahin *et al.*, 2011). Results obtained in our study could be related to the reduction in oxygen

availability and nutrient diffusions by forming microstructure on food surface (Baka *et al.*, 2016, Yosra Ben-Fadhel *et al.*, 2018). In fact, cells grow in the aqueous phase of food. The presence of fat increases the complexity in microstructure. As fat droplets are dispersed in the continuous phase of the emulsions and gelled emulsions, this phenomena decreases the amount of water available necessary for cell growth (Baka *et al.*, 2016).

On the other hand, the effectiveness time of many natural antimicrobial compounds applied on food matrix is short and their effectiveness is limited during storage time. This is mainly due to the instability of their ingredients *i.e.* polyphenols and their sensitivity to physical and chemical conditions, such as light, heat, some pH conditions and oxidation (Wei Lu *et al.*, 2016, Maier *et al.*, 2009). Wei Lu *et al.* (2016) showed that the encapsulation of polyphenols in biopolymer matrix *i.e* poly (lactic acid), poly(lactic-co-glycolic acid) can achieve high encapsulation efficiency, and encapsulated polyphenolic compounds showed a large increase in their solubility and physical/chemical stability. Ong *et al.* (2015) demonstrated that the use of a gelled emulsion using nonionic surfactants (Tween 80/Span 20), alginate and palm oil prevented phase separation and therefore improved the stability of the emulsion. Severino *et al.* (2014a) also encapsulated mandarin EO in gelatin microemulsions containing chitosan by mechanical homogenization and microfluidization in the presence of Tween 80. This process improved the bioactivity and the physical stability of the bioactive compounds and minimized their impact on the aroma and taste of the food. Han *et al.* (2008) have also demonstrated that the encapsulation of ferrous fumarate, ascorbic acid and β-carotene in alginate/chitosan microbeads increased their stability and protected these bioactive molecules against temperature, humidity and acidic pH. This improvement of bioactivity seems to be intimately related to the functional properties of polymer matrix. Otherwise, contrary to what was obtained in this study, Skandamis *et al.* (2000) revealed a high reduction in the antimicrobial inhibitory activity of oregano EO against *S. Typhimurium* in presence of gelatin gel. Different polymers could reduce the diffusion rate of active compounds by the structure of gel matrix which may explain these results. Antwi *et al.* (2006) demonstrated that the firmness of the gel has an influence on the dynamics growth of bacteria. Firmer gels with higher concentration of gelatin inhibited the growth of *Listeria innocua***,** in contrast to less firm gels with lower gelatin concentration.

The emulsions and gelled emulsions evaluated as bioactive coatings in this study had a significant effect ($P \leq 0.05$) in reducing *L. monocytogenes* and *P. chrysogenum* population in carrot samples and extending their shelf-life (**Fig. 7.5-7.6**). This efficiency could be related to a better controlled release, a higher protection and a high stability preservation of bioactive ingredients (Biosecur F440D, EOs) during storage, especially against oxidation.

4. Conclusions

In this study, it was illustrated that two assessed microstructural systems (emulsion and gelled emulsion) had significant inhibiting properties on the microbial growth of *L. monocytogenes* and *P. chrysogenum*, the TMF and Y/M populations in pre-cut carrots*.* The stabilization of bioactive ingredients such as polyphenolic and terpenic compounds during time and their controlled release is the main cause of this bioactivity improvement. In the case of emulsion systems, it can be concluded that the formulation of EOs and of citrus extracts can control *L. monocytogenes*, *P. chrysogenum* and total flora during the first days of storage. However, they lose rapidly their bioactivity during storage. This study proposes gelled emulsion as bioactive formulations based on pectin as an encapsulation matrix, giving a suitable microstructure to influence microbial dynamics and to improve the bioactivity of both EOs and citrus extracts. In order to understand the influencing mechanics of such an encapsulation method, FTIR, controlled release and stability studies were conducted. All characterizations showed that pectin matrix improved the stability of bioactive compounds and generated a better controlled release.

Acknowledgements

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CHAPITRE 8: NATURAL EXTRACTS- LOADED NANO-LIPOSOME: PREPARATION AND CHARACTERIZATION

Nano-liposome chargé en extraits naturels: préparation et caractérisation

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CONTRIBUTIONS DES AUTEURS

Ce travail de recherche a été réalisé sous la supervision du Pr. Monique Lacroix. Toutes les expériences ont été réalisées en laboratoire par Yosra Ben Fadhel et la discussion et les protocoles ont été assistés par Stéphane Salmieri. L'article a été écrit par Yosra Ben Fadhel, tandis que les corrections et révisions ont été effectuées par Stéphane Salmieri, Dr Behnoush Maherani, et Pr. Monique Lacroix.

RÉSUMÉ

Des extraits antimicrobiens naturels hydrophiles ou hydrophobes encapsulés dans des liposomes de grade alimentaire ont été développés par sonication. Les paramètres de sonication et le type et la concentration de la lécithine ont été étudiés afin d'optimiser les conditions de production pour obtenir des nanoliposomes contenant des extraits naturels hydrophiles et hydrophobes avec de bonnes propriétés physicochimiques, une bonne stabilité et une haute efficacité d'encapsulation. Les extraits antimicrobiens naturels hydrophiles et hydrophobes sont constitués respectivement de Biosecur F440D et d'un mélange d'huiles essentielles. De plus, l'interaction entre les composés bioactifs et le liposome a été caractérisée par spectroscopie infrarouge à transformée de Fourier (FTIR) et titrage calorimétrique isotherme (ITC).

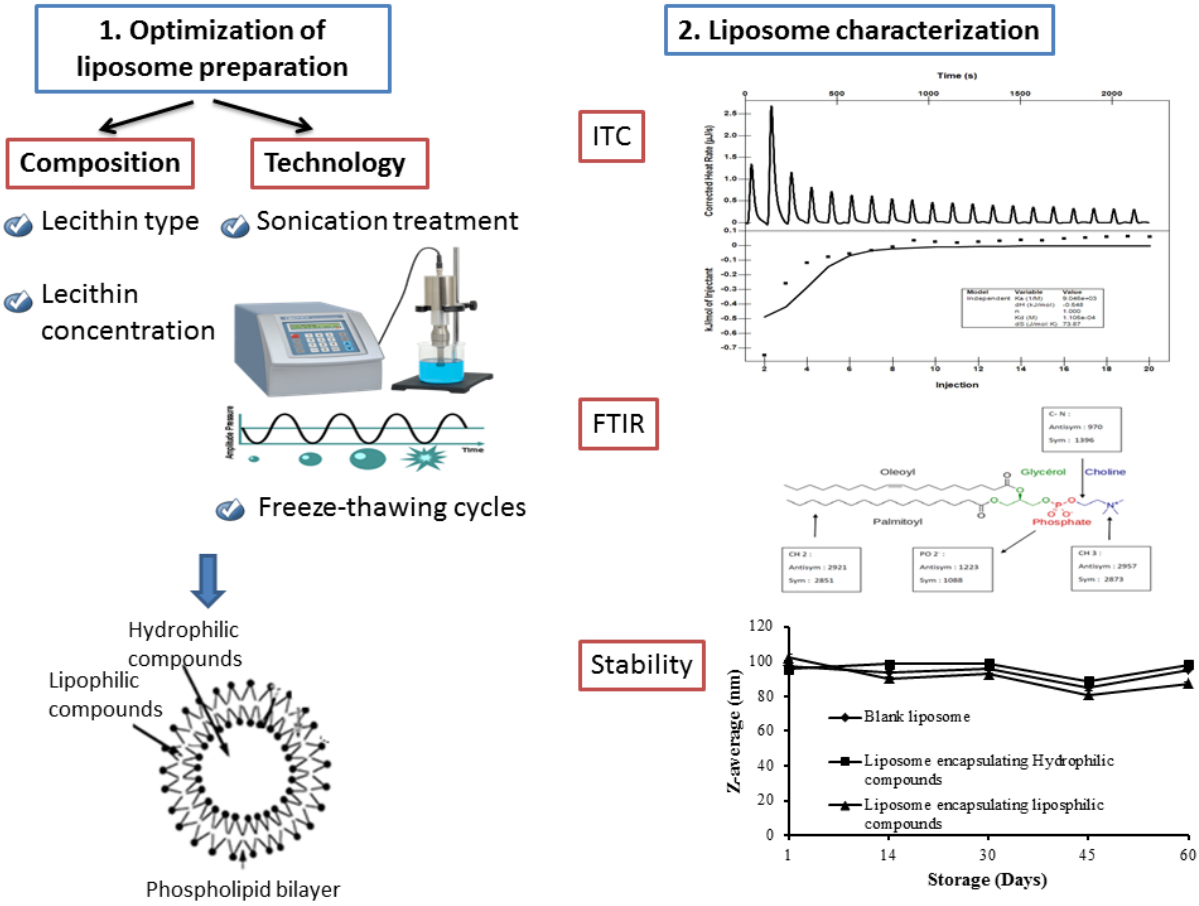
Les résultats ont montré que l'utilisation de lécithine déshuilée permettait une efficacité d'encapsulation plus élevée des extraits hydrophiles et hydrophobes. Un traitement aux ultrasons pendant 10 minutes à 70% de la pleine puissance et 1,5% de lécithine déshuilée a également permis la formation de liposomes les plus stables. De plus, le traitement avec 5 cycles de congélation (-80 °C) et de décongélation (+ 40 °C) a permis d'augmenter l'efficacité d'encapsulation des extraits hydrophiles. D'après une analyse ITC, le liposome a plus d'affinité pour encapsuler le Biosecur F440D que l'huile essentielle. L'incorporation de Biosecur F440D et d'une huile essentielle a été réalisée notamment par déformation antisymétrique éthyle, déformation symétrique méthyle, étirement P-O-C et étirement symétrique PO_2^-. Le présent système est respectueux de l'environnement en ce qui concerne la biocompatibilité et il est très approprié pour une application alimentaire et pour l'industrie alimentaire.

ABSTRACT

Food grade liposome nanoencapsulating hydrophilic or hydrophobic natural antimicrobial extracts were developed using sonication. Sonication parameters, lecithin type and concentration were investigated in order to optimize the conditions of production to obtain nanoliposomes containing hydrophilic and hydrophobic natural extracts with good physicochemical properties, good stability and high encapsulation efficiency. The hydrophilic and hydrophobic natural antimicrobial extracts consisted of Biosecur F440D and essential oils mixture respectively. Also, the interaction between bioactive compounds and liposome was characterized by Fourier-transform infrared spectroscopy (FTIR) and isothermal titration calorimetric (ITC).

Results showed that the use of deoiled lecithin allowed a higher encapsulation efficiency of both hydrophilic and hydrophobic extracts. A sonication treatment during 10 minutes at 70 % of full power and 1.5 % of deoiled lecithin allowed also the formation of most stable liposome. Also, the treatment with 5 cycles of freezing (-80 °C) and thawing (+ 40°C) allowed the increase of the encapsulation efficiency of hydrophilic extracts. Based on ITC analysis, liposome has more affinity to encapsulate Biosecur F440D than essential oil. The incorporation of Biosecur F440D and essential oils was conducted especially by ethyl antisymmetric deformation, methyl symmetric deformation, P-O-C stretching and PO_2^- symmetric stretching. The present system is environmentally friendly with respect to biocompatibility and it is very suitable for food application and for food industry.

Keywords: liposome, lecithin, sonication, bioactive compounds, encapsulation


1. Optimization of liposome preparation
Composition
Technology
Lecithin type
Sonication treatment
Lecithin concentration
Freeze-thawing cycles
Hydrophilic compounds
Lipophilic compounds
Phospholipid bilayer
2. Liposome characterization
ITC
Time (s)
Corrected Heat Rate (µJ/s)
kJ/mol of injectant
Injection
FTIR
Oleoyl
Glycérol
Choline
Palmitoyl
Phosphate
Stability
Z-average (nm)
Blank liposome
Liposome encapsulating Hydrophilic compounds
Liposome encapsulating lipophilic compounds
Storage (Days)

1. Introduction

Encapsulation of bioactive compounds can improve their activity, improve their stability, assure a controlled release, prevent lipid peroxidation, increase the food shelf-life and reduce the cost of production of the food (Ghosh *et al.*, 2014, Salvia-Trujillo *et al.*, 2014). Encapsulating plant extracts can also prevent volatilization and oxidation of their active compounds. Moreover, encapsulation could mask the strong aroma and prevent the degradation of the active compounds (MIS Santos *et al.*, 2017). Among different encapsulation techniques, liposomes have promising advantages because of their ability to encapsulate the hydrophilic and the hydrophobic compounds simultaneously (Maherani *et al.*, 2012b). Liposomes are vesicles composed by one or more phospholipid bilayers formed following an interaction between phospholipids and water molecules. The exposition of the hydrophilic headgroups of phospholipids to the aqueous phases begets the alignment of the hydrophobic hydrocarbon tails to face each other in a bilayer. This mechanism allows forming vesicles known as liposome able to encapsulate hydrophilic molecules inside the core of the vesicle and hydrophobic compounds between their hydrophobic tails.

The formation of liposome mainly consists of (a) bulk methods, based on the transfer of phospholipids from an organic phase into an aqueous phase by using high energy and (b) film methods, in which lipid films are first formed using organic solvent and subsequently hydrated to give liposomes (Yogita P. Patil *et al.*, 2014). Liposomes could have several sizes, polydispersity and lamellarity by subjecting them to homogenization, sonication, extrusion or freeze–thawing cycles (Yogita P. Patil *et al.*, 2014). Although liposomes preparation methods are well documented in the literature, their application was almost restricted to the pharmaceutical field for drug delivery, vaccine adjuvants or as diagnostic tools. Few investigations were done for their application in the food industry (Benita, 2005, Jemal *et al.*, 2003). Cui *et al.* (2015) investigated liposome-encapsulated clove oil in tofu and showed a reduction in the growth of bacteria that secrete pore-forming toxins (*ie*. *S. aureus*). According to the same authors, pore-forming toxins would insert into the liposome membranes and form pores which activated clove oil release from liposome. Laridi *et al.* (2003) encapsulated nisin Z in liposome for application on Cheddar cheese during fermentation and sh0owed a long term stability of the antimicrobial peptide (27 days at 4°C). Other studies showed that liposomes encapsulating vitamins E and C maintained the heat stability of vitamins and their activity without modifying the sensory property of the orange juice (Marsanasco *et al.*, 2011). Liposomal system was also used to encapsulate fish oil and nisin for application in yogurt (Ghorbanzade *et al.*, 2017, Imran *et al.*, 2015). The liposome-encapsulated nisin coated by chitosan showed higher Tg (glass temperature), improved thermal stability at 123 °C and less mass loss when nisin was encapsulated and coated with chitosan. This nisin encapsulated into liposome and coated with chitosan showed higher antimicrobial properties against *S. aureus*, *E. faecalis* and *L. monocytogenes* with better controlled release of nisin over time (Niaz *et al.*, 2018, Tan *et al.*, 2016). Whey protein isolate was also evaluated as a coating matrix for liposome to encapsulate quercetin and showed an improvement in the sensory profile of a whey protein based drink, an increase in storage stability and a better stability under stomach conditions (M. Frenzel *et al.*, 2015a).

In this study, an optimization of the encapsulation of a mixture of both hydrophilic and hydrophobic active ingredients such as Biosecur F440D and essential oil mixture respectively was done. The characterisation of the interaction between active ingredients and liposome was evaluated by Fourier-transform infrared spectroscopy (FTIR) and Isothermal titration calorimetry (ITC). Finally the stability of the developed liposome during storage was also evaluated.

2. Material and methods

2.1. Material

Two sunflower lecithins were used in this study in order to select the lecithin allowing the formation of a more stable liposome having the highest encapsulation efficiency: deoiled lecithin provided from Austrade Inc. (Palm beach Gardens, FL, USA) and fluidic lecithin provided from Compass Foods Company (Singapore). Their phospholipid composition is detailed in **Table 8.1**.

Table 8.1. Phospholipid composition of sunflower lecithin's used in this study *.

		Lecithin type	
Phospholipids	**Chemical structure**	**Deoiled lecithin**	**Fluidic lecithin**
Phosphatidylcholine (PC)	$C_{46}H_{84}NO_8P$	23	8
Phosphatidylethanolamine (PE)	$C_{40}H_{80}NO_8P$	18	3
Phosphatidylinositol (PI)	$C_{47}H_{83}O_{13}P$	8	11
Phosphatidic acid (PA)	$C_5H_9O_8P$	2	2

* Data provided by the suppliers of lecithin

Mediterranean, pan tropical and citrus essential oils (EOs) were provided by Zayat Aroma (Bromont, Qc, Canada) and their main constituents are presented in **Table 8.2**. Biosecur F440D (a hydrophilic citrus extracts) was provided by Biosecur Inc. (Mont St-Hilaire Québec, Canada). Triton 100x and Folin Ciocalteu were purchased from Sigma Inc. (Oakville, ON, Canada). Sodium carbonate was analytical grade and was purchased from Labmat Inc. (Québec, QC, Canada).

Table 8.2. List of organic essential oils and their composition

Common name	Compositions (%)*
Pan tropical EO	E-cinnamaldehyde (58.88), cinnamyl acetate (3.14), eugenol (6.52)
Citrus EO	Citral B (30.66), citral A (39.48), geraniol (7.75), geranyl acetate (4.42)
Mediterranean EO	Carvacrol (39.45), thymol (14.55), γ-terpinene (11.2), p-cymene (21.05)

* Data provided by the supplier of essential oils
EO: essential oil

2.2. Optimization of liposome preparation

2.2.1. Sonication Method

To prepare blank liposome, deoiled lecithin was dissolved in deionized water under magnetic stirring to obtain a final concentration of 1% (w/v). Sonication treatment was evaluated in order to determine the optimal parameters to obtain the most stable liposomes. To optimize the sonication method the lecithin suspension was subjected to different times of sonication 5, 10, 20 and 30 min (5 on, 2 off) with varying sonication intensities at 25, 50 and 70 % of full power to form liposome vesicles. Lecithin based solutions were kept in ice bath to control their temperature during treatment.

2.2.2. Selection of lecithin type and concentration

Three groups of liposomes (blank liposomes, liposomes encapsulating Biosecur F440D and liposome encapsulating essential oils mixture) prepared with two types of lecithin (deoiled and fluidic sunflower lecithin) were studied: Aqueous phase contains lecithin dissolved in deionized water (1% w/v) prepared under magnetic stirring. Then after, for liposome encapsulated hydrophobic compounds, the essential oils mixture (750 ppm) was added to aqueous phase. For liposome encapsulating hydrophilic compound, 1000 ppm of Biosecur F440D were added to aqueous phase. Prepared solutions were magnetically stirred and then treated during 10 minutes with sonication at 70 % of amplitude (5 on, 2 off) in an ice bath renewable each 5 minutes of treatment without considering the stop time.

To determine the optimal encapsulation efficiency conditions, different concentrations of lecithin were tested (1 %, 1.5 %and 2 %) and the preparation of liposomes was done as previously described.

2.2.3. Freeze thawing process

In order to evaluate the effect of freeze-thawing process on properties of prepared liposomes, five cycles of freezing at -80 °C and thawing at + 40 °C were carried out on blank liposomes and liposomes encapsulating hydrophilic and hydrophobic compounds. Vesicles were then treated 10 min with sonication at 70% of full power as described previously.

2.3. Liposome characterization

2.3.1. Particle size, PDI and zeta potential determination

The mean diameter, size distribution and ζ-potential of liposomes were determined upon empirical dilution of the liposome and by using the dynamic light scattering (DLS) technique with a Malvern Zetasizer Nano-ZS (ZEN3600) (Malvern Instruments Inc., Westborough, MA) equipped with a DTS Nano software (version 6.12) (Malvern Instruments Inc., Westborough, MA, USA). To avoid multiple scattering effects, liposomes were diluted (1:50), and then, the sample was put into a standard capillary electrophoresis cell equipped with gold electrodes for the evaluation of the particle size, the polydispersity index (PDI) and the zeta potential. The results (n = 3) were obtained after 3 runs. Results were presented as an average diameter of particle (z-average) with the PDI and ζ-potential of the liposomes allowing to judge the stability of the liposome preparation (Maherani *et al.*, 2012b).

2.3.2. Entrapment efficiency

The liposomal suspension was centrifuged at 60,000 rpm at 4 °C for 2 h to discard the encapsulated active compounds. Then, the amount of total phenolic compounds added to liposome suspension (initial active compounds) and present in the supernatant (unloaded active compounds) was measured using the Folin–Ciocalteau assay. Entrapment efficiency was calculated by the following equation:

$$\mathrm{EE}\ (\%) = \frac{\mathrm{C\ intial} - \mathrm{C\ sup}}{\mathrm{C\ initial}} \times 100$$

Where:

$C_{initial}$: The initial total phenol added to the liposome after disruption with 1% Triton® X-100 to release the encapsulated active compounds.

C_{sup}: the unloaded phenolic compounds determined in the supernatant.

The measurement of the total phenol content was carried out using a Folin-Ciocalteu colorimetric method according to Dewanto *et al.* (2002). Measurements were done at 760 nm versus the blank prepared similarly with water and ethanol for hydrophilic and hydrophobic compounds respectively. All values were expressed as mean (milligrams of gallic acid equivalents per g of antimicrobial compounds). Gallic acid was used as standard.

2.3.3. Isothermal titration calorimetry (ITC) evaluation

The ITC was carried out to evaluate the interaction between antimicrobials and liposome. Blank liposome was first prepared by solubilizing deoiled lecithin sunflower at 2.3 mM under magnetic agitation. The solution was then treated with sonication using the same parameters as described previously. The major essential oil used in the essential oil mixture, pan tropical essential oil, was solubilized in DMSO at a concentration of 6.3 mM. Biosecur F440D was prepared in Milli-Q water at a concentration of 40 mM. All solutions were degassed using Degassing station before analysis.
The experiments were performed at 20 °C with an isothermal titration calorimeter (Nano ITC, TA instrument, New Castle, DE, USA). For the titration of Biosecur F440D, experiment consisted of 20 injections of 2.02 µL of Biosecur F440D at 40 mM into 300 µL of blank liposome at 2.3 mM with an interval of 120 seconds between injections.

For pan tropical essential oil titration, experiment consisted of 40 injections of 2.5 µL of essential oil dissolved in DMSO (6.3 mM) into 300 µL of blank liposome suspension at 2.3 mM of lecithin supplemented with the same quantity of DMSO as used in essential oil solution. For both titration experiments, the blank liposome suspension in the cell was stirred at 150 rpm. Data was analyzed and fitted using the NanoAnalyze software v2.3.6 (TA Instruments). Results were expressed as the heat rate released upon each injection. The major thermodynamic parameters: binding affinity (Ka), dissociation constant (Kd), enthalpy of the interaction (ΔH) and entropy of the interaction (ΔS) were determined. Binding affinity (Ka) represent the strength of the binding interaction between a single biomolecule to its binding partner. Dissociation constant (Kd) is the inverse of Ka. Thus, the smaller Kd value (the higher Ka), the greater the binding affinity of the molecule for its target. The larger the Kd value (the lower Ka), the more weakly the target molecule and ligand are attracted to and bind to one another. The yields of binding enthalpy (ΔH) and the binding entropy (ΔS) for the reaction provide unique information about the forces that dominate the binding reaction. Polar interactions tend to contribute favorably to the enthalpic component, whereas entropically favored interactions tend to be more hydrophobic.

2.3.4. FTIR study

The FTIR spectra of blank liposomes, liposomes encapsulating Biosecur F440D and liposomes encapsulating essential oils mixture were recorded using a Spectrum One spectrophotometer (Perkin-Elmer, Woodbridge, ON, Canada) equipped with an attenuated total reflectance (ATR) device for solids analysis and a high-linearity lithium tantalate detector. Spectra were analyzed using Spectrum 6.3.5 software within the spectral region of 650–4000 cm^{-1} with 64 scans recorded at a 4 cm^{-1} resolution. To remove interference with water and free antimicrobials, the liposomal suspensions were centrifuged at 60,000 rpm at 4 °C for 2 h and the supernatant containing the non-loaded active compounds were discarded. The lower phase has been recovered and analyzed by FTIR. After attenuation of total reflectance and baseline correction, spectra were normalized with a limit ordinate of 1.5 absorbance units.

2.4. Stability analysis

Stability of the developed liposomes encapsulating hydrophilic or hydrophobic antimicrobial extracts was evaluated during 60 days of storage at 4 °C for the vesicle size, PDI and ζ-Potential (mV) determination as compared to blank liposome.

2.5. Statistical analysis

Each experiment was done in 3 repetitions. For each repetition, a triplicate was analyzed. Analysis of variance (ANOVA), Tukey's multiple range tests for equal variances and

Tamhane's test for unequal variances were performed for statistical analysis using SPSS 18.0 software (SPSS Inc, USA). Differences between means were considered significant when the confidence interval was lower than 5 % ($P \leq 0.05$).

3. Results and discussion

3.1. Optimization of sonication parameter

Results obtained for the determination of the optimal sonication treatment are shown in **Table 8.3**. Results showed that vesicle sizes were reduced by increasing i) the amplitude (sonication power) and ii) the sonication time. Vesicle sizes reduced from 749.1 nm for non-treated lecithin suspension to 81.9, 70.4 and 61.4 nm for sonicated lecithin for 30 minutes treatment at respectively 25%, 50% and 70% of full power. Obviously, high-power sonication induced stronger mechanical vibrations and produced more powerful shock waves, thereby resulting in smaller vesicle size than those produced in low-power sonication. Vesicle produced by 50% and 70 % of full power showed an initial size reduction period (transition) and a levelling – off period (equilibrium) which was not observed when sonication was done at 25 % of full power. In fact, the obtained results highlighted that after 20 min of sonication, the vesicle sizes were only weakly affected by the sonication treatment for both 50 % and 70 % of full power which was not the case for 25% of full power. On the basis of these results, a sonication treatment during 20 min at 70% of amplitude allowed to obtain a liposome with a vesicle size < 70 nm as compared to 749.1 nm for untreated liposome. However, the polydispersity index (PDI) was highly increased reaching 0.331 which contributes to the non-stability of the liposome during storage. In fact, results showed that PDI was not affected by the amplitude; however, it was highly affected by treatment duration. The PDI indicates the homogeneity of the vesicle size distribution. A lower PDI corresponds to a more homogeneous the vesicle size distribution. PDI higher than 0.3 indicates that the distribution of particle size is non uniform. In this study, results showed that PDI increased significantly depending on the sonication duration. With a full power of 70%, the PDI remained stable during 10 minutes of treatment showing a value of 0.222 and 0.251 for respectively 5 and 10 min, afterwards, a significant increase was observed reaching a PDI of 0.331 and 0.330 for respectively 20 and 30 minutes of treatment. These observations suggest a fusion and agglomeration of liposomes and the whole dispersion became inhomogeneous after 20 minutes of sonication treatment at 70 % of full power (Pistone *et al.*, 2017, Wieber *et al.*, 2012).

Table 8.3. Droplet diameter (nm) and interfacial ζ-potential (mV) of liposomes produced by sonication at different processing amplitude (µm) and sonication times (s).

Amplitude (µm)	Sonication time (s)	Vesicles diameter (nm)	PDI	ζ-Potential (mV)
0	**0**	749.1 ± 25.5	0.268 ±0.048	-52.9 ± 1.56
25% = 30 µm	**5**	138.2 ± 0.8 ^{d}	0.235 ±0.022 ^{a}	-46.9 ± 0.3^{b}
	10	119.5 ± 0.6 ^{c}	0.240 ± 0.022 ^{a}	-48.4 ± 0.6^{c}
	20	100.6 ± 1.2 ^{b}	0.244 ± 0.009 ^{a}	-43.4 ± 0.7^{a}
	30	81.9 ± 1.1 ^{a}	0.251 ± 0.019^{a}	-46.2 ± 0.1^{b}
50% = 60 µm	**5**	117.2 ± 0.08 ^{d}	0.215 ± 0.025^{a}	-45.3 ± 0.5^{b}
	10	100.8 ± 2.4 ^{c}	0.242 ± 0.020^{ab}	-46.5 ± 1.4^{b}

	20	79.5 ± 1.7 [b]	0.288 ± 0.045[bc]	-52.9 ± 1.1 [c]
	30	70.4 ± 0.4 [a]	0.332 ± 0.030[c]	-42.5 ± 0.5[a]
70% = 84 µm	**5**	104.2 ± 1.5[d]	0.222 ± 0.017[a]	-42.3 ± 0.4[a]
	10	90.5 ± 1.63 [c]	0.251 ± 0.018[a]	-49.5 ± 1.0[c]
	20	65.5 ± 1.5 [b]	0.331 ± 0.22[b]	-41.5 ± 1.4[a]
	30	61.4 ± 1.4 [a]	0.330 ± 0.061[b]	-44.9 ± 0.4[b]

Several parameters could be used to predict the stability of liposomes. Viral H. Shah *et al.* (2018) showed that critical quality attributes which would affect the efficiency and performances of the liposomes are PDI, zeta potential and the entrapment efficiency. Results in **Table 8.3** showed that for all prepared liposomes, zeta potential was higher than |30 mV| suggesting a stable liposome.

On the basis of these results, sonication treatment during 10 minutes at 70% of full power was more efficient to give a nano-sized and stable liposome.

3.2. Optimization of liposome composition

3.2.1. Selection of lecithin type

Two sunflower lecithins (deoiled lecithin and fluidic lecithin) at a concentration of 1 % were studied. Results of particle size, PDI, and zeta potential (**Table 8.4**) showed that, for both lecithin, zeta potential was < -30 mV and the PDI was < 0.25 allowing forming liposomes with high surface charge, more homogeneous size distribution and high stability index. In fact, when the zeta potential is higher than |30 mV|, the liposome surface charge is high enough to provoke a repulsion of the vesicles avoiding their fusion and aggregation. However, a significant difference on vesicle sizes ($P \leq 0.05$) was observed between the two lecithins. Deoiled lecithin showed the lowest vesicle size with 87.2 nm as compared to 162.7 nm obtained with fluidic lecithin. This could be related to the lecithin composition especially on saturated and unsaturated phospholipids.

Table 8.4. Effect of lecithin type on the characteristics of blank liposomes.

	Particle size (nm)	**PDI**	**Zeta potential**
Deoiled lecithin	87.2 ± 0.2[a]	0.247±0.011[a]	-44.0±0.8 [a]
Fluidic lecithin	162.7 ±2.1[b]	0.244±0.022[a]	-40.3±0.6 [b]

In fact, previous researches studied the relation between vesicle sizes and the saturation level of phospholipids that are involved in liposome preparation. The vesicle size was intimately related on saturated and unsaturated phospholipids which could orient the increase or the decrease of liposomes size. In fact, Maherani *et al.* (2012b) showed that liposome size increases when more unsaturated fatty acids were incorporated on phospholipid chains such as oleic acid in the liposomal formulation. This was related to the increase of liposome membrane fluidity and lead then to the improved packing of phospholipid bilayer (Maherani *et al.*, 2012b, Zhao *et al.*, 2015). On the other hand, a phospholipid with unsaturated fatty acyl chains resulted in increased asymmetry of liposome (Zhao *et al.*, 2015)

The results of entrapment efficiency are presented in **Figure 8.1**. The results showed that the lecithin type affects significantly the entrapment efficiency. The entrapment efficiency of hydrophilic compounds passed from 35.7 % for fluidic lecithin to 44.1 % with the deoiled

lecithin. Deoiled lecithin was also effective in encapsulating essential oils mixture showing a significantly ($P \leq 0.05$) higher encapsulation than fluidic lecithin with an increase from 29.5% to 50.9%. The improvement of the entrapment of hydrophilic and lipophilic ingredients is also related to the composition in unsaturated fatty acid such as linolenic acid with 3 unsaturation allowing the formation of more space in the liposome bilayer where essential oils could be encapsulated. The encapsulation efficacy could be improved by adding cholesterol to the liposome formulation (Coderch *et al.*, 2000). On the basis of the obtained results, deoiled lecithin allowed a better encapsulation efficiency and thus a better antimicrobial activity (Gao *et al.*, 2013).

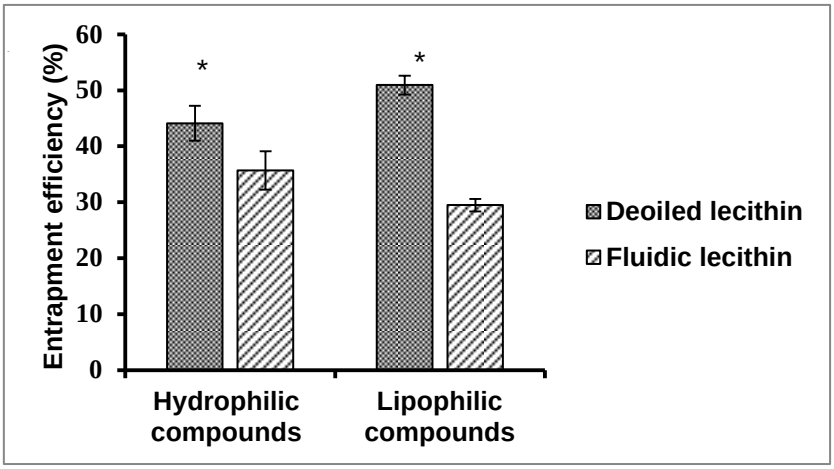


Figure 8.1. Lecithin type effect on the characteristics of obtained liposomes.

* Significantly different ($P \leq 0.05$)

3.2.2. Effect of lecithin concentration

Results of the effect of the lecithin concentration on vesicle size, PDI and zeta potential are presented in **Table 8.5**. Results showed that the increase of the deoiled lecithin concentration up to 2 % was followed by a significant increase on the mean size of blank liposome. An increase from 91.5 nm to 104.8 nm was observed when the lecithin concentration was from 1% to 2% ($P \leq 0.05$). However, no effect on the PDI and zeta potential of the prepared liposomes was observed. No significant difference ($P > 0.05$) was also observed up to 1.5 % of lecithin. Trucillo *et al.* (2017) showed also that the increase of lipid concentration allowed an increase of the mean diameter of liposome. However, Naeem *et al.* (2016) and Zhao *et al.* (2015) demonstrated that the increased phospholipid concentration was favorable for the formation of smaller size vesicles with higher uniformity and also the mixtures of dipalmitoylphosphatidylcholine (DPPC) and phosphatidylserine (PS) as phospholipids give smaller particle size especially at higher concentrations.

Table 8.5. Lecithin concentration effect on the characteristics of obtained with blank liposomes.

Lecithin concentration	Size (nm)	PDI	ζ-Potential (mV)
1 %	91.5 ± 4.8 [a]	0,255 ± 0,015 [ab]	-41.7 ± 2.7 [a]
1.5 %	97.3 ± 1.2 [a]	0.231± 0.009 [a]	-44.8 ± 3.3 [a]
2 %	104.8 ± 2.2 [b]	0.277 ± 0.024 [b]	-38.9 ± 0.4 [a]

The effect of deoiled lecithin concentration on the EE showed that 1% of deoiled lecithin gave the lowest encapsulation efficiency of both hydrophilic and hydrophobic compounds and showed 35% and 50% of EE respectively (**Figure 8.2**). When the concentration of deoiled lecithin was ≥1.5%, the EE increased significantly and reached 43 % and 77 % of EE for respectively hydrophilic and lipophilic compounds. It is interesting to note that developed liposome encapsulating hydrophobic compounds showed better EE than hydrophilic ones. This could be related to the electrostatic interaction between bioactive compounds and the surface of the lipidic wall of the liposome (Colletier *et al.*, 2002).

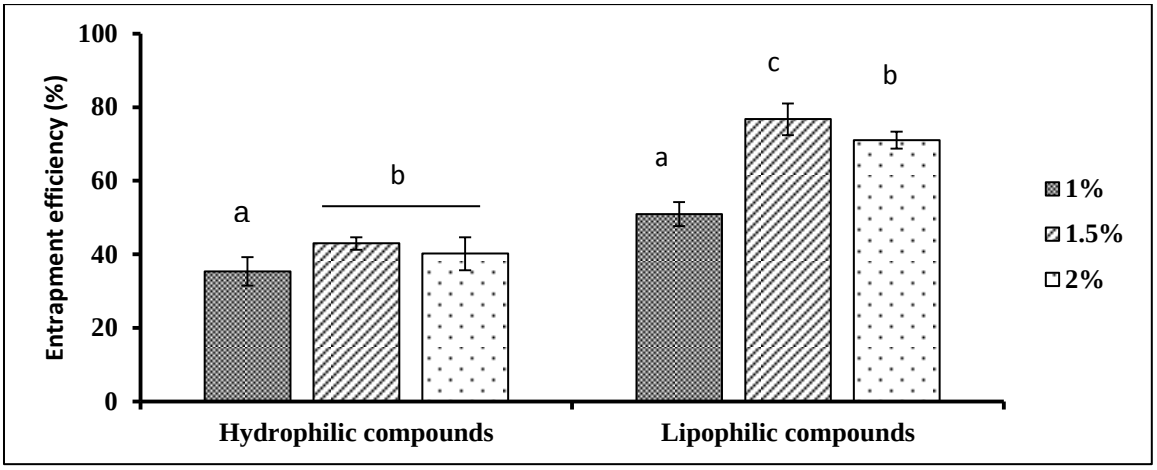


Figure 8.2. Effect of lecithin concentration on the EE of hydrophilic and lipophilic compounds
* For each data followed by a different letter, a significant difference ($P \leq 0.05$) is observed.

3.2.3. Effect of freeze thawing on the encapsulation efficiency of hydrophilic and hydrophobic compounds

The effect of treatment with freeze thawing on the EE (**Figure 8.3**) showed that five cycles of freezing at -80 °C and thawing at +40 °C did not significantly improve the EE of hydrophobic compounds. However, the EE of Biosecur F440D was significantly increased after a freeze-thawing treatment and reached 92% as compared to 45.7 % when Biosecur F440D was encapsulated in liposome without freeze-thawing. Similar results were obtained by Ohsawa *et al.* (1985), where an improvement of the EE of water-soluble drugs in liposomes by freeze-thawing was observed. According to Castile *et al.* (1999), during freezing, internal ice formation begets the damage of liposome bilayers and this may occur by breaking off or partially fragmentation. However, due the hydrophobic effect, damaged bilayers could re-assemble and new liposomes could be formed at different sizes. This process may involve fusion with other liposomes in each freeze–thaw cycle for both unaffected liposomes and those damaged by a previous freeze–thaw cycle (Castile *et al.*, 1999).

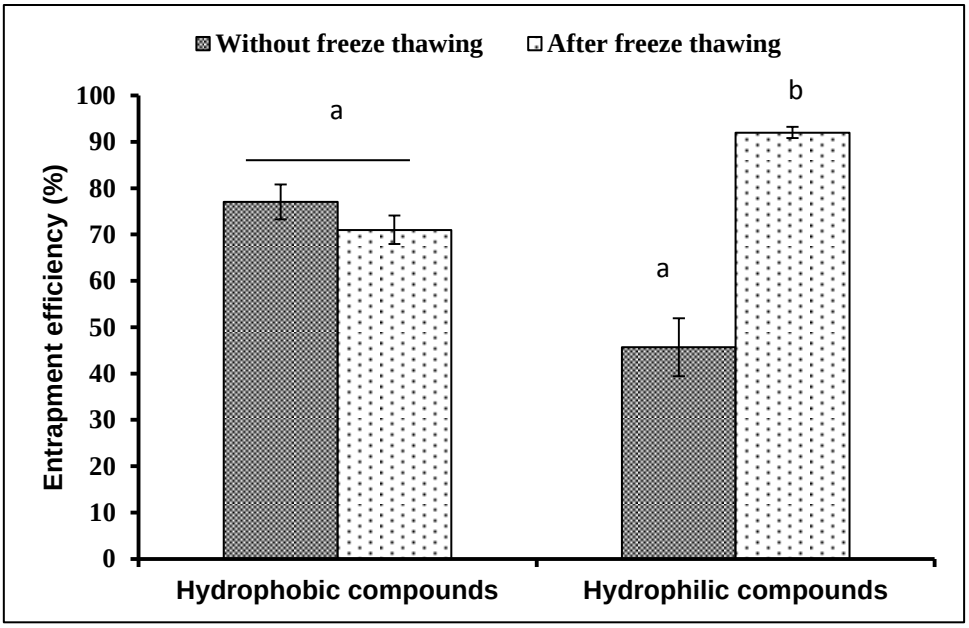


Figure 8.3. Effect of freeze thawing on the encapsulation efficiency of liposome**.**
* For each data followed by a different letter, a significant difference ($P \leq 0.05$) is observed.

3.3. Liposome characterization

3.3.1. Isothermal titration calorimetry

Isothermal titration calorimetry was done in order to evaluate the interaction of Biosecur F440D (**Figure 8.4**) and the interaction of pan tropical essential oil with the liposome membrane (**Figure 8.5**). Upon subtracting the contribution from the control experiments, the

data have been analyzed using a simple one-site binding model.

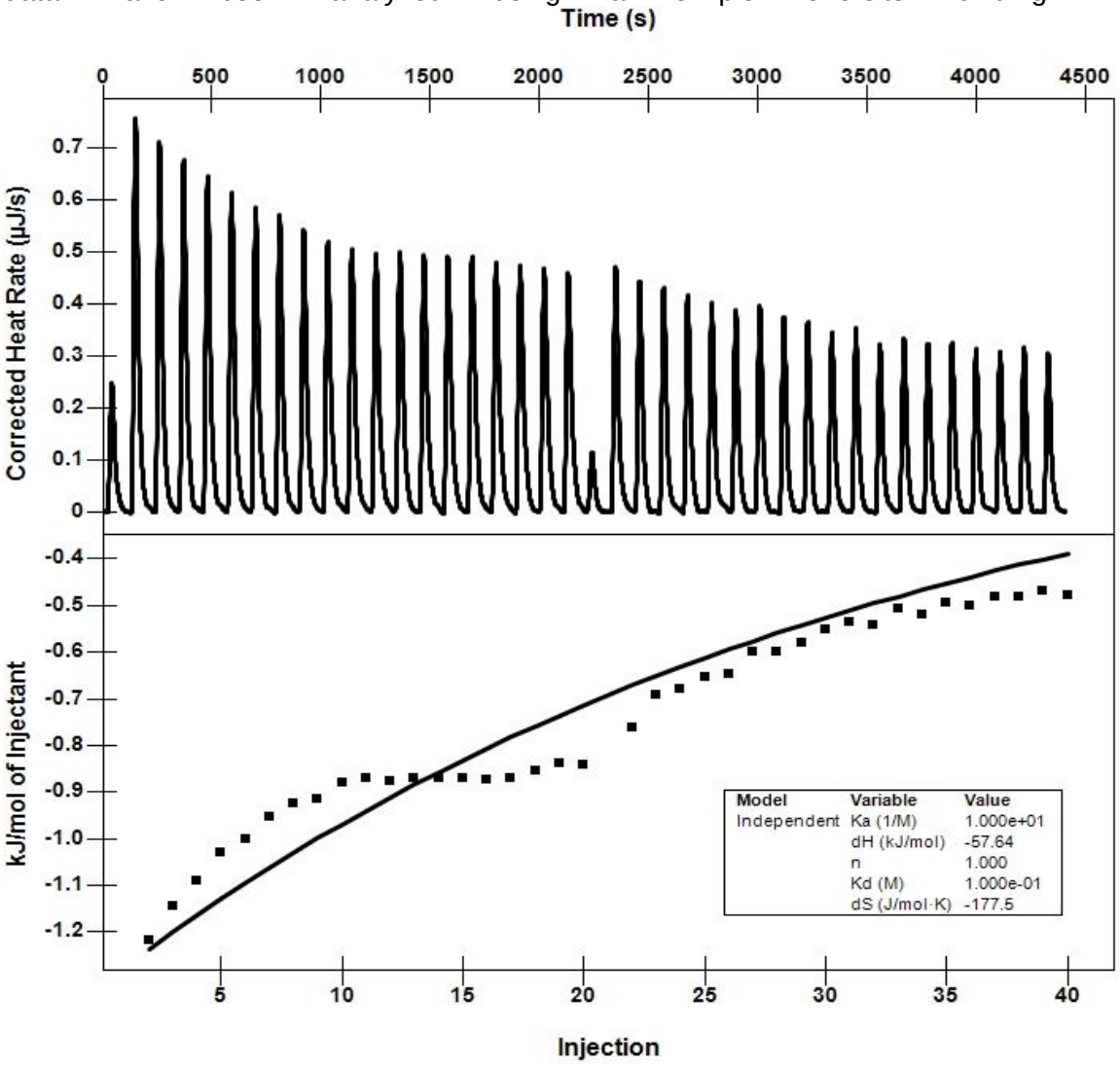


Figure 8.4. Titration of pan tropical essential oil into 300 µL of blank liposome at 20 °C.

The obtained results (**Figures 8.4-8.5**) represent the whole set of peaks given by the titration curve. The major thermodynamic parameters Ka, Kd, ΔH and ΔS are also presented in **Table 8.6**. Results showed that the heat rate decreased during the titration until reaching constant values, indicating a saturation of liposomes after 32 injections for essential oil and after the 7th injection for Biosecur F440D. The binding constant (Ka) obtained with the association of Biosecur F440D and liposome was 900 times higher (Ka = 9.046 10^3 M^{-1}) than the Ka obtained for essential oil (Ka= 10 M^{-1}). These results suggest that Biosecur F440D has more affinity to bind into liposome than essential oil.

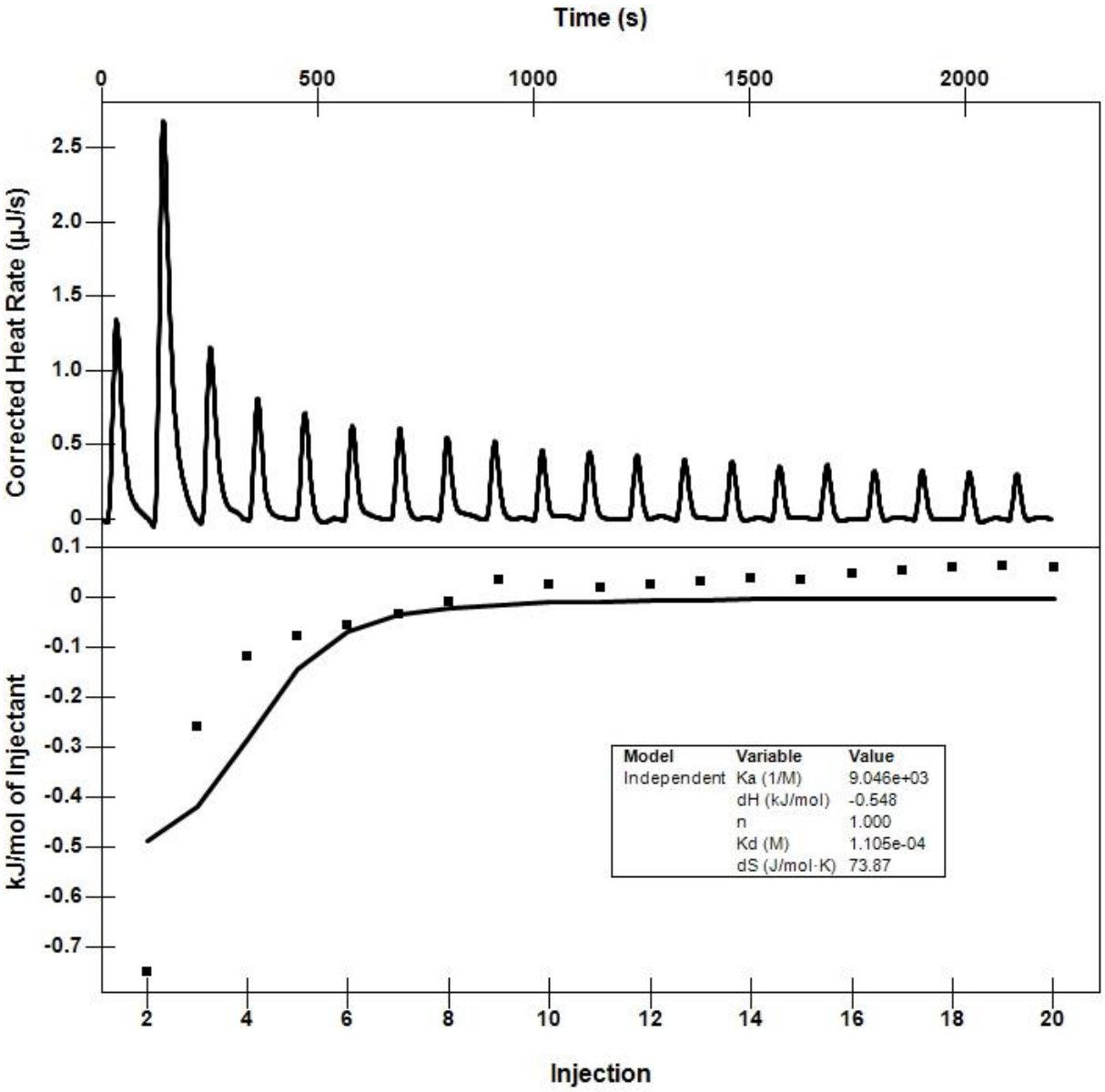


Figure 8.5. Titration of Biosecur F440D into 300 µL of blank liposomes at 20 °C.

From the data presented in **Table 8.6** of both Biosecur F440D and essential oil, the interaction with liposome was through heat-releasing exothermic peaks. Similar results were also observed by previous studies where cinnamic acid was encapsulated into liposome vesicles (Cury *et al.*, 2015). These authors have observed that the interaction curve between phosphatidylcholine liposomes and cinnamic acid resulted in a ΔH ≈ -5.3 kJ/mol and Ka ≈$4.310^{3}M^{-1}$. Likewise, the interaction between cinnamic acid and phosphatidylcholine:phosphatidylserine presented a ΔH ≈-16.0 kJ/mol and Ka of $8.4\ 10^{3}M^{-1}$ (Cury *et al.*, 2015).

Table 8.6. Thermodynamic parameters for the interaction of liposome with Biosecur F440D and pan tropical essential oil.

	Liposome - Biosecur F440D	Liposome – EO
Ka (M^{-1})	$9.046\ 10^{3}$	10
Kd (M)	$1.105\ 10^{-4}$	0.1
dH (kJ/mol)	-0.548	-57.64
dS(J/mol.K)	73.87	-177.5

Ka: binding affinity
Kd: dissociation constant
dH: enthalpy of the interaction
dS : entropy of the interaction
EO: Essential oil

On the other hand, our study showed that the interaction of liposome with pan tropical essential oil allowed higher heat releasing than with Biosecur F440D (ΔH = -57.64 kJ/mol Vs ΔH = -0.548 kJ/mol). The reduction in the enthalpy was caused by the terpenes (ie. eugenol, caryophyllene oxide, α-humulene, α-phellandrene, and α-pinene) present in pan tropical essential oil suggesting that pan tropical essential oil takes the place of lipid molecules in the lipid vesicles (Yoshida *et al.*, 2010).

These results showed that liposome has more affinity to encapsulate Biosecur F440D than pan tropical essential oil. This could be related to the high internal surface of contact of Biosecur in the core of the vesicle as compared to the tight bilayer phospholipids where the essential oils are incorporated.

3.3.2. FTIR

Figures 8.6-8.7 showed the spectrum of the sunflower lecithin based liposome encapsulating essential oils and Biosecur F440D respectively.

FTIR spectrum of blank liposome

The spectrum of blank liposome is consistent with intact liposomes. The principal band between 2800 and 3000 cm^{-1} represents the C-H stretching modes with the maxima of peaks at 2854 cm^{-1} and at 2925 cm^{-1}, corresponding to the symmetric and antisymmetric stretching in the CH_2 groups of alkyl chains, respectively, with minor contribution from the symmetric and antisymmetric stretching vibration in CH_3 groups at 2959 cm^{-1}. The broad band centered at 3369 cm^{-1} represents the O-H stretching in water molecules associated with the membranes via hydrogen bonds. The band at 1729 cm^{-1} corresponds to the stretching vibrations of the ester carbonyl groups of phosphatidylcholine (Pawlikowska-Pawlęga *et al.*, 2013).

FTIR spectrum of liposome encapsulating essential oils or Biosecur F440D

It is clear from **Figures 8.6-8.7** that for almost all absorption peaks of interest there is no shift in peak positions for the liposome- essential oils complex or liposome- Biosecur F440D complex compared to blank liposome or Biosecur F440D or essential oils alone. This suggests that there is no detectable structural change in either of liposome or essential oils /Biosecur F440D liposome complex. Similar result was also observed by Mady *et al.* (2011) when they encapsulated insulin in phosphatidylcholine liposome. This was explained as the association of insulin with phosphatidylcholine liposomes and the formation of liposome

insulin complex is probably far beyond the induction of a major change that would affect the structure and function of either insulin or liposomal membrane.

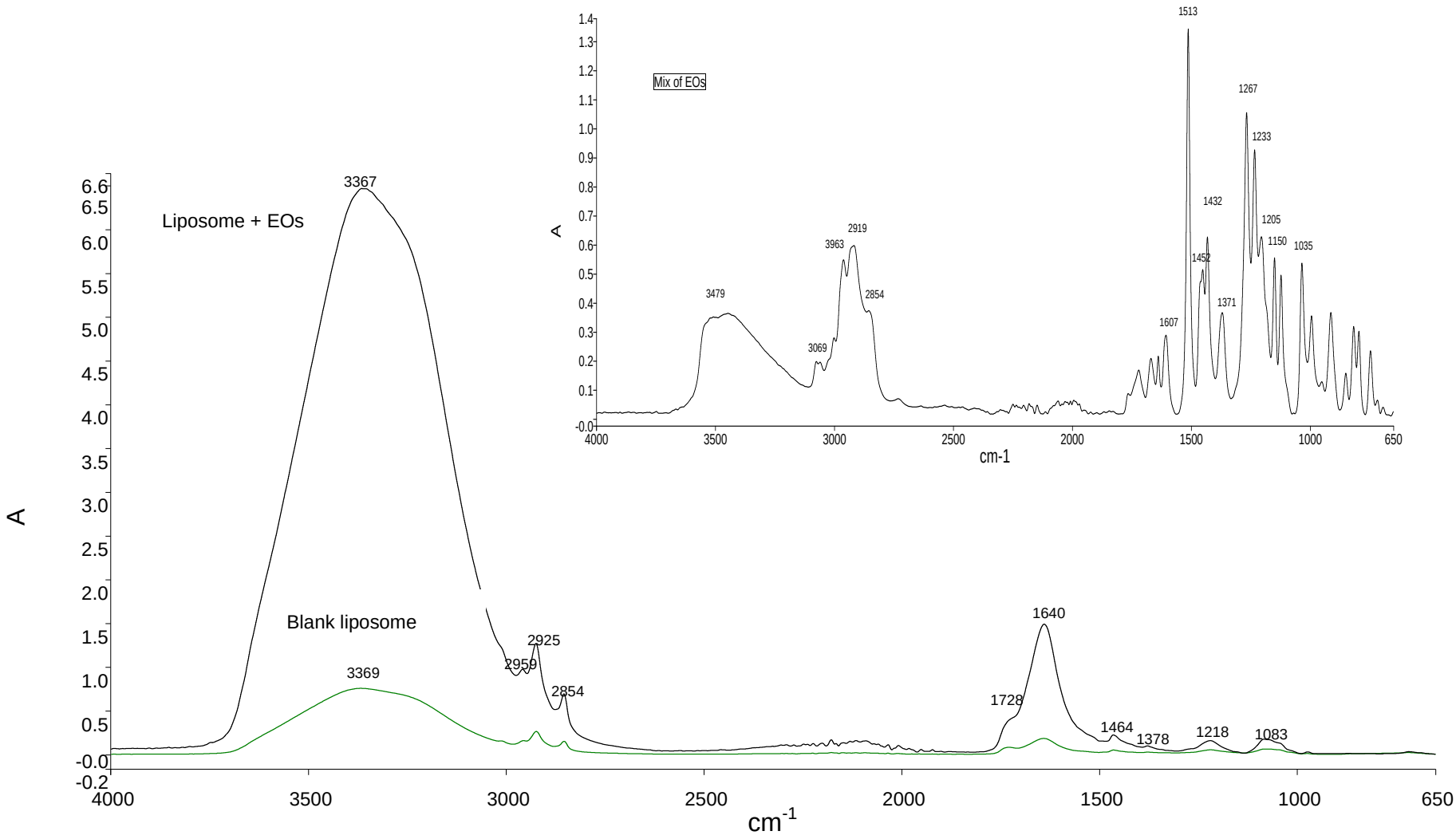


Figure 8.6. IR spectra of blank liposomes and liposomes encapsulating essential oils (EOs)

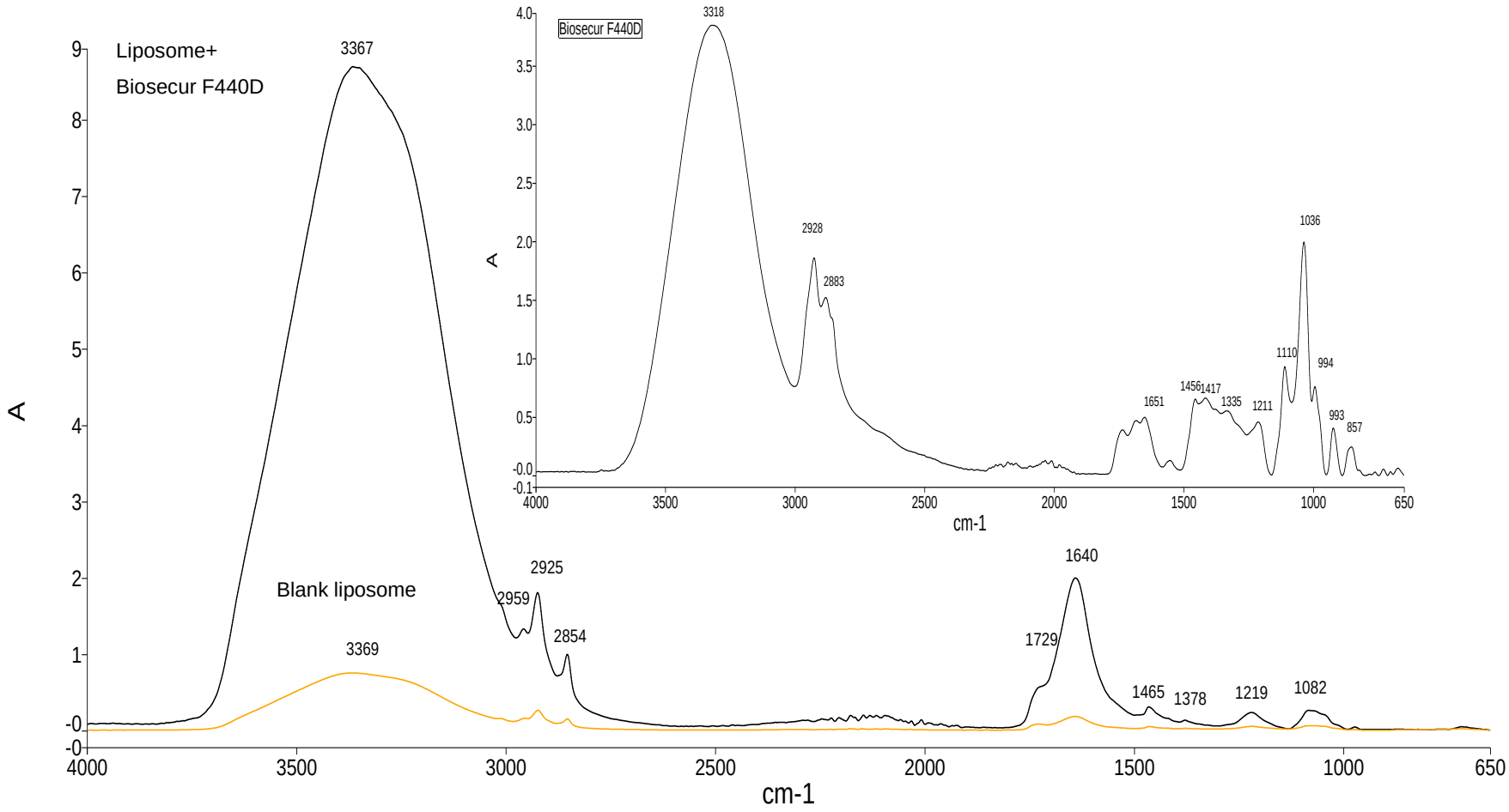


Figure 8.7. IR spectra of blank liposomes and liposomes encapsulating Biosecur F440D

In the current study, the incorporation of Biosecur F440D and a mix of essential oils allowed the increase of the absorbance of all peaks and the appearance of new peaks including 1465 cm^{-1}, 1378 cm^{-1}, 1219 cm^{-1} and 1082 cm^{-1} corresponding respectively to methyl antisymmetric deformation, methyl symmetric deformation, P-O-C stretching and PO_2^- symmetric stretching (**Figure .8.8**) (Changfeng Chen *et al.*, 2008).

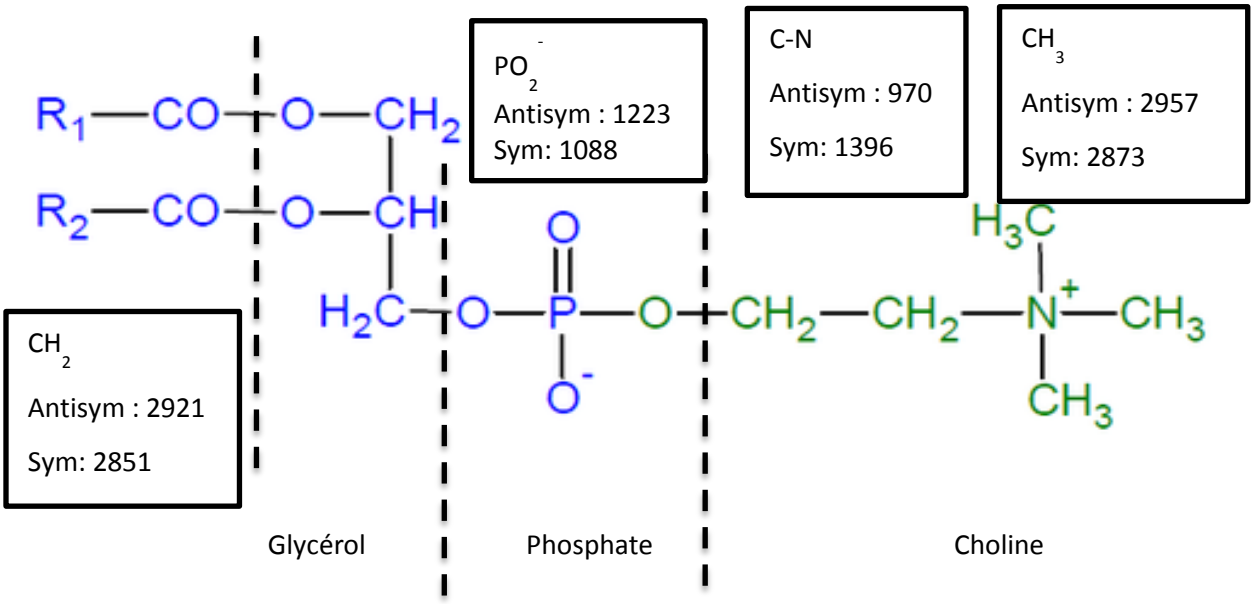


Figure 8.8. Scheme of phospholipid and the major absorbance bands.

These results suggest that the major change observed in liposome structure is on the polar head group of phospholipids and further confirms that the compounds of Biosecur F440D or essential oils and the phosphatidylcholine were combined through chemical reactions contrary to what was observed with the encapsulation of garlic extract on liposome where a physical interaction was observed (Pinilla *et al.*, 2017).

3.3.3. Stability

Results (**Table 8.7**) showed that on day 1, the incorporation of hydrophilic or lipophilic compounds in liposome did not affect the vesicle size which remained ≈ 100 nm.
Blank liposome vesicles and liposome encapsulating hydrophilic compounds were stable during 60 days of storage at 4 °C. However, the incorporation of lipophilic compounds begets an increase of the vesicle size reaching 102 nm as compared to 97 nm and 96 nm for respectively blank liposome and liposome encapsulating Biosecur F440D. During storage a significant reduction on vesicle size was observed starting from day 14 to reach a vesicle size of 87 nm on day 60. The results of PDI showed no significant difference during storage for all prepared liposome.
On the basis of these results, the developed liposome remained stable during the whole storage period keeping nanometric size.

Table 8.7. Stability of vesicle size and PDI of blank liposomes and liposomes encapsulating hydrophilic or lipophilic compounds during storage at 4 °C.

	Days	Blank liposome	Liposome encapsulating hydrophilic compounds	Liposome encapsulating lipophilic compounds
Z-average (nm)	**1**	97.3±1.2^{b}	95.6±2.6^{b}	102.2±2.0^{d}
	14	93.7±2.3^{b}	98.6±2.2^{b}	90.3±0.2^{c}
	30	95.9±1.1^{b}	99.1±1.1^{b}	92.6±0.4^{c}
	45	85.0±1.8^{a}	88.6±1.1^{a}	80.9±0.3^{a}
	60	95.2±1.3^{b}	98.3±1.1^{b}	87.3±1.1^{b}
PDI	**1**	0.231±0.009^{a}	0.301±0.023^{b}	0.242±0.022^{a}
	14	0.250±0.020^{a}	0.225±0.036^{a}	0.233±0.021^{a}
	30	0.235±0.020^{a}	0.226±0.017^{a}	0.228±0.012^{a}
	45	0.254±0.040^{a}	0.242±0.031^{ab}	0.250±0.022^{a}
	60	0.232±0.024^{a}	0.238±0.017^{ab}	0.240±0.014^{a}

4. Conclusion

Based on the obtained results, vesicle size of liposome depends not only on the physical parameters of technique such as sonication time and the amplitude but also on liposome composition such as phospholipid composition and especially saturated and unsaturated fatty acid composition.

It was observed that sonication is a feasible technology to reduce the vesicle size of plant oil/citrus extracts loaded liposome up to nanometric scale. Encapsulation increased the bioavailability of active components in liposome by vesicle size reducing and homogenously distribution of active molecules in liposome. From a point of view in the commercialization of natural food preservatives, these results are very promising.

ACKNOWLEDGEMENTS

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CHAPITRE 9: FOOD GRADE NANOEMULSION DEVELOPMENT TO CONTROL FOOD SPOILAGE MICROORGANISMS

Développement de nanoémulsion de grade alimentaire pour contrôler les micro-organismes d'altération des aliments

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Contributions des auteurs

Ce travail de recherche a été réalisé sous la supervision du Pr. Monique Lacroix. Toutes les expériences ont été réalisées en laboratoire par Yosra Ben Fadhel avec l'aide de Mélinda Aragones et la discussion et les protocoles ont été assistés par Stéphane Salmieri. L'article a été écrit par Yosra Ben Fadhel, tandis que les corrections et révisions ont été effectuées par Stéphane Salmieri et Pr. Monique Lacroix.

RÉSUMÉ

L'effet du mélange d’émulsifiants et de leurs concentrations sur le développement de la nanoémulsion a été étudié. L'impact des conditions de traitement par sonication et microfluidisation sur l'activité physicochimique et antimicrobienne *in vitro* a été évalué. La formulation optimale de nanoémulsion a ensuite été évaluée pour sa capacité à contrôler *B. subtilis* et *A. flavus* sur la surface du pain. Les résultats ont montré que l'utilisation d'émulsifiants à un équilibre hydrophile-lipophile de 12 et un rapport de 1 permettait la formation d'une nanoémulsion la plus stable. De plus, la microfluidisation et la sonication ont toutes deux permis la formation d'émulsion à l'échelle nanométrique sans aucun effet significatif sur les phénols totaux et les flavonoïdes. L'émulsion à l'échelle nanométrique a montré une meilleure activité antimicrobienne *in vitro* contre *L. monocytogenes* et *E. coli*. En outre, La sonication a permis d'obtenir le plus bas indice de polydispersité (PDI) suggérant une nanoémulsion plus stable. L'application de la nanoémulsion sur la surface du pain inoculé avec *B. subtilis* a montré un retard de la dégradation. Cependant, aucun effet n'a été observé contre la croissance d'*A. flavus*.

Abstract

The effect of emulsifier mixture and concentrations on the development of nanoemulsion was studied. The impact of sonication and microfluidization processing conditions on the physicochemical and on the *in vitro* antimicrobial activity was evaluated. The optimal nanoemulsion formulation was then evaluated for its ability to control *B. subtilis* and *A. flavus* on bread surface. Results showed that using emulsifiers at hydrophilic-lipophilic-balance of 12 and a ratio of 1 allowed the formation of a most stable nanoemulsion. Also, both microfluidization and sonication allowed the formation of nanoscale emulsion without any significant effect on the total phenol and flavonoids. Nanoscale emulsion showed a better *in vitro* antimicrobial activity against *L. monocytogenes* and *E. coli.* Furthermore, sonication allowed obtaining the lowest polydispersity index suggesting more stable nanoemulsion. The application of nanoemulsion on bread surface inoculated with *B. subtilis* showed a delay of the decay. However, no effect was observed against *A. flavus* growth.

Keywords: Food safety, antimicrobial activity, nanoemulsion, stability, encapsulation.

1. Introduction

Evidence and recorded history have both shown that the use of aromatic plants may be dated to 10000 BC. The most important document containing 700 formulations of aromatic plants is so-called “Papyrus Ebers” (Baser *et al.*, 2015). Egyptians exploited the virtues of plants to cure diseases and relieve pain and discomfort. These aromatic plants have also been used in food as spices, preservatives, flavorings and a diverse range of plants has been discovered and used leading to the welfare of the human being (Petrovska, 2012). Presently, their use in food as preservatives is still limited, first because of their hydrophobicity which reduces their bioavailability on food surface and also because of their strong aroma and toxicity at high concentrations (Ait-Ouazzou *et al.*, 2011, Dima *et al.*, 2015). Chemical additives were intensively used such as parabens, sulfite and nitrite because of their high efficiency and low effect on the sensory property of food. Currently, in the 21st century, the negative impacts of chemical additives continue to appear, including risks in the mid-long-term such as allergies, endocrinian perturbation and in some case cancer risks (Linke *et al.*, 2018, Rahman, 2007). This has contributed to an increased interest in natural products. These products have already proven their effectiveness but also are "safe" when used correctly (Hashemi *et al.*, 2017). In the food industry, real trend of change towards natural additives like antimicrobials and aromas of natural origin is emerging (Aschemann-Witzel *et al.*, 2019). To maintain their efficiency on food, natural antimicrobial should be incorporated in an adequate matrix involving their stability and homogeneity.

Nanoemulsion could be a good encapsulation form to stabilize hydrophobic natural antimicrobials. It can act as carriers or delivery systems for lipophilic compounds (Hélder Daniel Silva *et al.*, 2012). Generally, nanoemulsions are highly stable to gravitational separation and against droplet aggregation (de Oca-Ávalos *et al.*, 2017). However, it is important to use the adequate emulsifiers and the adequate emulsification methods. Emulsifiers are able to adsorb at the interface between the hydrophilic and hydrophobic phases, decreasing the interfacial tension and preventing or slowing down the aggregation of particles of the dispersed phase by increasing repulsion forces between them (Hélder Daniel Silva *et al.*, 2012). The preparation of nanoemulsion consists mainly on low or high energy approaches (Suyanto *et al.*, 2019). Low energy methods are based on the selection of favorable interfacial properties and require significantly less energy input (Badruddoza *et al.*, 2018). High energy includes several methods such as sonication and high-pressure homogenization (ie. microfluidization). The principle of sonication consists of the transformation of the electrical energy into an electrical signal which is converted into a mechanical vibration. In liquid medium, the rapid vibration causes cavitation, formation and violent collapse of microscopic bubbles which releases considerable energy in the cavitation field (Gupta *et al.*, 2016). For the microfluidization, the fluid to be treated passes from the tank to the interaction chamber under high pressure at high speeds through microchannels producing high shear forces. This applied force generates disruptive forces to form fine droplets (Panagiotou *et al.*, 2008). Due to the high pressure (up to 25,000 psi), the treated fluid is heated. So, it will go through a cooling circuit. This technique has the advantages of (i) producing a smaller particle size with a narrower distribution, (ii) easy to extrapolate on a large scale and (iii) reproducible (Yukuyama *et al.*, 2016). However, the microfluidizer is difficult to clean and it is highly energy consuming.

Bread and other bakery products are subjected to various spoilage problems, physical, chemical and microbial; the latter is the most serious one particularly bacterial (*Bacillus* sp.) and mold growth. *Bacillus subtilis* is the major problem of the bread quality deterioration (Rosenkvist *et al.*, 1995). This microorganism, by contaminating raw materials (for example, flour, sugar and

yeast), survives the cooking process, ends during cooling, and develops under both conditioning conditions aerobic and anaerobic. *Bacillus* spores are able to survive a heat treatment (100 °C, 10 min) which corresponds to the cooking process (Rosenkvist *et al.*, 1995). *Bacillus subtilis* is responsible for the phenomenon of "ropiness". *A. Flavus* could also contaminate bread raw materials (Rosenkvist *et al.*, 1995). *A. flavus* could also contaminate bread. According to Halt (1994), isolates of *A. Flavus* were capable of producing aflatoxin B_1 under favorable conditions. Lethal doses of aflatoxin cause the death of experimental animals in 72 hours and, in smaller doses they cause carcinogenic changes of the liver. They also inhibit the synthesis of RNA, DNA and proteins (Halt, 1994).

The aim of this study is to evaluate the effect of the stabilization of an antimicrobial formulation based on natural antimicrobial ingredients into nanoemulsion using microfluidization and sonication method on its bioactivity.

The nanoemulsion composition was first optimized by selecting the best hydrophilic and lipophilic balance (HLB) and oil: emulsifier ratio. Then, the evaluation of the effect of sonication and microfluidization on the physicochemical and antimicrobial activity was carried out. The effect of nanoemulsion on the decay of bread as food model inoculated with *A. flavus* or *B. subtilis* were also evaluated.

2. Material and methods

2.1. Material

Cinnamon essential oil was bought from Biolonreco, Inc. (Dorval, QC, Canada). Biosecur F440D® was provided by Biosecur Inc. (Mont St-Hilaire Québec, Canada). Sucrose monopalmitate and sunflower lecithin were kindly provided from Compass Foods (Singapore). Folin Ciocalteu was purchased from Sigma Inc. (Oakville, ON, Canada). Sodium carbonate was purchased by Labmat Inc. (Québec, QC, Canada).

2.2. Preparation of nanoemulsions

The oil in water (o/w) emulsion contains cinnamaldehyde based essential oil and Biosecur F440D® with ratio of 1:6 (w/w) was optimized with different emulsifier: oil phase ratios (0.3 to 1.25) and various HLB values in presence of lecithin (HLB ~ 7), sucrose monopalmitate (HLB~18) and lecithin–sucrose monopalmitate (HLB = 7-18) as emulsifiers. The mix was vigorously magnetically stirred then passed through Ultra-Turrax T25 high-shear homogenizer (IKA Works Inc., Wilmington, NC, USA) at 10,000 rpm during 1 minute to obtain the coarse emulsion. The coarse emulsion containing plant extracts with emulsifiers dispersed in water, were subjected to sonication or microfluidization treatment at different amplitudes, duration, pressures and cycles to obtain the homogeneous nanoemulsion.

2.3. Sonication method

The coarse emulsion was subjected to sonication in an ice bath at 40 kHz, and 70% of full power for 10, 20 and 30 min (5 s ON and 2 s OFF) to obtain a homogeneous nanoemulsion (Sonicator Vibra cell 75115, Fisher Scientific Co.). Each 5 min, the ice bath was renewed to maintain the some conditions during all the treatment.

2.4. Microfluidization method

The coarse emulsion was subjected to microfluidization, using an electric-hydraulic M-110P Microfluidizer® equipped with a Diamond Interaction Chamber for emulsions downstream (Microfluidics International Corp., Newton, MA, USA). Optimization was performed at pressures of 10,000, 15,000 and 20,000 psi in 1-2-3 cycles (at 25 °C).

2.5. Size and polydispersity index (PDI)

The mean diameter and size distribution of emulsions were determined upon empirical dilution of the samples using dynamic light scattering (DLS) technique employing a Zetasizer Nano ZS (Malvern Instruments Ltd., Germany). The software used is DTS Nano, version 6.12 supplied by the manufacturer (Malvern Instruments Inc., MA, USA). To avoid multiple scattering effects, nanoemulsions were diluted (1:50) in MilliQ water, and then the sample was put into a standard capillary electrophoresis cell equipped with gold electrodes (DTS1060, Westborough, MA). All measurements were carried out at 20 °C by considering a medium viscosity of 1.33 and medium refractive index of 1.333. For each sample, diameter average size and polydispersity index (PDI) were measured. Three values were obtained for each sample.

2.6. Turbidity (τ)

Optical turbidity of the emulsions was determined by measuring their absorbance at 600 nm (λ= 600 nm) using a UV-Visible spectrophotometer (Scinco S-3100 (Betatek Inc., ON, Canada) at room temperature. The samples were contained within 1 cm path length optical cells, and deionised water was used as a control. Triplicate measurements of turbidity were carried out on each sample.

2.7. Total phenols and total flavonoids content

The total phenols content was determined using a Folin-Ciocalteu colorimetric method according to Y. Ben-Fadhel *et al.* (2019b) with a standard curve ranging between 0-200 µg of gallic acid mL^{-1}. A quantity of 125 µL of the standard gallic acid solution or emulsions was mixed with 0.5 mL of distilled water in a test tube followed by addition of 125 µL of Folin-Ciocalteu reagent (FCR). Samples were mixed well with vortex at room temperature. Six minutes after, 1.25 mL of a 7% sodium carbonate aqueous solution was added to the mix and the final volume was adjusted to 3 mL by adding water. Samples were allowed to stand for 90 min at room temperature before measurement at 760 nm versus the blank prepared similarly with water. All mean values were expressed as milligrams of gallic acid equivalents.

Total flavonoid content was determined by using a colorimetric method (Dewanto *et al.*, 2002). Briefly, 0.25 mL of diluted emulsions or (+) catechin standard solution was mixed with 1.25 mL of distilled water followed by addition of 75 µL of a 5% $NaNO_2$ solution. After 6 min, 150 µL of a 10% $AlCl_3$ $6H_2O$ solution was added and allowed to stand for 5 min at room temperature before 0.5 mL of 1 M NaOH was added. The mixture was brought to 2.5 mL with distilled water and mixed well. The absorbance was measured immediately against the blank at 510 nm in comparison with the standards prepared similarly with known (+)-catechin concentrations. The mean results are expressed as micrograms of catechin equivalents per gram of emulsion.

2.8. Effect on the bioactivity

2.8.1. Preparation of bacterial cultures

Emulsions were evaluated for their antimicrobial activity against *L. monocytogenes* HPB2812 (Health Canada, Health Products and Food Branch, Ottawa, Canada), *E. coli* O157:H7 (EDL 933, isolated from contaminated meat, provided by Pr. Charles Dozois), *B. subtilis* and *A. flavus*. *E. coli* O157:H7, *B. subtilis* and *L. monocytogenes* were kept each at -80 °C in Tryptic Soy Broth (TSB; Becton-Dickinson, Sparks, MD, USA) containing glycerol (10%; v/v). Before each experiment, stock cultures were propagated through two consecutive 24 h at 35 °C ± 2 °C

growth cycles (10^{-1} dilution) in TSB. For fungal evaluation, *A. flavus* were propagated through 72 h growth cycle in potato dextrose agar (PDA, Difco, Becton Dickinson) at 28 °C ± 2 °C. Conidia were isolated from the agar media using sterile platinum loop, suspended in sterile peptone water, and filtrated through sterile cell strainer (Fisher scientific, Ottawa, ON, Canada). The filtrate was adjusted to 10^4 conidia/mL for the MIC determination

2.8.2. Minimal inhibitory concentration (MIC) determination

The MIC value of emulsions was determined in sterilized flat-bottomed 96-well microplate according to the two fold microdilution modified method of Yosra Ben-Fadhel *et al.* (2017). Briefly, serial two fold dilutions (100:100 µL) of the antimicrobial compounds were made in Mueller Hinton Broth (MHB, Difco, Becton Dickinson) and dispensed into 96-well microplates. The concentrations ranges of emulsions were 0.0049–5 %. Then, a volume of 100 µL of bacteria and fungi suspension (10^4 CFU/mL or conidia/mL) was added to 100 µL of each antimicrobial serial dilution and the microplates were incubated on the shaker at 80 rpm at respectively 37 °C and 28 °C for 24 h and 48 h respectively. The absorbance was measured at 595 nm in a BioTek ELx800® absorbance microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The MIC is the lowest concentration of antimicrobial agent demonstrating the complete inhibition of bacterial and fungal growth and showing equal absorbance as blank.

2.8.3. *In situ* evaluation

2.8.3.1. Bacterial and spores preparation

B. subtilis 23857 and *A. flavus* were selected for the evaluation of the effectiveness of the antimicrobial treatments. These microorganisms were chosen as they have been determined to be the main spoilage agents of bread. Nutrient agar (NA), supplemented with $MnSO_4$ (10 mg/L) and K_2HPO_4 (2 g/L) to induce sporulation, was inoculated with 2 ml of 24 h culture grown of *B. subtilis* in tryptic soy broth at 30 °C. After incubation at 30 °C for 5 days, spores were collected by flooding the agar plate with sterile double distilled water, scratching the surface with a glass spatula. After harvesting, spores were washed four times with saline water (0.85% w/v) by centrifugation at 4400× g for 15 min and the resultant pellet was re-suspended in sterile double distilled water. Spore suspension was heat treated in a water bath at 80 °C for 10 min to kill remaining vegetative cells. The concentration of spore suspension was estimated by spread-plating 100 µL on plates of tryptic soy agar (TSA, BD Difco, Mississauga, ON, Canada), which were incubated at 30 °C for 24 h. The spore suspension was then maintained at 4 °C until used (S. Ayari *et al.*, 2012).

For *A. flavus* spore preparation, *Aspergillus* was propagated through 7 days growth cycle on potato dextrose agar (PDA, Difco, Becton Dickinson) at 28 °C ± 2 °C. Spores were isolated from the agar media using sterile platinum loop, suspended in sterile peptone water, and filtrated through sterile cell strainer (Fisher scientific, Ottawa, ON, Canada). The filtrate was adjusted to 10^4 conidia/mL using a microscope (Qayyum *et al.*, 2019).

2.8.3.2. Challenge test of par-baked bread

White bread paste was purchased from Costco (Laval, Qc, Canada). Bread was treated with 5 µL of *B. subtilis* or *A. flavus* spores at 10^4 CFU/mL on 4 face of bread. Bread was then divided into groups: untreated bread, treated bread with nanoemulsion and treated bread with coarse emulsion. Treatment was applied by spraying breads during 2 seconds each surface. Bread was then baked in convection oven for 14 minutes at 200 °C (10 breads per treatment). All bread balls were individually packed in Whirl-Pak™ Sterile Filter Bags (Nasco, Whilpack®, Fort Atkinson, WI, USA) under atmospheric air and placed at room temperature. Bread balls were

evaluated for the surface contamination and the results were expressed as percentage of contamination over time.

2.9. Statistical analysis

Each experiment was done in triplicate. For each replicate, three samples were analyzed. Analysis of variance (ANOVA), Duncan's multiple range tests for equal variances and Tamhane's test for unequal variances were performed for statistical analysis using SPSS 18.0 software (SPSS Inc, New York, USA). Differences between means were considered significant when the confidence interval was lower than 5 % ($P \leq 0.05$).

3. Results and discussion

3.1. Preparation of nanoemulsions

3.1.1. Effect of HLB

In order to prepare a stable nanoemulsion showing low PDI and mean particle size, lecithin (HLB = 7) was combined with sucrose monopalmitate (HLB = 18) to prepare nanoemulsions with different HLB values. Nanoemulsions were prepared using 1 cycle of microfluidization treatment at a pressure of 15000 psi. Results of mean particle size, PDI, turbidity and shape of emulsions were respectively detailed in **Figure 9.1**. Results showed that, particle size and PDI **(Figure 9.1 A-B)** had similar parabolic behavior showing a minimum particle size and PDI of 39.11 nm and 0.44 respectively for HLB 12 (mix of lecithin: sucrose monopalmitate = 55:45). Afterwards, z-average and PDI increased significantly and reached 68 nm and 0.84 suggesting a non-stable nanoemulsion. Reducing the droplet size is very important factor in nanoemulsion preparation because it can result in higher retention of encapsulated components in emulsion systems (Wen-Chien Lu *et al.*, 2018). On the other hand, PDI value ($0 < PDI < 1$) indicates the uniformity and the stability of the droplet size distribution in the nanoemulsion. When the PDI ≤ 0.3, the nanoemulsion system has narrow size distribution and it is homogeneous. When the PDI ≥ 0.5, the system is heterogeneous and called broad size distribution (Pongsumpun *et al.*, 2020). Generally, large particle size and large size distribution indicate the instability of the nanoemulsion.

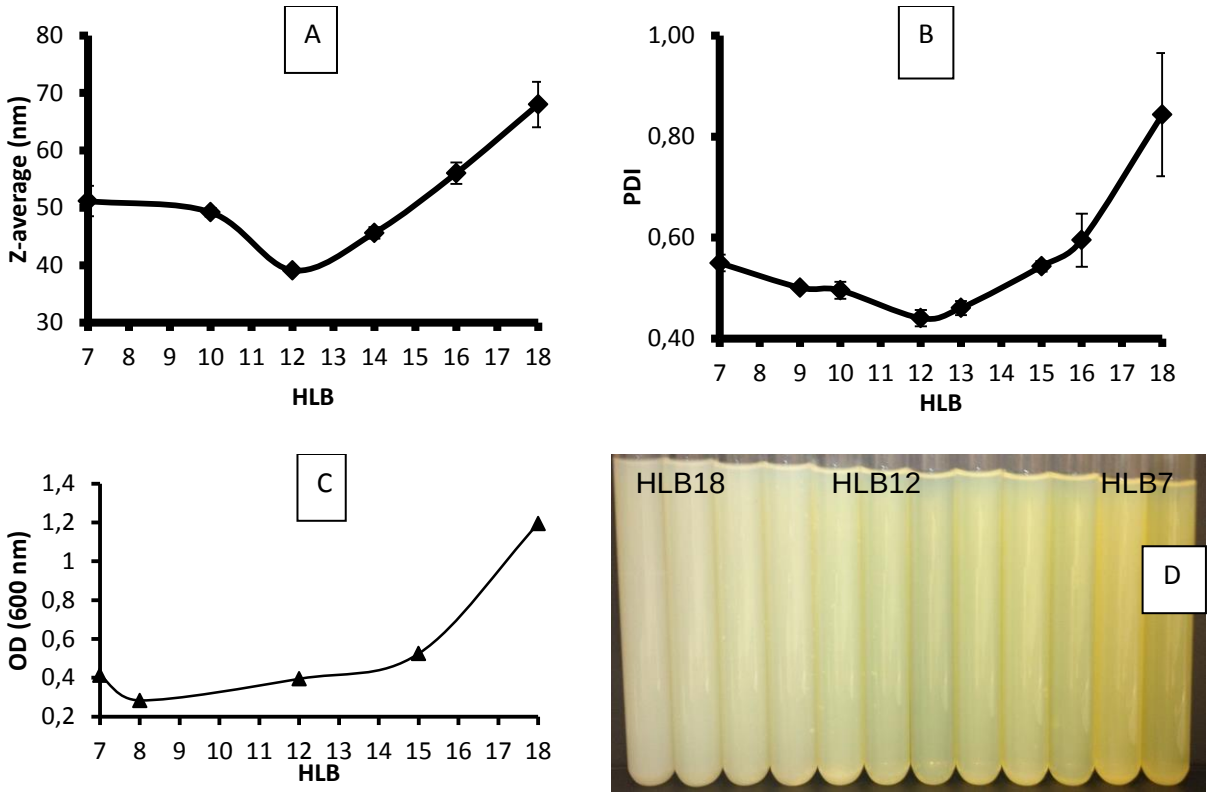

Figure 9.1: Effect of HLB on (**A**) particle size, (**B**) PDI, (**C**) turbidity and (**D**) appearance of prepared nanoemulsion with microfluidization 1 cycle at 15000 psi and at ratio oil: emulsifier = 1:1.

The turbidity and the color of nanoemulsion (**Figure 9.1 C-D**) seems to correlate with the observation of droplet size and PDI showing an increased turbidity starting from HLB 15 (OD_{600}= 0.53) with a maximum observed at HLB=18 with OD_{600}= 1.2. Similar results were also observed by Carpenter *et al.* (2017) where mustard o/w nanoemulsion was prepared using span 80 and tween 80 showing a minimum droplet size observed with HLB 10-11 and reporting this to the packing strength of emulsifiers at the droplet interface which had reached its saturation state at HLB 10. At higher HLB, sucrose monopalmitate molecules are present in excess and therefore will occupy maximum active sites. Wen-Chien Lu *et al.* (2018) also demonstrated that emulsions appear to be opaque (white) at low HLB values and are transparent or translucent at HLB value = 12.

3.1.2. Effect of ratio

By using a HLB 12, the ratio of emulsifiers and oily phase were varied from 0.3:1, 0.5: 1, 0.75:1, 1:1 to 1.25:1. Results (**Figure 9.2**) showed that the use of low concentration of emulsifiers affects significantly the particle size of nanoemulsion which reached 91.3 nm on day 0 as compared to 56.9 nm for ratio of 0.5:1, 43.2 nm for ratio 1:1 and 39.1 nm for both ratios 0.75:1 and 1.25:1. Similar results were also observed by Wen-Chien Lu *et al.* (2018) where the ratio emulsifier: citral of 0.4-0.6 showed the lowest droplet size. During storage also, an increase of z-average was observed for the ratio 0.3:1 and the z-average reached 107.6 nm on day 15. However, the z-average of both 0.75-1.25: 1 seems to be able to make the most stable nanoemulsion during storage. Bai *et al.* (2016) showed that the type of emulsifier and its concentration significantly affect the mean droplet diameter and the

interfacial tension. Depending on the emulsifier type, soy lecithin and gum Arabic showed the lowest droplet diameter at a ratio of 1:1 contrary to WPI where only a ratio 0.1:1 was enough to reduce droplet diameter. Qian *et al.* (2011) showed similar results for tween 20, SDS and casein. This was explained by the presence of the emulsifier to cover any new droplet surfaces formed during homogenization, and because the droplet surfaces will be covered more rapidly by a layer of emulsifier molecules. However, with β-lactoglobulin after a ratio of 0.5:1, the droplet diameter increased and this was explained with denaturation of some protein during the high-pressure homogenization process.

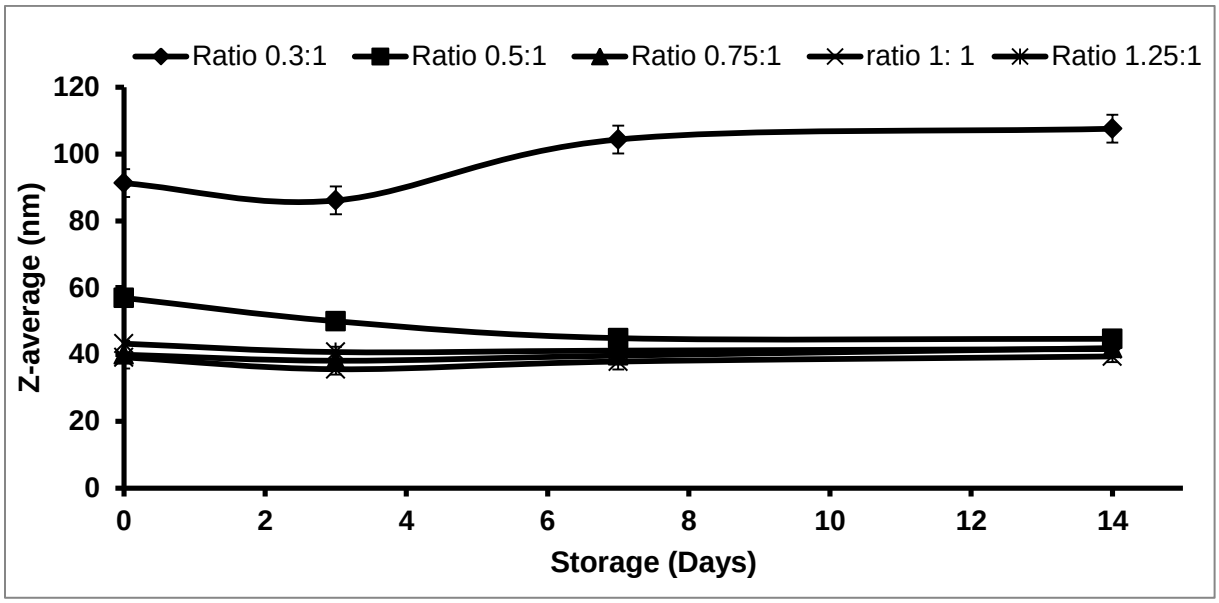

Figure 9.2. Effect of the ratio emulsifier: oil on the z-average of emulsions

Increasing emulsifier: oil ratio allowed the decrease in droplet size. This was explained by Wen-Chien Lu *et al.* (2018) as the presence of enough surfactant to stabilize newly formed droplets. However, by increasing more the surfactant ratio, droplet size would increase again due to much residual surfactant which interferes with the stability and appearance of emulsion.

3.1.3. Effect of the preparation method

3.1.3.1. Particle size, PDI and turbidity determination

Results of the effect of microfluidizatin and sonication parameters on z-average, PDI and turbidity of nanoemulsion are detailed on **Table 9.1**.

Table 9.1. Particle size, PDI and turbidity of prepared emulsions

Treatments			Particle size (nm)	PDI	Turbidity
Coarse emulsion			189.5±28.3	0.399±0.044	9.56±0.5
Microfluidization	**10000 psi**	**1 cycle**	45.9 ± 0.6	0.430±0.008	4.54±0.35
		2 cycles	35.3 ±0.1	0.404±0.002	3.76±0.47
		3 cycles	28.3±0.7	0.321±0.006	2.6±0.46
	15000 psi	**1 cycle**	38.6±0.2	0.425±0.006	4.39±0.81
		2 cycles	28.0±0.4	0.331±0.010	1.84±0.04
		3 cycles	28.6±0.4	0.332±0.009	1.39±0.22
	20000 psi	**1 cycle**	41.1±2.1	0.488±0.73	4.72±0.01
		2 cycles	27.2±0.1	0.340±0.014	2.25±0.10
		3 cycles	25.7±0.7	0.313±0.007	2.64±0.29
Sonication	**10 min**		34.1±0.4	0.283±0.024	1.05±0.11
	20 min		27.4±0.4	0.304±0.011	0.43±0.12
	30 min		27.6±0.5	0.350±0.017	0.56±0.05

Results showed that both methods affect significantly the z-average and the turbidity showing a z-average reduction from 189.5 nm to 45.9, 38.6 and 41.1 nm for treated emulsion by microfluidization for 1 cycle at 10,000, 15,000 and 20,000 psi respectively and to reach 34.1, 27.4 and 27.6 nm for treated emulsion with sonication during 10, 20 and 30 min respectively. Treatments allowed also the reduction of the turbidity to reach ≈ 4.5 for microfluidization treatment and < 1.1 for sonication treatment as compared to ≈ 9.6 for the coarse emulsion. These results suggest that both methods were efficient to reduce droplet mean diameter and turbidity. However, sonication method seems to be the most efficient especially for reducing the turbidity and the PDI.

In fact, PDI was not as sensitive to the applied treatment as the droplet size parameter. The lowest PDI value of 0.283 was obtained by treatment with sonication during 10 min. contrary to what was expected, a longer sonication treatment increased the PDI. This phenomenon can be referred to an “over-processing” with an increase in the Brownian motion, hence higher probability of collision and coalescence at higher energy input (Wen-Chien Lu *et al.*, 2018, Mahdi Jafari *et al.*, 2006). For microfluidization method, increasing microfluidization pressure did not decrease the PDI. However, PDI decreased with the number of cycles applied and it remained > 0.3 suggesting a non-stable nanoemulsion.

3.1.3.2. Effect on total phenol and flavonoid contents

Results of total phenol content (**Figure 9.3a**) showed that microfluidization method did not reduce significantly total phenol content of the emulsion. In contrast, sonication treatment, even for 10 minutes, reduced significantly total phenol content of nanoemulsion as compared to the coarse emulsion. These results confirmed the study of Maherani, Khlifi, Salmieri, and Lacroix (2018) where sonication method reduced total phenol content especially during storage and was explained as the result of polymerization and oxidation of phenolic compounds thereby the loss of their activity and solubility. Thus, total phenol seems to be more sensitive to sonication treatment than microfluidization.

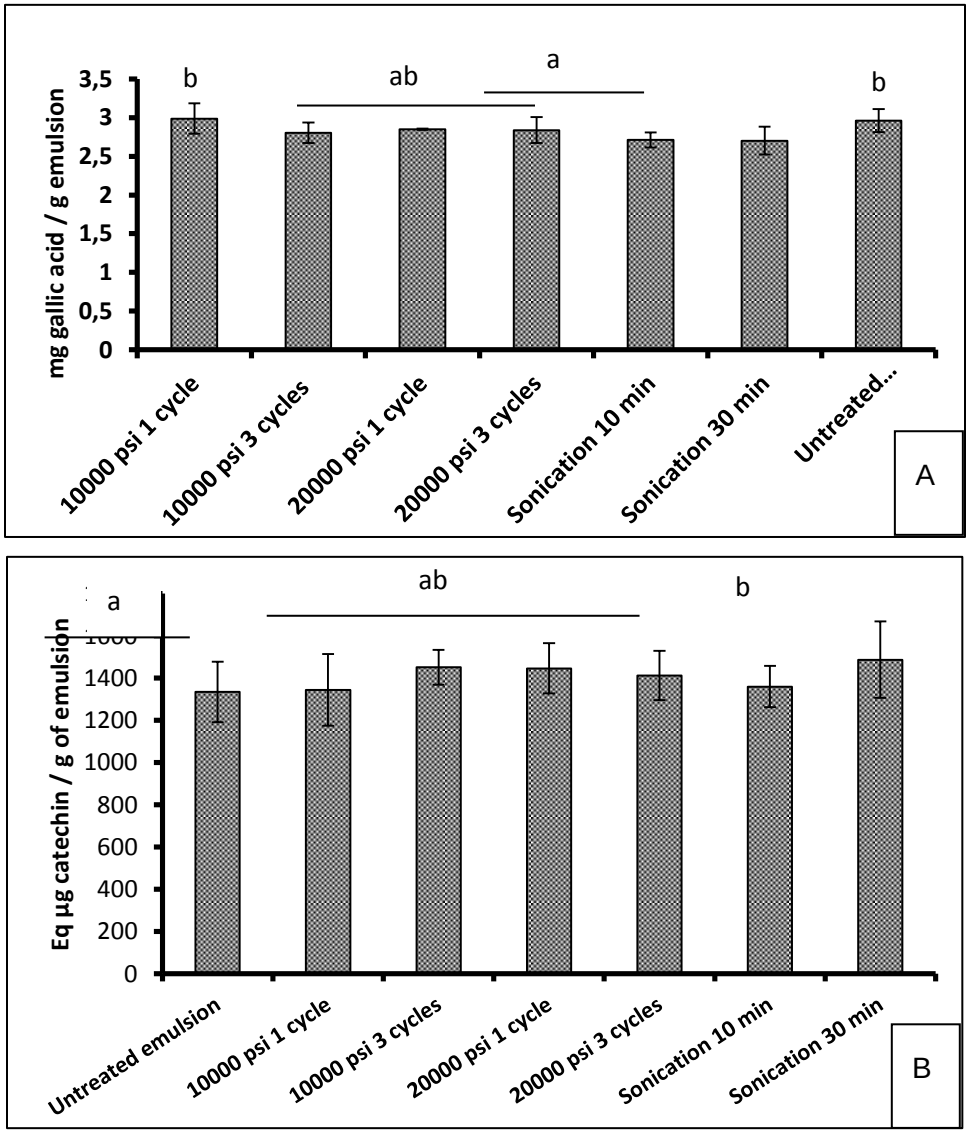

Figure 9.3. Effect of sonication and microfluidization on **(A)** total phenol and **(B)** flavonoid contents

* Means with the same letter are not significantly different (P > 0.05).

Results of total flavonoids (**Figure 9.3b**) showed that sonication treatment during 30 minutes increase significantly the total flavonoids content of the nanoemulsion as compared to the coarse emulsion. Similar results were also observed by Maherani *et al.* (2018) where coarse emulsions presented lower total flavonoids content than other nanoemulsions.

Overall, both sonication and microfluidization treatments did not have a drastic impact on the total phenol and total flavonoid contents of prepared nanoemulsion as compared to the coarse emulsion.

3.1.4. **Effect on the bioactivity**

3.1.4.1. ***In vitro* analysis**

Results of the effect of sonication and microfluidization methods on the MIC of the prepared nanoemulsions are presented in **Table 9.2**. Results showed that for *B. subtilis* and *A. flavus*, the MIC was respectively 0.0024% and 0.07813% for coarse emulsion. No effect was observed with the application of sonication and microfluidization treatments. On the other hand, The MIC of the coarse emulsion against *E. coli* and *L. monocytogenes* were 1.25% and 0.354% respectively. Contrary to what was observed with *B. subtilis* and *A. flavus*, sonication and microfluidization treatments allowed the reduction of the MIC against *L. monocytogenes* to reach < 0.049% and *E. coli* to reach < 0.078% for both treatments.

Table 9.2. MIC of prepared emulsions against 4 microorganisms

	E. coli	***L. monocytogenes***	***B. subtilis***	***A. flavus***
Coarse emulsion	1.25	0.354	0.0024	0.07813
10 minutes	0.078	0.029	0.0024	0.07813
20 minutes	0.029	0.024	0.0024	0.07813
30 minutes	0.039	0.024	0.0024	0.07813
10000 psi 1 cycle	0.039	0.029	0.0024	0.07813
10000 psi 3 cycles	0.078	0.029	0.0024	0.07813
20000 psi 1 cycles	0.039	0.049	0.0024	0.07813
20000 psi 3 cycles	0.039	0.029	0.0024	0.07813

These results suggest that reducing droplet mean diameter affects significantly the efficiency of the emulsion against *L. monocytogenes* and *E.coli* which was not observed with *B. subtilis* and *A. flavus* probably because they are spore forming microorganisms. In fact, the inhibitory mechanism of action differs from the non-spore forming microorganisms. Previous studies demonstrated the effect of nanoscale droplets on the antimicrobial activity against *E. coli* (Salvia-Trujillo *et al.*, 2014). The same authors demonstrated that sonication causes a significant loss in terms of antimicrobial activity against *E. coli* depending on the amplitude applied. Treatment at 100 µm for 3 minutes causes a total loss of bactericidal activity of lemongrass EO, which is not the case of microfluidization. Also, a sonication treatment for 1 h can esterify more than 5% of lipids. In the current study, both microfluidization and sonication were able to improve the antimicrobial activity of *Listeria* and *E. coli.* This could be related to a better control of the temperature of the treated sample with sonication by renewing the ice bath. In the current study, treated nanoemulsions were maintained at 30 °C as compared to 47 °C for the study of Salvia-Trujillo *et al.* (2014). These results suggest that the efficiency of sonication treatment and maintaining of the antimicrobial bioactivity is highly related to the control of the treatment conditions especially the temperature of the sample during treatment.

3.1.4.2. *In situ* study

Results of the *in situ* study on bread surface are detailed in **Figure 9.4a** for *B. subtilis* and **Figure 9.4b** for *A. flavus*. Results of *B. subtilis* showed that for untreated samples, 100 % of decay was observed on day 14 of storage at room temperature. Applying coarse emulsion on the bread surface delayed the decay by 12 more days to reach 26 days. It is interesting to note, that applying a sonication treatment during 10 min to the coarse emulsion allowed the improvement of microbial quality of bread by 21 days as compared to the control and by 4 days as compared to coarse emulsion to reach 35 days.

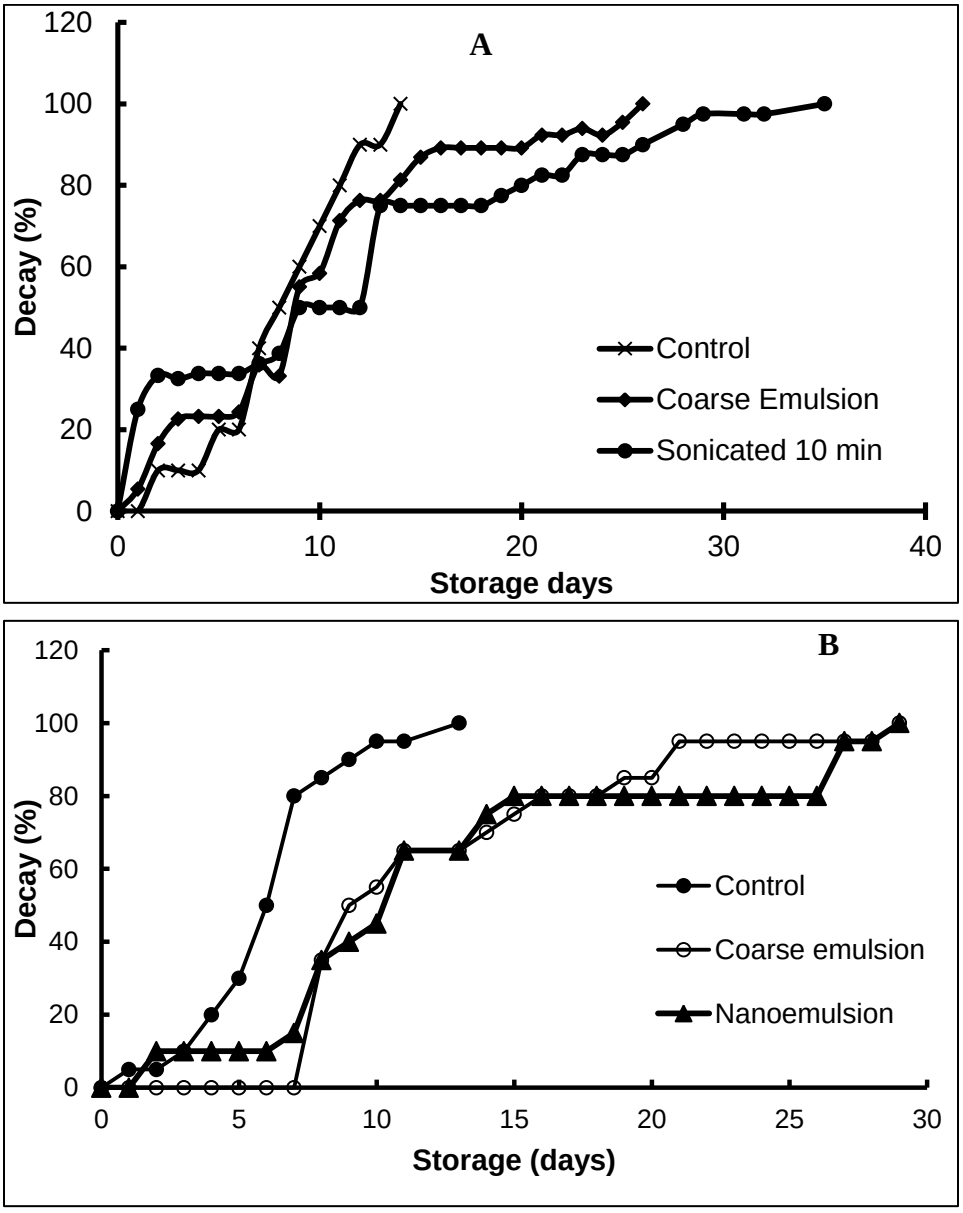

Figure 9.4. Effect of coarse emulsion and nanoemulsion on the bread decay inoculated with (**A**) *B. subtilis* and (**B**) *A. flavus* and stored at room temperature.

Based on *A. favus* results (**Figure 9.4b)**, untreated bread reached the 100% of decay on day 13 as compared to day 29 for both coarse and nanoemulsions. Previous studies demonstrated the impact of reducing droplet size on the antimicrobial activity of antimicrobial loaded

nanoemulsion to control *A. niger* in orange juice (Maherani *et al.*, 2019). This was related to a faster delivery of plant extracts in nanoemulsion rather than coarse emulsion due to the decreased droplet size, with a higher surface area exposed to the microbial cells.

4. Conclusion

In this study, it was illustrated that microfluidization and sonication treatments allowed the formation of nanoscale emulsion. Sonication gave the most stable nanoemulsion with lower PDI and turbidity, higher total flavonoid content and a slight decrease on the total phenol content. Nanoemulsion improved the *in vitro* antimicrobial activity against *E. coli* and *L. monocytogenes.* When applied on bread surface, nanoemulsion allowed a better control of *B. subtilis.* This study exploited the stabilization of hydrophobic antimicrobials of natural origin to eventually replace synthetic preservatives in the food field.

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CHAPITRE 10: INFLUENCE OF ENCAPSULATION OF NATURAL ANTIMICROBIALS INTO NANOLIPOSOME, NANOEMULSION AND GELLED EMULSION FOR FOOD APPLICATION

Influence de l'encapsulation d'antimicrobiens naturels en nanoliposome, nanoémulsion et émulsion gelée pour application alimentaire

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CONTRIBUTIONS DES AUTEURS

Ce travail de recherche a été réalisé sous la supervision du Pr. Monique Lacroix. Toutes les expériences ont été réalisées en laboratoire par Yosra Ben Fadhel avec l'aide de Carolina Martinez et la discussion et les protocoles ont été assistées par Stéphane Salmieri. L'article a été écrit par Yosra Ben Fadhel, tandis que les corrections et révisions ont été effectuées par Stéphane Salmieri et Pr. Monique Lacroix.

RÉSUMÉ

L'effet de l'encapsulation de la formulation antimicrobienne dans une nanoémulsion, une émulsion gélifiée ou dans un nanoliposome a été évalué. Premièrement, l'efficacité d'encapsulation (EE) de la formulation bioactive dans le nanoliposome a été optimisée par le plan central composite (CCD). Ensuite, les formes d'encapsulation ont été évaluées pour leur stabilité, leur efficacité et le contrôle de la libération des ingrédients bioactifs et l'activité antimicrobienne. Les résultats ont montré que le CCD permettait de modéliser l'EE des nanoliposomes et de sélectionner la concentration optimale de lécithine et d'antimicrobiens (2.7% et 0.52% respectivement). Un comportement de stabilité similaire a été observé pour tous les systèmes colloïdaux. Le nanoliposome a particulièrement retardé la libération de la formulation antimicrobienne au cours du temps et maintenu la teneur totale en phénol. Les résultats *in vitro* ont montré que l'émulsion gélifiée a amélioré l'activité antimicrobienne contre les micro-organismes évalués. Lorsqu'elles sont appliquées sur la surface du pain, l'émulsion gélifiée et le nanoliposome sont plus efficaces contre *A. flavus* et *B. subtilis* sans affecter les propriétés sensorielles du pain cuit.

Mots-clés: nanoliposome, biopolymère, nanoémulsion, émulsion gélifiée, stabilité, encapsulation.

ABSTRACT

The effect of the encapsulation of antimicrobial formulation into nanoemulsion, gelled emulsion or nanoliposome was evaluated. First, the encapsulation efficiency (EE) of bioactive formulation into nanoliposome was optimized by a central composite design (CCD). Then, encapsulation forms were evaluated for their stability, the efficiency and the release control of bioactive ingredients and the antimicrobial activity. Results showed that the CCD allowed modelling the EE of nanoliposome and the selection of the optimal lecithin and antimicrobial concentrations (2.7 % and 0.52 % respectively). Similar stability behavior was observed for all colloidal systems. Nanoliposome has particularly retained the antimicrobial formulation release over time and maintained the total phenol content. The *in vitro* results showed that gelled emulsion has improved the antimicrobial activity against the evaluated microorganisms. When applied on bread surface, gelled emulsion and nanoliposome were more efficient against *A. flavus* and *B. subtilis* without affecting the sensory properties of baked bread.

Keywords: nanoliposome, nanoemulsion, gelled emulsion, stability, encapsulation, antimicrobial activity.

1. Introduction

Microencapsulation involves trapping very small bioactive molecules that are generally sensitive to temperature, humidity, microorganisms or other components of the food system, in a more convenient matrix. Currently, this technology is increasingly used in agri-food applications with the encapsulation of flavoring agents, antioxidants, dyes, sweeteners, antimicrobial agents, bioactive and nutraceutical molecules, probiotics, enzymes, etc. Encapsulation allows better protection of their biological activity, improved targeting of encapsulated ingredients and improved solubility and bioavailability (Fathi *et al.*, 2012b). In fact, some bioactive compounds are poorly soluble in water which reduces their efficiency and the homogeneity of their distribution in the food from where the interest of their encapsulation in a matrix more convenient to a food application (Davidov-Pardo *et al.*, 2014). Encapsulation also allows better control of the release of bioactive compounds over time (Madene *et al.*, 2006). The advantage of the control of the release is to assure the presence of active compounds and to protect their bioactivity over a prolonged period and avoiding vitamins and minerals losses during the process (Pothakamury *et al.*, 1995).

Microencapsulation could take different forms. Nanoemulsion is belong the most basic encapsulation forms where two non-miscible phases are stabilized with the use of emulsifiers characterized by a hydrophilic head and a lipophilic tail and by a hydrophilic and lipophilic balance (HLB). Emulsifiers are able to adsorb at the interface between the two phases, lowering the interfacial tension and preventing or slowing down the aggregation of particles of the dispersed phase (Hélder Daniel Silva *et al.*, 2012). They can act as carriers or delivery systems for lipophilic compounds (Hélder Daniel Silva *et al.*, 2012). Nanoemulsions are highly stable to gravitational separation and to droplet aggregation (David Julian McClements, 2011). It has been demonstrated that the entrapment of bioactive ingredients into nanoemulsions allowed the improvement of their bioactivity (Salvia-Trujillo *et al.*, 2014).

Lipids could also be used to encapsulate bioactive molecules to form liposome vesicles composed mainly of phospholipids containing an aqueous volume in the center which makes it possible to trap hydrophilic molecules. The hydrophobic tail makes it possible to seal the liposomes in aqueous media and also to trap lipophilic compounds. Despite their interesting properties, liposomes are not used on a large scale in food because of their low physical stability in the environmental conditions of many food products (Monika Frenzel *et al.*, 2015b). In addition, they generally have poor EE for hydrophilic bioactive substances and the interaction with the food matrix during the product life chain for determining the stability of the liposome in the food matrix is little known (McClements, 2015).

Biopolymers could also be used to encapsulate bioactive ingredients as a beads or coatings. This improved the stability of anthocyanins in the jaboticaba bark extract against light and heating when encapsulated by "rapid expansion of supercritical solution" or by trapping in calcium-alginate beads as compared to the free extract (Diego T. Santos *et al.*, 2013).

In the current study, the optimization of the nanoliposome formulation was first performed. Then, the evaluation of nanoliposome, nanoemulsion and gelled emulsion previously optimized and characterized was carried out based on the release of bioactive ingredients, the stability, the physicochemical properties and the *in vitro* antimicrobial activity. The evaluation of the impact of such treatments on microbial quality, the sensory and the texture property of bread was also performed.

2. Material and methods

2.1. Material

Deoiled lecithin was provided from Austrade Inc. (Palm beach Gardens, FL, USA) and sucrose monopalmitate and high hydrolyzed lecithin were provided from Compass Foods (Singapore). Three different essential oils (EOs) were provided by Zayat Aroma (Bromont, Qc, Canada) and their main constituents are presented in **Table 10.1**. Biosecur F440D (a hydrophilic citrus extract) was provided by Biosecur Inc. (Mont St-Hilaire Québec, Canada).

Table 10.1. List of organic EOs and their main composition.

	Botanic name	**Compositions (%)***
1	*Cinnamomum verrum*	E-cinnamaldehyde (58.88), cinnamyl acetate (3.14), eugenol (6.52)
2	*Cymbopogon winterianus*	Citral B (30.66), citral A (39.48), geraniol (7.75), geranyl acetate (4.42)
3	*Origanum compactum*	Carvacrol (39.45), thymol (14.55), γ-terpinene (11.2), p-cymene (21.05)

*Composition was provided by Zayat Aroma Inc. and was determined by by FAST GCFID; identifications validated by GC-MS
* Essetial oils were selected based on disk diffusion method

2.2. Development of nanoliposome formulation

As shown in **Table 10.2**, a central composite design (CCD) in the form of 2^2 full factorial design was used, in which two independent variables were converted to dimensionless ones (x_1, x_2) with the coded values at 3 levels: −1, 0, +1 allowing the arrangement showed in **Table 10.3**. The response is the EE measured according to Y. Ben-Fadhel *et al.* (2019b).

Table 10.2. Independent variables and their levels for the central composite design used in the present study

Variables	**Symbol**	**Coded variable levels**		
		-1	**0**	**+1**
Lecithin concentration (%)	x_1	1	2	3
Antimicrobial concentration (%)	x_2	0.175	0.35	0.525

Table 10.3. Arrangement of the CCD for the two independent variables used in the present study

			Factor 1	Factor 2
Std	**Run**	**Type**	**A:Lecithin concentration** %	**B:Antimicrobial concentration** %
17	1	Center	2	0.35
21	3	Center	2	0.35
19	5	Center	2	0.35
18	13	Center	2	0.35
20	18	Center	2	0.35
9	2	Axial	0.59	0.35
14	4	Axial	2	0.10
11	8	Axial	3.41	0.35
15	11	Axial	2	0.60
10	14	Axial	0.59	0.35
12	17	Axial	3.41	0.35
13	20	Axial	2	0.10
16	21	Axial	2	0.60
8	6	Fact	3	0.525
3	7	Fact	3	0.175
2	9	Fact	1	0.175
1	10	Fact	1	0.175
7	12	Fact	3	0.525
5	15	Fact	1	0.525
6	16	Fact	1	0.525
4	19	Fact	3	0.175

2.3. Preparation of solutions

Deoiled lecithin suspension was dissolved in deionized water under magnetic stirring. The antimicrobial formulation based on a mix of three EOs and Biosecur F440D was added and stirred. Sonication treatment was then applied during 10 min (5 on, 2 off) at 70 % of full power. Lecithin based solutions were kept in an ice bath to control it temperature during treatment.

Gelled emulsion developed by Y. Ben-Fadhel *et al.* (2019b) was slightly modified and prepared by dissolving high ester pectin in MilliQ water at 2 % under magnetically stirring (O/N) at room temperature. The antimicrobial compounds were added and the mix was magnetically stirred. Sonication treatment was then applied during 10 min (5 on, 2 off) at 70 % of full power. Gelled based solutions were kept in an ice bath to control their temperature during the treatment.

The oil in water (o/w) nanoemulsion containing the antimicrobial compounds was also previously optimized and characterized and was prepared by using high hydrolyzed lecithin (HLB ~ 7) and sucrose monopalmitate (HLB ~ 18) as emulsifiers. The mix was vigorously magnetically stirred then passed through a Ultra-Turrax T25 high-shear homogenizer (IKA Works Inc., Wilmington, NC, USA) at 10,000 rpm for 1 min. The colloidal suspension was subjected to sonication to obtain a homogeneous nanoemulsion.

2.4. Characterization

2.4.1. Size, polydispersity index (PDI), zeta potential and viscosity

The mean diameter, PDI and ζ-potential of nanoliposomes, gelled emulsion and nanoemulsion were determined upon empirical dilution (1:50) to avoid multiple scattering effects, and by using the dynamic light scattering (DLS) technique with a Zetasizer Nano ZS (Malvern Instruments Ltd., Germany) equipped with a DTS Nano software (version 6.12) (Malvern Instruments Inc., Westborough, MA, USA). For the evaluation of the particle size, the PDI and the zeta potential, sample was put into a standard capillary electrophoresis cell equipped with gold electrodes, at 20 °C, by considering a medium viscosity of 1.33 cP and medium refractive index of 1.333. Sample was equilibrated during 120 seconds and the values (n = 3) were obtained after 3 runs. The viscosity of samples was determined using a Brookfield LVDV-II+ viscometer (Brookfield Eng. Lab., MA, USA). The viscosimeter was equipped with an Ultra-Low adapter using the "closed tube" method to control the temperature. The absolute viscosity (cP) of the samples (16 mL) was determined at room temperature.

2.4.2. Stability

The stability was evaluated during 30 days at 4 °C in order to determine the size, the PDI, the zeta potential (mV) and the total phenol content (TPC) using Folin-ciocalteu method.

2.4.3. Release of antimicrobial compounds

The release of antimicrobial compounds was evaluated *in vitro* according to Y. Ben-Fadhel *et al.* (2019b) by measuring TPC using UV-Vis spectroscopy and by using 10 % ethanol as a simulated medium for bread according to the European Commission (2011) No 10/2011 standard method (2011) and Kim (2011). The cumulative release and the release kinetics parameters were determined according to Y. Ben-Fadhel *et al.* (2019b)

2.5. Effect of the different encapsulation forms on the antimicrobial efficiency

2.5.1. *In vitro* evaluation

The minimal inhibitory concentration (MIC) value of nanoemulsion, nanoliposome and gelled emulsion was determined using the two fold microdilution method (100:100 µL) against *E. coli* O157:H7, *L. monocytogenes* 2812, *B. subtilis* 23857, *C. albicans* and *A. flavus* (Yosra Ben-Fadhel *et al.*, 2019a). A volume of 100 µL of bacteria and fungi suspension (10^4 CFU/mL) was added to 100 µL of each antimicrobial serial dilution. The microplates were incubated under agitation at respectively 37 °C and 28 °C for 24 h and 48 h respectively. The absorbance was measured at 595 nm in a BioTek ELx800® absorbance microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The MIC is the lowest dilution of solution demonstrating complete inhibition of bacterial and fungal growth and showing equal absorbance as blank.

2.5.2. *In situ* antibacterial activity

2.5.2.1. Bacterial and spores preparation

B. subtilis 23857 and *A. flavus* were selected for the evaluation of the effectiveness of the antimicrobial treatments. These microorganisms were chosen as they have been determined to

be the main spoilage agents of bread. Spores of *B. subtilis* were prepared according to S. Ayari *et al.* (2012).

Spores of *A. flavus* were isolated after 7 days of growth cycle on potato dextrose agar (PDA, Difco, Becton Dickinson) at 28 °C ± 2 °C using sterile platinum loop, suspended in sterile peptone water, and filtrated through sterile cell strainer (Fisher scientific, Ottawa, ON, Canada). The filtrate was adjusted to 10^4 conidia/mL using a microscope.

2.5.2.2. Challenge test of bread dough

White bread (≈ 50 g each, Costco, Laval, Canada) was defreeze and raised for 2 h at room temperature. Each bread ball was then challenged with 10 µL of 10^4 spores/mL of *B.subtilis* or *A. flavus* on the bread paste surface. Each bread was then vaporized with, nanoemulsion, nanoliposome or gelled emulsion and baked in convection oven for 14 minutes at 200 °C (10 breads per treatment). All bread balls were individually packed in bags under air atmosphere and placed at room temperature. On each day, bread was observed and the percent of contaminated bread was noticed.

2.5.3. Sensory evaluation

The sensory evaluation was carried out by comparing the control to treated bread with encapsulated formulation into nanoemulsion, nanoliposome and gelled emulsion and by using a hedonic test with nine points of level of appreciation (1 = dislike extremely; 5 = neither like nor dislike; 9 = like extremely). Samples were coated with the loaded antimicrobial formulation and baked under convection oven during 14 minutes at 200 °C. The sensory evaluation was done by a panel of 15 untrained people after the treatment application. For each panelist, 3 pieces of bread were served to evaluate the appearance, the odor, the taste, the texture and the global appreciation.

2.5.4. Texture

The texture analysis of bread was performed using texturometer Stevens-LFRA (model TA-1000, Texture Technologies Corp., Scarsdale, NY, USA). The whole bread balls (≈ 9 cm thick with a flat surface) were used for compression force measurement. A probe (d = 35 mm) was used with speed and penetration depth of 2 mm s^{-1} and 12 mm, respectively. The instrument was calibrated with 1000 g before starting the measurements. The maximum compression force in bread balls was recorded. Five bread balls were used for each treatment.

2.6. Statistical analysis

Each experiment was done in triplicate. For each replicate, three samples were analyzed. Analysis of variance (ANOVA), Duncan's multiple range tests for equal variances and Tamhane's test for unequal variances were performed for statistical analysis using SPSS 18.0 software (SPSS Inc, New York, USA). Differences between means were considered significant when the confidence interval was lower than 5 % ($P \leq 0.05$).

3. Results

3.1. Central composite design (CCD)

3.2. Central composite design (CCD)

The EE as function of significant effects is represented by the **equation (1)**:

EE = 32.65914 + 19.56284 * Lecithin concentration + 23.76479 *Antimicrobial concentration - 3.13169* Lecithin concentation^2

The obtained results were then analyzed by ANOVA to assess the “goodness of fit” (**Table 10.4)**. The obtained model had high R^2 value and showed no lack of fit. The Model F-value of 27.58 implies that the model is significant. Values of "Prob > F" lower than 0.05 indicates significant model terms. In this case A, B, A^2 are significant model terms. The "Pred R²" of 0.80 is in reasonable agreement with the "Adj R²" of 0.87. "Adeq Precision" measures the signal to noise ratio and the model ratio of 14.9 indicates an adequate signal. This model can be used to navigate the design space considering that a ratio > 4 is desirable.

Table 10.4. Statistical parameters obtained from the analysis of variance for the reduced models

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	673.84	5	134.77	27.58	< 0.0001	significant
A-Lecithin concentation	475.43	1	475.43	97.31	< 0.0001	
B-Antimicrobial concentration	79.72	1	79.72	16.32	0.0011	
A^2	101.24	1	101.24	20.72	0.0004	
Residual	73.29	15	4.89			
Lack of Fit	13.16	3	4.39	0.88	0.4808	not significant
Pure Error	60.12	12	5.01			
Cor Total	747.12	20				

Std. Dev.	2.210	**R²**	0.902
Mean	61.613	**Adj R²**	0.869
C.V. %	3.588	**Pred R²**	0.800
PRESS	149.589	**Adeq Precision**	14.935

Results showed that at low lecithin and antimicrobial concentration, the EE was the lowest with 45%. By increasing the antimicrobial concentration, an increase of the EE (57 %) was observed. The maximum EE was observed by increasing both concentrations to reach an EE of ≈ 70 %. The CCD analysis provided a correlation between the EE and the lecithin and the antimicrobial concentrations. The obtained results suggest that the optimal formulation was determined to lecithin and antimicrobial concentration of 2.7 % and 0.52 % respectively for an EE of 68%.

3.3. Characterization of particles

Results (**Table 10.5)** showed that nanoliposome had the lowest diameter size and PDI with 91.86 nm and 0.23 respectively as compared to 99.61 nm and 0.243 for nanoemulsion and 458 nm and 0.47 for gelled emulsion. In this study, results showed that PDI increased significantly with polymer addition. With nanoliposome and nanoemulsion, the PDI remained stable showing a value of 0.230 and 0.243 respectively. Also, the viscosity of nanoemulsion and nanoemulsion were 1.47 and 1.54 cP respectively and increased significantly to 13.4 cP for gelled emulsion.

Table 10.5. Particle size (nm), PDI, interfacial zeta potential (mV) and viscosity of particles produced by sonication

	Particle size (nm)	PDI	Zeta potential (mV)	η (cP)
Nanoliposome	91.86 ± 1.61	0.230 ± 0.006	-36.0±1.7	1.54
Nanoemulsion	99.61±1.50	0.243 ± 0.029	-14.5 ±1.37	1.47
Gelled emulsion	458±47.02	0.38±0.09	-30.8±0.78	13.4

PDI : Polydispersity index

3.4. Release of antimicrobial compounds

The releases of phenolic compounds, an important antimicrobial compound from the EOs and Biosecur extract present in nanoliposome, nanoemulsion or gelled emulsion, through the dialysis membrane immersed in ethanol (10 %) at room temperature are shown in **Fig. 10.1**. Results showed that the release level of phenolic compounds was similar between all forms of encapsulation during the early stage of 30 min. Afterwards, the phenolic compounds release increased and reached 50 % of cumulative release at 60 min, 90 min and 140 min for nanoemulsion, gelled emulsion and nanoliposome respectively. Stabilization was observed after 250 min showing a respective release of 99.2 and 84.4 and 79.8 % for gelled emulsion, nanoemulsion and nanoliposome respectively. A 100% of cumulative release was observed at 360 min, 1200 and 1400 min for gelled emulsion, nanoemulsion and nanoliposome. The release of phenolic compounds was faster from gelled emulsion than nanoemulsion and nanoliposome. These results suggest that the release of bioactive ingredients from formulated antimicrobials to simulated media got delayed in formulations entrapped in nanoliposome matrix when compared to nanoemulsions and gelled emulsion, allowing a slower release during storage time.

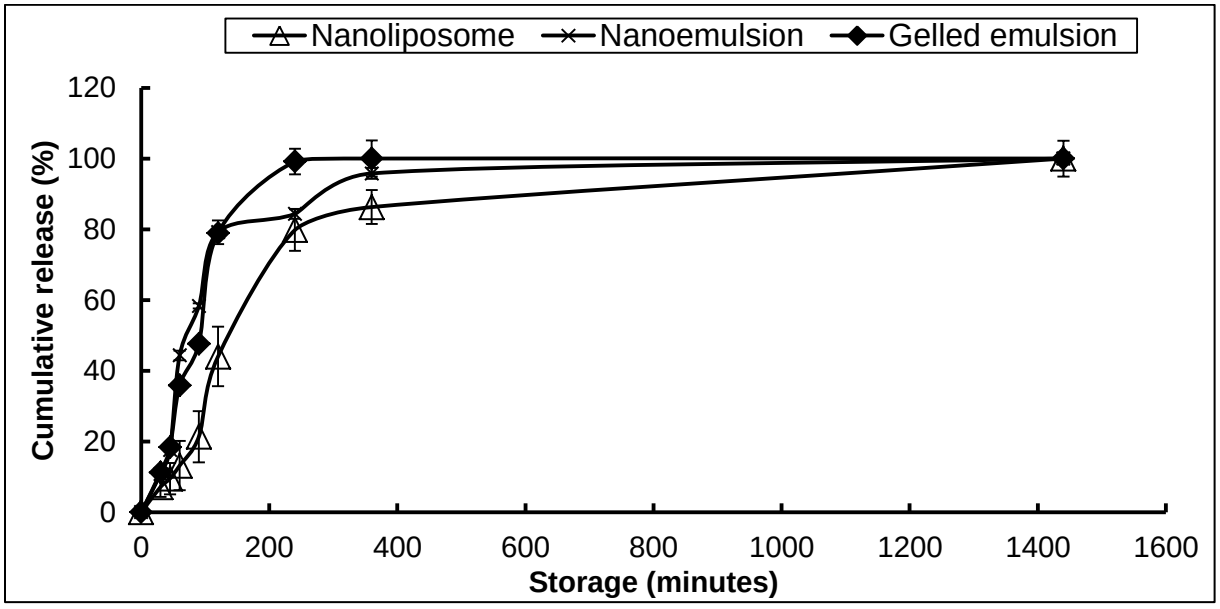


Figure 10.1. Effect of the entrapment of antimicrobial formulation on their release profile.

To analyze kinetics and the mechanism of release, the parameters k, n and R^2 values were determined (**Table 10.6**). The R^2 value for all samples was > 0.8 demonstrating that the Korsmeyer-Peppas model fitted the release of phenolic compounds from antimicrobial

ingredients. The value of n > 0.89 for all encapsulation forms corresponding to a sigmoidal release pattern and ascribed to a Super Case II transport, in which phenolic compounds release seemed to be controlled by the polymer relaxation (Wei *et al.*, 2006).

Table 10.6. Release kinetic studies of natural antimicrobials from nanoemulsion, nanoliposome and gelled emulsions

	n	k	R^2
Nanoliposome	1.28	0.045	0.9486
Gelled emulsion	1.38	0.051	0.9603
Nanoemulsion	1.65	0.033	0.9353

n : release exponent
k: constant of apparent release
R^2: regression coefficient

The values of '*k*' were found to vary with the encapsulation form. The constant of apparent release showed 0.033, 0.045 and 0.051 for nanoemulsion, nanoliposome and gelled emulsion confirming previous observations that nanoliposome and nanoemulsion showed the slowest release as compared to gelled emulsion.

3.5. Stability study

Results of TPC (**Table 10.7**) showed that liposome gave the highest TPC on day 0. A level of 2710 GAE µg/mL was observed as compared to 2185 and 1886 GAE µg/mL for gelled emulsion and nanoemulsion respectively. After 30 days, the level of TP of liposome samples showed also the highest value of 2189 GAE µg/mL as compared to 1539 and 1221 GAE µg/mL respectively for gelled emulsion and nanoemulsion. These results suggest that the encapsulation of bioactive molecules into nanoliposome and gelled emulsion allowed respectively 19.23 % and 29.6 % of TP loss from day 0 as compared to 35.23 % of TPC loss for nanoemulsion.

Table 10.7. Particle size, PDI, zeta potential and total phenol content of encapsulation forms during storage at 4 °C.

Mode of encapsulation	Days	Particle size (nm)	PDI	Zeta potential (mV)	Total phenol GAE (µg/mL)
Nanoemulsion	**0**	99.6±1.6^{a}	0.243 ±0.029^{a}	-14.47±1.37^{b}	1886 ± 13^{b}
	15	102.9±4.3^{a}	0.257±0.042^{a}	-17.67±0.12^{b}	1252 ± 21^{a}
	30	104.9 ±3.1^{a}	0.278±0.013^{a}	-22.3±2.82^{a}	1221 ± 15^{a}
Nanoliposome	**0**	91.9±1.6^{a}	0.230±0.006^{a}	-36.0±1.71^{b}	2710 ± 118^{b}
	15	103.6±5.1^{b}	0.246±0.010^{a}	-38.43±1.10^{ab}	2277 ± 141^{a}
	30	108.1±2.0^{b}	0.291±0.041^{a}	-39.47±1.55^{a}	2189 ± 125^{a}
Gelled emulsion	**0**	458.5±47.0^{a}	0.380±0.090^{a}	-30.80±0.78^{a}	2185 ± 132^{b}

15	500.7±9.7 [ab]	0.383±0.015 [a]	-30.83±0.72 [a]	1593 ± 165 [a]
30	549.2±29.5 [b]	0.369±0.104 [a]	-30.57±0.96 [a]	1539 ± 23 [a]

Based on particle size results, the gelled emulsion had the highest droplet size. A particle size of 458.5 nm for gelled emulsion was observed as compared to 99.6 and 91.9 nm for respectively nanoemulsion and nanoliposome. After 30 days of storage, particle size remains statistically stable for nanoemulsion as compared to a significant increase for both gelled emulsion and nanoliposome to reach respectively 549.2 and 108.1 nm. These results suggest that nanoemulsion was highly stable to agglomeration than nanoliposome and gelled emulsion.

Based on the zeta potential results, nanoliposome had the lowest zeta potential. A zeta potential of -36 mV was observed on day 0 as compared to -30.8 and -14.5 mV for gelled emulsion and nanoemulsion respectively. This is due mainly to the presence of lecithin and pectin having an anionic charge. After 30 days of storage, zeta potential of gelled emulsion remains statistically stable, however, a significant decrease of zeta potential was observed on for nanoliposome and nanoemulsion showing respectively -39.5 and -22.3 mV.

Based on PDI results, gelled emulsion had the highest PDI showing 0.380 on day 0 as compared to 0.243 and 0.0230 for respectively nanoemulsion and nanoliposome. After 30 days, no significant effect on the PDI was observed for all tested solutions.

3.6. Antimicrobial efficiency

3.6.1. In vitro study

Results of MIC (**Table 10.8)** obtained from broth dilution assay showed that the MIC of nanoliposome was ≥ 3000 ppm for all evaluated microorganisms. At similar antimicrobial concentrations, the nanoemulsion showed lower MIC with 3000 ppm, 1500 ppm, 375 ppm and 156.25 ppm for *A. flavus*, *E. coli*, *L. monocytogenes* and *B. subtilis* respectively. The entrapment of the antimicrobial formulation in pectin, matrix allowed to reduce the MIC to reach 500 ppm, 187.5 ppm, 93.75 ppm, 46.88 ppm and 46.88 ppm for *E. coli*, *A. flavus*, *L. monocytogenes*, *B. subtilis* and *C. albicans*. In summary, gelled emulsion showed the highest antimicrobial efficiency for all evaluated microorganisms and the efficiency was from 16 to 64 time higher as compared to nanoliposome.

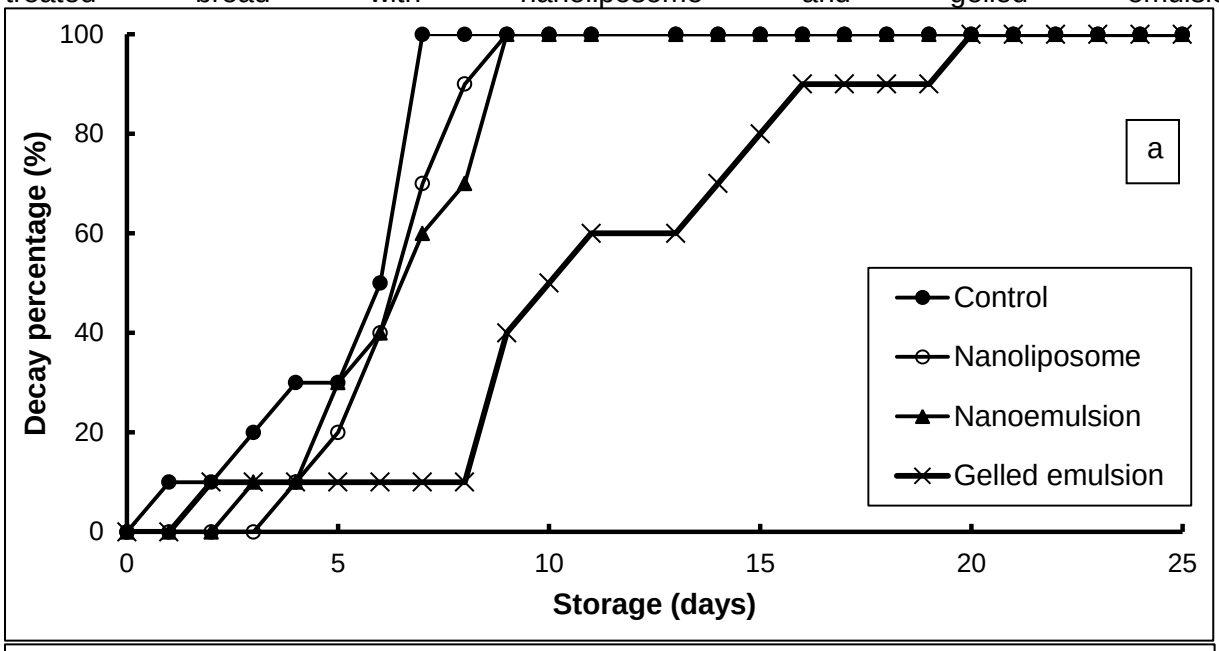
Table 10.8. MIC of antimicrobial formulations evaluated against evaluated microorganisms.

	MIC (ppm)		
	Nanoliposome	**Nanoemulsion**	**Gelled emulsion**
E. coli	> 3000	1500	500
L. monocytogenes	3000	375	93.75
B. subtilis	> 3000	156.25	46.88
C. albicans	> 3000	375	46.88
A. flavus	> 3000	3000	187.5

3.6.2. In situ study

A. flavus. The obtained results (**Fig.10.2a**) showed that for untreated bread, the growth of *A. flavus* reached 100 % at day 7 as compared to day 9 for nanoliposome and nanoemulsion and day 20 for treated bread with gelled emulsion. These results suggest that the encapsulation of a mix of EOs and Biosecur F440D into nanoliposome vesicle was efficient to reduce the growth of both *A. flavus* and *B. subtilis* as compared to the control.

B. subtilis. The obtained results (**Fig.10.2b**) showed that for untreated bread and treated bread with nanoemulsion, the growth of *B. subtilis* reached 100 % of growth at day 14 of storage as compared to day 25 for treated bread with nanoliposome and at day 36 for treated bread with gelled emulsion. This represents a 11 days and 22 days of shelf-life extension for respectively treated bread with nanoliposome and gelled emulsion.



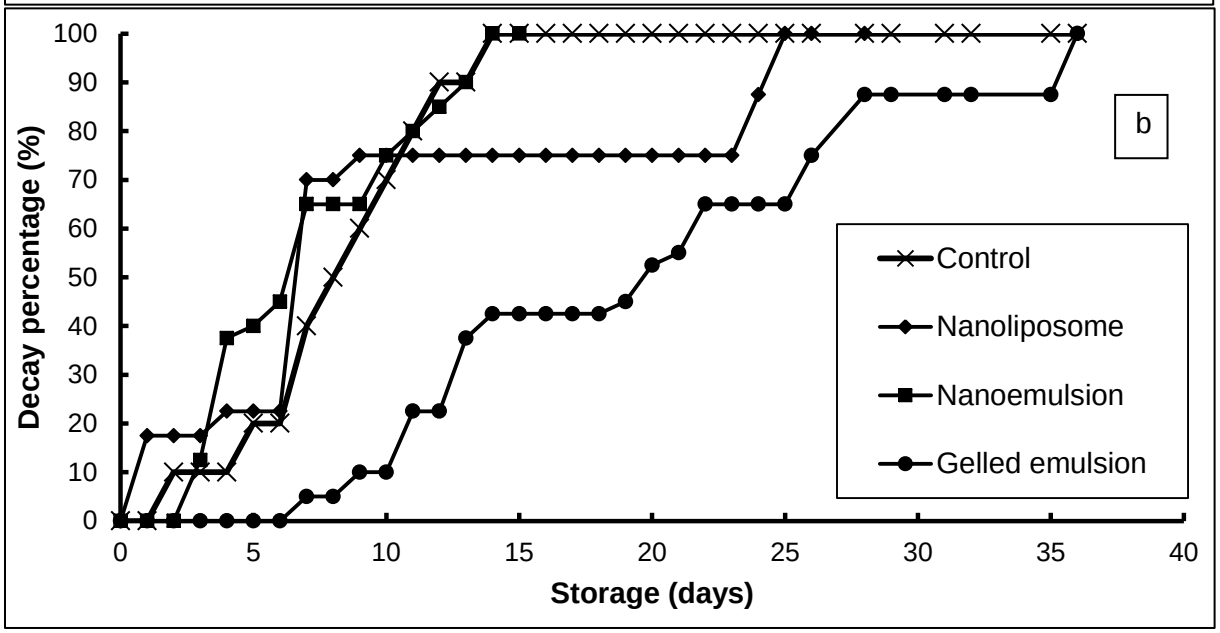


Figure 10.2. Effect of antimicrobial coating application on the growth of (**a**) *A. flavus* and (**b**) *B. subtilis* on bread surface during storage at room temperature.

3.7. Sensory evaluation

Results (**Fig.10.3)** showed that the antimicrobial treatment encapsulated under different forms did not have any detrimental effect on the sensorial quality of the baked bread. The values of the appearance, the odor, the taste, the texture and the global appreciation were 7.4, 6.8, 7.3, 7.9 and 7.3 for the bread balls treated with the antimicrobial formulation loaded nanoliposome and 7.3, 7.3, 6.7, 7.9 and 6.8 antimicrobial formulation loaded nanoemulsion and 8, 7.2, 7.2, 7.9 and 7.3 for antimicrobial formulation loaded gelled emulsion as compared to 8.1, 7.7, 7.5, 8.2 and 7.5 for the control samples. Overall, no significant negative effect ($P > 0.05$) was observed and all the attributes were ≥ 6.7.

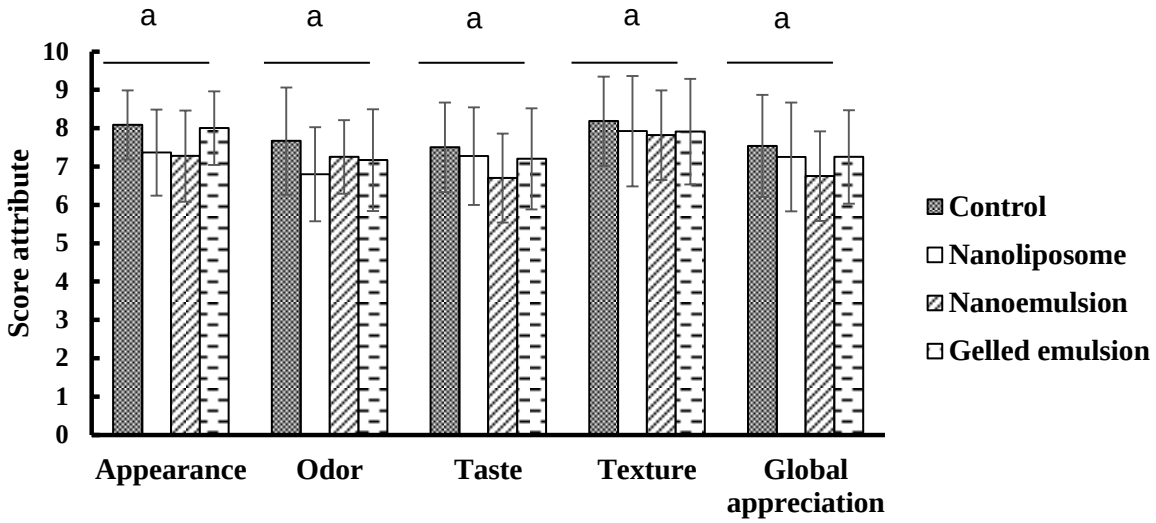


Figure 10.3. Sensory attributes of white bread precook-treated with different coatings

3.8. Texture

Results (**Fig.10.4)** showed no significant effect (**P** > 0.05) between untreated bread and treated bread with nanoliposome regarding the hardness value (**Fig.10.4**). However, a significant reduction of the hardness was observed for treated bread with nanoemulsion and gelled emulsion showing 249 and 230 g respectively as compared to 296 g for the control ($P \leq 0.05$). These results suggest that applying nanoemulsion or gelled emulsion on bread surface allowed softer bread.

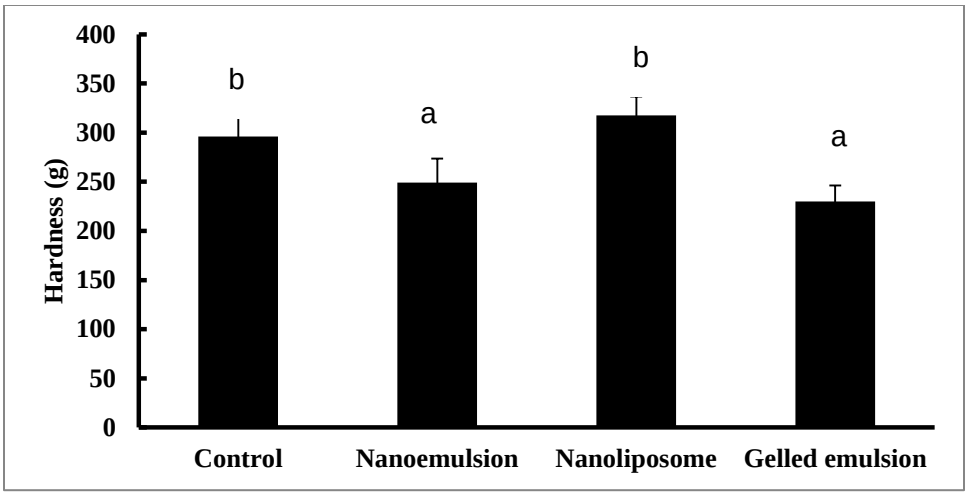


Figure 10.4. Texture of treated bread with different coating.

4. Discussion

Encapsulation of bioactive ingredients can provide advantages of controlled delivery for efficient bioactive ingredients release capable of overcoming various parameters related to physicochemical properties, process and storage conditions of the commodity including pH, a_w and temperature. However, several limitations need to be addressed before their use in the food. One of the difficulties is the instability and the low EE of the bioactive ingredients. In the present study, we utilized various colloidal systems to encapsulate an antimicrobial formulation based on hydrophobic (EOs mixture) and hydrophilic (Biosecur F440D) bioactive ingredients under the form of nanoemulsion, gelled emulsion and nanoliposome. The optimization of nanoliposome composition in order to have optimal EE allowed the maximization of the antimicrobial concentration into liposome vesicles. This allowed to achieve better efficacy especially against *B. subtilis* on bread matrix, allowed delayed release of phenolic ingredients and a better protection of phenolic compounds. Similar results were also observed with the encapsulation of thymol, carvacrol and mix of Carvacrol/Thymol and Carvacrol/γ-terpinene in liposome (C. C. Liolios *et al.*, 2009b, Maherani *et al.*, 2012b). This could be related to a better stabilization of bioactive ingredients as well as a very high retention ability of bioactive ingredient inside liposome nanovesicle. It is surprising to note that the *in vitro* antimicrobial activity was low suggests that bioactive ingredients were highly protected which reduced their bioavailability during a short contact time with the microorganisms. In fact, nanoliposome was more effective than nanoemulsion to control *B. subtilis in situ* which confirm the hypothesis of the efficiency of nanoliposome during a long storage allowing the release of more bioactive ingredients from the core of nanoliposome.

The EE in nanoliposomes could be affected principally by the properties of both nanoliposomes (aqueous volume, membrane rigidity, surface area and preparation methods) and the hydrophilic or lipophilic properties of encapsulated bioactive ingredient (Maherani *et al.*, 2012a). Nanoliposome also allowed to protect the TPC during storage due to their ability to isolate the encapsulated ingredients from the surrounding food environment, protecting them from conditions that would otherwise impede activity (Reza Mozafari *et al.*, 2008). The release behavior of phenolic compounds from nanoliposome was governed by a super Case II transport. Park *et al.* (2013) demonstrated that liposome encapsulating quercetin and rutin followed a diffusion behavior and that the use of liposome hydrogel allowed the increase of the diffusion rate. Also, the release of sulfanilamide or curcumin from liposome was governed by only diffusion and both diffusion and dissolution from phospholipid bilayer respectively (Liu *et al.*, 2015, Petrović *et al.*, 2017).

However, the rapid release observed on the gelled emulsion did not correlate with previous studies. In fact, by using the same pectin matrix, Y. Ben-Fadhel *et al.* (2019b) showed a delayed release of polyphenolic ingredients as compared to their respective emulsions. This suggests that the control of the release of phenolic compounds is not only related to the choice of the polymer matrix but mainly to the preparation method. Thus, to achieve to a better controlled release, it is important to have stable emulsion first followed by a coating which demonstrated a specific interaction allowing the retention of phenolic compounds (Y. Ben-Fadhel *et al.*, 2019b). Similarly to nanoliposome, a non-Fickian behavior (case II transport) was also observed for gelled emulsion and nanoemulsion. This was also confirmed by Paula *et al.* (2010) and Kulkarni *et al.* (2000) where EOs and natural pesticide were released from angico gum/chitosan nanoparticles or from alginate beads crosslinked with glutaraldehyde respectively. Also a non-Fickian or anomalous diffusion was obtained for the release of oleanolic acid and its isomer ursolic acid from nanoemulsion (Alvarado *et al.*, 2015). Similar results were observed by El-Badry *et al.* (2014) where the antifungal activity of Croconazole was higher in gelled emulsion than liposome.

The stability of the colloidal system is a very important factor that could be predicted by following several parameters. Viral H. Shah *et al.* (2018) showed that critical quality attributes which would affect the efficiency and performances of the liposomes are PDI, zeta potential and the EE. In the current study, the zeta potential of nanoliposomes and gelled emulsion was highly negative which can be attributed to the presence of partially charged carboxylic acid groups along the pectin chain at pH 3.5 suggesting stable colloidal systems (Chang *et al.*, 2012). Electrostatic repulsion plays an important role in maintaining the stability of polymer complex (Chang *et al.*, 2012). However, this is not always true. Although gelled emulsion has high zeta potential, the increase of the particle size during storage suggests some instability as compared to nanoemulsion and nanoliposome. In fact, zeta potential depends mainly on pH and the formulation especially the use of anionic or cationic ingredients. When zeta potential is high, particle surface are enough charged to create repulsion force and avoid aggregation and sedimentation. Other parameters can affect the stability of polymer systems ie. polymer concentration, presence of salt (Dickinson *et al.*, 1998, Kang *et al.*, 2011).

The determination of the TPC during storage clearly demonstrated that nanoliposome and pectin matrix led a higher TPC and suggesting the presence of some phenolic compounds present in sugar-beet pectin and in soy lecithin (Y. Ben-Fadhel *et al.*, 2019b, Sun *et al.*, 1998). It is interesting to note that gelled emulsion and nanoliposome were the most efficient to maintain the TPC during storage. Similar observations were also obtained by Assis *et al.* (2014) and Y. Ben-Fadhel *et al.* (2019b) where a better protection of phenolic compounds encapsulated in nanoliposome or gelled emulsion respectively was also observed. This was related to the formation of specific interactions between antimicrobial compounds and polymer or to rigid cross-link network of the polymer at oil-water interface (Y. Ben-Fadhel *et al.*, 2019b, Kang *et al.*, 2011). In current study, the stabilization of TPC allowed also the improvement of the *in vitro* antimicrobial activity of gelled emulsion to control Gram positive, negative, yeasts and molds evaluated. Nanoemulsion and gelled emulsion showed a good *in vitro* antimicrobial activity. Gelled emulsion when applied on bread surface kept also a very good antimicrobial efficiency against *A. flavus* and *B. subtilis*. These results suggest that encapsulation of bioactive compounds into gelled emulsion allowed an improvement of the bioactivity of active ingredients. Also, nanoliposome encapsulation form seems to be efficient during long term storage. These results suggest that the encapsulation of bioactive compound enhance their bioactivity and extend their release during time allowing a better control of bacterial growth (Y. Ben-Fadhel *et al.*, 2019b). The application of the different coatings on bread surface did not affect the sensory properties and only nanoemulsion and gelled emulsion reduced slightly the hardness of the bread without any effect on panelist's appreciation.

5. Conclusion

This study was conducted to compare the different encapsulation forms (nanoemulsion, nanoliposome and gelled emulsion). This study has shown that nanoliposome allowed a better controlled release and a good *in situ* antimicrobial activity against *B. subtilis* on bread surface. Gelled emulsion has particularly the best *in vitro* and *in situ* antimicrobial activity against all the evaluated microorganisms. Nanoliposome was also efficient during long term food storage. Both of them have shown a good TPC protection during storage. From the viewpoint of the sensory and texture quality, all colloidal systems did not have a detrimental effect.

Acknowledgements

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CHAPITRE 11 : DISCUSSION GÉNÉRALE ET CONCLUSIONS

Encore aujourd'hui, les denrées alimentaires peuvent se retrouver contaminées par des microorganismes pathogènes mettant en péril la santé humaine et animale. Plusieurs études de recherche se sont intéressées à l'amélioration de la qualité microbiologique des aliments (Gould, 2012, Matthews *et al.*, 2019, Sung *et al.*, 2013, GH Zhou *et al.*, 2010). Les antimicrobiens naturels représentent une des méthodes prometteuses grâce à leur pouvoir inhibiteur des pathogènes alimentaires et leur capacité à prolonger la durée de conservation des aliments (Sung *et al.*, 2013, Tongnuanchan *et al.*, 2014). Cependant, ces antimicrobiens sont très peu utilisés en industrie alimentaire principalement à cause de leur goût et leur instabilité. Les études de recherche se sont principalement concentrées sur le potentiel antimicrobien que ces antimicrobiens possèdent sans proposer des solutions pour une application en industrie.

Dans cette thèse, nous avons, tout d'abord, confirmé que les antimicrobiens naturels ont un grand potentiel antimicrobien. Par l'intermédiaire des contributions réalisées, nous avons démontré que ce potentiel varie selon la nature hydrophobe ou hydrophile de l'antimicrobien utilisé. En effet, nous avons démontré dans le **Chapitre 6** que les antimicrobiens hydrophiles présentent une activité bactéricide contre toutes les souches bactériennes évaluées (Yosra Ben-Fadhel *et al.*, 2019a). Les antimicrobiens lipophiles présentent par contre une activité bactériostatique et fongicide. Ceci est intéressant puisque cela nous permet de mieux sélectionner les antimicrobiens pour viser les microorganismes en question. Selon Mourey *et al.* (2002), l'activité bactériostatique ou bactéricide des huiles essentielles dépend principalement de leur composition. En effet, des composés des huiles essentielles tels que α-pinène β-pinène R-limonène, S-limonène ont un effet bactériostatique ce qui n'est pas le cas de 1,8-cinéole et bornéol qui ont un effet bactéricide contre *Listeria monocytogenes* (Mourey et al., 2002). En se basant donc sur nos résultats obtenus et présentés au **Chapitre 6** et la composition des huiles essentielles, il sera plus facile de prédire l'activité bactériostatique/ fongistatique ou bactéricide/ fongicide d'une huile essentielle. D'autre part, nous avons développé des formulations à base d'un mélange de ces antimicrobiens à des concentrations inférieures à la CMI de chacun d'entre eux (**Annexe I**), ce qui a permis de réduire les concentrations nécessaires pour inhiber les bactéries et les moisissures cibles et ainsi de réduire l'impact sur les propriétés sensorielles de l'aliment.

Bien que les antimicrobiens naturels sélectionnés dans le **Chapitre 6** avaient séparément des activités bactéricides et fongicides, des études antérieures ont démontré qu'une fois appliqué sur la surface de l'aliment, certains de ces antimicrobiens, comme les huiles essentielles, peuvent perdre leur activité antimicrobienne avec le temps dû principalement à l'altération de leurs composants par les interactions avec les composants de la matrice alimentaire, tels que les graisses, l'amidon et les protéines (Hyldgaard *et al.*, 2012, Prakash *et al.*, 2015). De plus, à cause de leur volatilité, il y a plus de risque de perte durant le processus de fabrication et le transport (Prakash *et al.*, 2015). Les huiles essentielles sont aussi sensibles à l'oxydation ce qui dégrade les composés mono et sesquiterpéniques (Hyldgaard *et al.*, 2012, Isman, 2000). Ce phénomène est beaucoup plus rapide en présence de facteurs tels que la température et la lumière. Notre contribution dans la deuxième partie de cette thèse est ainsi de présenter une méthodologie pour l'incorporation des antimicrobiens naturels d'origine végétale dans des matrices adéquates capables de protéger ces antimicrobiens lors d'une application alimentaire. Nous avons, tout d'abord, proposé une technique d'encapsulation à base d'un biopolymère capable de stabiliser l'activité antimicrobienne des composés actifs naturels et de la maintenir plus longtemps au cours de l'entreposage de l'aliment. Un criblage préliminaire des polymères a

permis de sélectionner la pectine hautement méthylée qui s'est avérée la plus efficace à contrôler la flore totale des carottes (**Annexe II**). Dans le **Chapitre 7,** nous avons démontré que la pectine était, de plus, efficace à contrôler la croissance de *L. monocytogenes* et *P. chrysogenum* dans les carottes (Y. Ben-Fadhel *et al.*, 2019b). Suite à la caractérisation des capsules formées, nous avons pu associer cette efficacité à une meilleure rétention des composés actifs due principalement à des liaisons hydrogène et des élongations -CH avec l'encapsulation de Biosecur F440D et des élongations asymétriques fortes -CH_3 dans les groupes alkyl pour l'encapsulation des huiles essentielles dans la matrice de pectine (Y. Ben-Fadhel *et al.*, 2019b). Donc, l'utilisation de la pectine comme matrice d'encapsulation a effectivement protégé les composés actifs des HE et de Biosecur F440D contre la dégradation et a induit une nette amélioration de l'activité antimicrobienne sur le modèle alimentaire.

Par la suite, une deuxième stratégie a été développée en encapsulant les antimicrobiens naturels dans des vésicules de nanoliposome. En effet, dans la littérature, les liposomes ont démontré un grand potentiel à encapsuler les composés bioactifs (enzymes, vitamines, etc.) mais aussi à protéger leur bioactivité. Cependant, comme mentionné dans la section 4.2.2, les liposomes sont très peu appliqués en industrie alimentaire à cause de leur faible efficacité d'encapsulation et le peu de connaissances sur les interactions entre la membrane phospholipidique et les composés bioactifs d'une part et l'interaction entre les vésicules et la matrice alimentaire (McClements, 2015). Dans cette thèse, nous avons développé un nanoliposome encapsulant des antimicrobiens naturels et ceci en optimisant l'efficacité d'encapsulation et en s'intéressant plus particulièrement à la caractérisation des interactions entre la couche phosphilipidique des liposomes et les antimicrobiens hydrophiles ou hydrophobes par FTIR et ITC. Ceci a permis de mieux comprendre le système développé. Les liposomes ont, en effet, démontré une plus grande affinité à encapsuler Biosecur F440D que les huiles essentielles et ceci via une déformation éthyl antisymétrique, déformation méthyl symétrique, élongation P-O-C et élongation symétrique PO_2 (**Chapitre 8**). De tels résultats permettront de mieux caractériser les vésicules développées et de pouvoir prédire le comportement des antimicrobiens avec le temps (libération des composés encapsulés) et ainsi prédire leur efficacité.

La 3ème stratégie que nous avons développée dans cette thèse est l'encapsulation des composés bioactifs en nanoémulsion (**Chapitre 9**). Dans ce chapitre, les deux techniques d'émulsification (sonication et microfluidisation) ont permis d'obtenir des gouttelettes de taille nanométrique. Nous avons également démontré l'importance de facteurs telsque le HLB et le ratio huile : émulsifiants dans la stabilité des nanoémulsions. L'impact de ces facteurs a déjà fait l'objet de plusieurs études (Carpenter et al., 2017, Mayer et al., 2013, Ševčíková et al., 2012). Dans la plupart des cas, un HLB entre 10 et 14 est requis pour stabiliser une émulsion o/w. Cependant, Takamura et al. (1979) ont démontré qu'une différence de HLB < 7 permettra l'obtention d'une stabilité optimale. Dans cette thèse une différence de HLB =11 n'a pas affecté la stabilité de la nanoémulsion durant la période de l'étude (14 jours). La turbidité est également affectée par la valeur de HLB et elle est est à sa valeur minimale (nanoémulsion transparentes ou translucides) à HLB = 12 ce qui est confirmé par l'étude de Wen-Chien Lu *et al.* (2018). La turbidité est également intimement reliée à la technique d'émulsification utilisée. Elle est affectée par le temps de sonication et aussi par le nombre de cycle et la pression du microfluidisateur. Ceci est du principalement à la réduction de la taille des particules. En effet, Zhang et al. (2016) a identifié la taille des gouttelettes, l'indice de réfraction des phases continues et dispersées, la composition de l'interface et la concentration des phases dispersées comme les principaux facteurs influençant la turbidité de l'émulsion. L'évaluation de l'activité antimicrobienne *in vitro* a permis de démontrer une amélioration de cette activité contre principalement *E. coli* et *L.monocytogenes.* Ceci est due principalement à la réduction de la taille des particules de l'émulsion (Salvia-Trujillo et al., 2014). Cependant, aucun impact n'a été

observé contre *B. subtilis* et *A. flavus in vitro* probablement parce que ce sont des micro-organismes sporulants, ainsi le mécanisme d'action inhibiteur diffère par rapport aux micro-organismes non sporulants. La nanoémulsion a également était efficace à améliorer la durée de conservation des aliments testés sans avoir d'impact négatif sur les propriétés physicochimiques de l'aliment (**Annexe III- IV**)

Finalement, dans le **Chapitre 10,** nous avons comparé les 3 stratégies développées (nanoémulsion, nanoliposome et émulsion gélifiée). Ceci a permis de démontrer que l'émulsion gélifiée était la plus efficace à contrôler *E. coli*, *L. monocytogenes*, *B. subtilis*, *C. albicans* et *A. flavus in vitro* et *in situ* quand elle est appliquée sur la surface du pain. Bien que la pectine utilisée dans cette étude a démontré une bonne teneur en phénol totaux dans des études précédentes (Sun *et al.*, 1998), les résultats *in vitro* obtenus dans l'annexe V ont permis d'écarter l'hypothèse que l'amélioration de l'activité antimicrobienne pourra être due à une éventuelle activité de la pectine. Cette amélioration est due plutôt à une meilleure stabilité des composés phénoliques encapsulés. Cependant, le test de libération des composés actifs contredit les résultats obtenus dans le **Chapitre 7**. Ceci suggère que le contrôle de la libération des composés phénoliques n'est pas seulement lié au choix de la matrice polymèrique mais principalement à la méthode de préparation. Ainsi, pour parvenir à un meilleur contrôle de la libération des composés bioactifs, il est important d'avoir d'abord une émulsion stable suivie d'un enrobage démontrant une interaction spécifique permettant la rétention des composés phénoliques.

D'autre part, les nanoliposomes malgré leur faible efficacité *in vitro*, ils ont démontré une meilleure efficacité contre *B. subtilis in situ* sur le pain. Ceci suggère que les nanliposomes sont capables de retarder la libération des composés phénoliques comme l'ont confirmé Maherani *et al.* (2012b) et de mieux protéger les composés phénoliques contre la dégradation. Ceci implique que l'efficacité des nanoliposomes pourra être observée plutôt pendant un long stockage ce qui donnera le temps de libérer les composés bioactifs de l'intérieure des vésicules.

11.1. CONTRIBUTIONS

Une première contribution importante de cette thèse présentée au **Chapitre 6** consiste à une démonstration que les antimicrobiens naturels (extraits de végétaux et huiles essentielles) présentent un large spectre d'inhibition contre les pathogènes alimentaires, de manière analogue ou supérieure aux additifs synthétiques.

Une deuxième contribution majeure de cette thèse, présentée aux **Chapitres 7-8 et 9** est la faisabilité industrielle des procédés de fabrication des systèmes colloïdaux de délivrance sans qu'aucun solvant ne soit utilisé ("chimie verte"), que les constituants de ces systèmes sont d'origine naturelle, que la technologie fait appel à des étapes simplifiées (équipements d'appoint comme les sonicateurs et homogénéisateurs), et que le procédé n'interfère pas avec la chaîne de production des ingrédients.

Une troisième contribution consiste à une démonstration d'une preuve de concept que les microcapsules gélifiées développées peuvent être appliquées sur différents modèles alimentaires solides (enrobage comestible sur légumes prêt-à-manger) et intermédiaires (pain) (**Chapitres 7-10**).

De plus, très peu de publications scientifiques ont fait l'objet de l'utilisation de nanoliposomes dans les applications alimentaires. La dernière contribution majeure consiste donc à la compréhension des mécanismes qui régissent les interactions ingrédients actifs/liposomes/matrice alimentaire (**Chapitres 8**-**10**).

11.2. RETOMBÉES ÉCONOMIQUES

La nature double des technologies qui ont été développées dans ce projet vise le marché des bio-ingrédients fonctionnels pour application alimentaire mais aussi celui des dispersions, nanoémulsions et nanoliposomes qui sont connues dans les domaines pharmaceutiques et cosmétiques, mais encore sous-utilisées pour des applications alimentaires et des aliments fonctionnels. Ceci permettra également de réduire les déchets alimentaires et plus précisément les pertes dues à la contamination.

Leur application sur des produits alimentaires de nouvelle génération se distingue par leur caractère "Clean Label", en comparaison avec les aliments qui comportent des additifs synthétiques, souvent jugés nocifs pour la santé.

Le fait d'utiliser des matrices d'encapsulation à base de biopolymères d'origine naturelle renforce encore leurs applications ciblant le marché en émergence des aliments "santé".

Enfin, ces technologies englobent le marché de l'industrie alimentaire reliée à divers produits tels que les fruits/légumes prêts-à-manger, les produits de boulangerie et les pains. Également, elles touchent les industries de traitement sanitaire des aliments comme les fruits et légumes frais après la récolte, avant l'emballage et la commercialisation

11.3. PERSPECTIVES ET RECOMMANDATIONS

Par la présente étude, nous avons démontré que l'encapsulation des antimicrobiens naturels peut être utilisée comme une nouvelle technologie pour la préservation des aliments et l'augmentation de leur durée de conservation sans altérer leurs propriétés sensorielles et physicochimiques. L'émulsion gélifiée, a particulièrement démontré une bonne efficacité à contrôler les pathogènes alimentaires. Cependant, il est recommandé de bien stabiliser l'émulsion avant de l'intégrer dans le biopolymère afin d'avoir une libération des antimicrobiens plus prolongée dans le temps.

Il est également recommandé de bien contrôler les conditions expérimentales lors de la préparation de la nanoémulsion et plus précisément, la température de la nanoémulsion durant le traitement de sonication ou de microfluidisation.

Il est également important de noter que les résultats des analyses *in situ* de cette thèse ont été obtenus en traitant les carottes pré-coupées par trempage et le pain par vaporisation.

Les défis actuels de l'application des antimicrobiens naturels sont multiples et les perspectives suivantes peuvent fournir plus d'informations pour d'autres défis futurs à relever.

a- La présente étude s'est concentrée sur l'activité bactéricide et fongicide contre 9 microorganismes (des bactéries Gram positif et négatif, des levures et des moisissures). Cette étude pourra être étendue contre d'autres microorganismes, spécifiquement ceux qui ont démontré dans la littérature une résistance particulière contre des antibiotiques. Les antimicrobiens naturels peuvent également être étudiés pour leurs propriétés antivirales et insecticides en vue d'identifier des sources potentielles de nouveaux composés antiviraux, capables de promouvoir la conservation des aliments.

b- Il sera intéressant d'explorer le mécanisme d'action des composés antimicrobiens sélectionnés dans cette étude lorsqu'ils sont appliqués seuls ou en mélange. Ceci

permettra d’évaluer la présence de synergie. Il sera intéressant d’étudier le mécanisme d’action des capsules développées telles que les nanoémulsions, les émulsions gélifiées et les nanoliposomes sur des microorganismes cibles en vue d'optimiser leur application dans la conservation des aliments.

c- La microencapsulation des antimicrobiens naturels a été étudiée pour l’amélioration de leur activité antimicrobienne. Avec les résultats promoteurs de l’utilisation de la pectine comme matrice d’encapsulation, il sera intéressant de développer un enrobage des liposomes qui pourra permettre un meilleur contrôle de la libération et une amélioration de l’activité antimicrobienne des composés encapsulés. Aussi, les complexes moléculaires peuvent être étudiés pour limiter l’oxydation et permettre une libération contrôlée dans la matrice alimentaire des antimicrobiens hydrophobes tels que les huiles essentielles

d- Il peut également être important de tester les capsules développées dans cette étude en combinaison avec d’autres méthodes de conservation des aliments telles que les méthodes de traitement thermique ou des méthodes d’emballage sous atmosphère modifiée ou des traitements à l’irradiation gamma (Annexe VI). Ceci permettra de réduire la dose nécessaire des antimicrobiens naturels et réduire ainsi le potentiel impact négatif sur les propriétés sensorielles des aliments. Il sera intéressant d'explorer les avantages de combiner les capsules développées à plusieurs de ces méthodes afin d’améliorer encore plus leur stabilité et d’amplifier leur effet protecteur.

ANNEXE I

Supplementary data 1

Determination of the synergy between antimicrobials agents

The checkerboard method was used in order to evaluate the possible synergy between compounds (Hossain et al., 2016). In this method, each of the two EOs was separately two-fold diluted with MHB in microplates of 96 wells. Then, the EOs dilutions were transferred into the main microplate which contained a serial concentration of 50 µL of AM_1 along the X axis and the serial concentration of same volume of AM_2 along the Y axis (6 × 6 matrix) to obtain the Fractional Inhibitory Concentration (FIC) index of antimicrobial in combinations. This study was divided into 2 parts. The first part was to evaluate the combined effect between two EOs against the most resistant microorganisms *P.chrysogenum* and *A. flavus*. The second part was to evaluate the antifungal activity of the EO mixture and Biosecur F440D against the same microorganisms.

Plates were incubated at 37 °C and 28 °C for 24 h and 48 h under agitation at 80 rpm for bacteria and fungi respectively and were read at 595 nm. FIC was calculated according to the following formula:

(1) FIC1=MIC1combined/MIC1alone

(2)FIC2=MIC2combined/MIC2alone

(3)FIC=FIC1+FIC2Where:

MIC_1 alone: the MIC value of AM_1 tested alone;

MIC_2 alone: the MIC value of AM_2 tested alone;

MIC_1 combined: the MIC value of AM_1 tested in combination with AM_2;

MIC_2 combined: the MIC value of AM_2 tested in combination with AM_1.

The results are considered as synergistic when $FIC \leq 0.5$, additive when $0.5 < FIC \leq 1$, not interactive when $1 < FIC \leq 4$ and antagonist when the $FIC > 4$.

Results

Table 1. FIC of combined essential oils against *A. flavus* and *P.chrysogenum*.

Test compound	*A. flavus*		*P. chrysogenum*	
	FIC	**Activity**	**FIC**	**Activity**
Cinnamon / lemongrass	0.6	AD	0.6	AD
Oregano /java citronella	0.5	S	0.6	AD

Table 2. FIC of combined essential oils and Biosecur F440D against *A. flavus* and *P.chrysogenum*.

Test compound	*A. flavus*		*P. chrysogenum*	
	FIC	**Activity**	**FIC**	**Activity**
Oregano/citronella/ cinnamon-lemongrass and Biosecur F440D	1	AD	1	AD

ANNEXE II

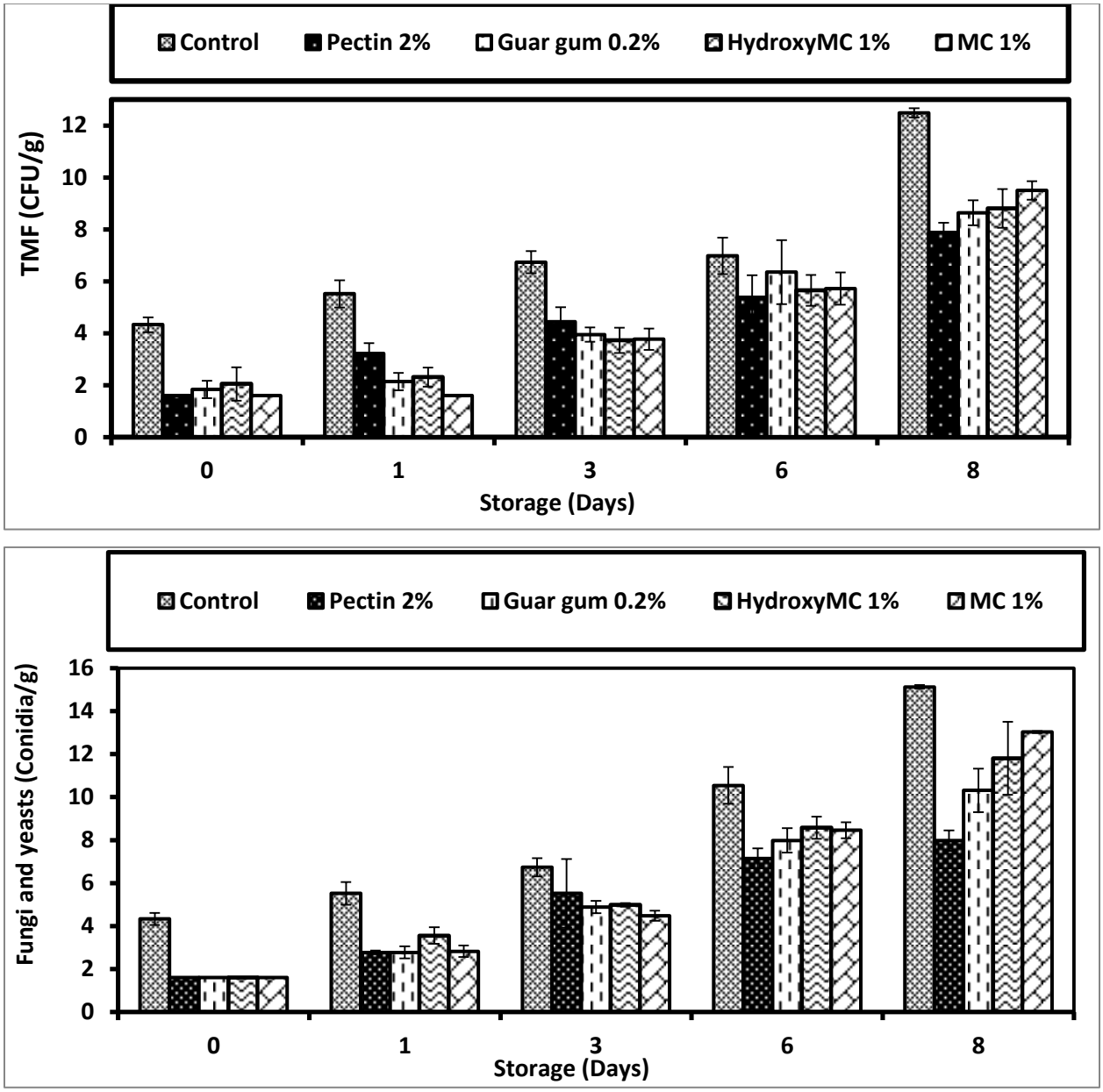

Figure. Effect of incorporation of antimicrobial formulation into polymer matrix on the growth of total mesophilic flora (TMF) and total fungi and yeasts of carrot.

ANNEXE III

Table. Texture of sliced carrots treated by developed capsules

	Days	Hardness (kg)	Cohesiveness	Elasticity (cm)	Chewiness (kg*cm)
Control	1	1.2	0.97	24.95	28.53
	15	1.2	1.18	24.96	34.19
	30	1.2	0.95	24.96	28.60
Nanoemulsion	1	1.2	0.96	25.00	29.39
	15	1.2	0.91	24.92	26.54
	30	1.2	0.99	24.91	30.13
Nanoliposome	1	1.2	0.99	24.97	29.95
	15	1.3	0.95	24.96	29.54
	30	1.2	0.98	24.88	29.60
Gelled emulsion	1	1.2	0.97	25.02	29.42
	15	1.2	0.88	25.10	25.88
	30	1.2	0.98	24.91	29.81

ANNEXE IV

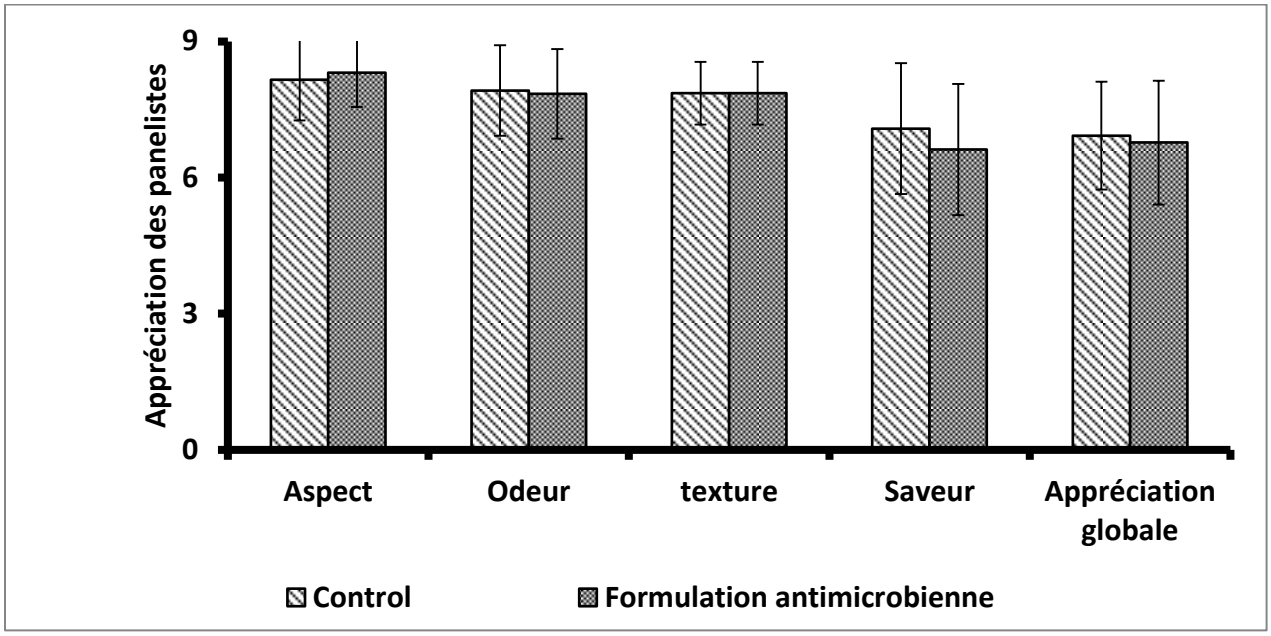

Figure. Effet de l'application de la nanoémulsion sur les propriétés sensorielles du pain

ANNEXE V

Table. MIC of pectin

	MIC (ppm)
E. coli	> 10000
L. monocytogenes	> 10000
B. subtilis	10000
C. albicans	> 10000
A. flavus	> 10000

ANNEXE VI

Effect of Gamma Irradiation on Film Properties and Effect of Combined Treatments Involving Γ-Irradiation and Bioactive Edible Coating On Carrot Preservation

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ABSTRACT

Irradiation was evaluated for its ability to improve the barrier properties of calcium caseinate films (Ca-Cas). Color, texture and water vapor (WVP) were assessed to characterize developed films. Then, pre-cut carrots were coated with antimicrobial-based nanoemulsion loaded in Ca-Cas and/or irradiated at 0.5 kGy. The impact on weight loss, texture, color and microbial quality of the pre-cut carrots stored at 4 °C was evaluated. Results showed that the irradiation of Ca-Cas solution at 32 kGy allowed the reduction of WVP, more yellow-green and elastic film. Nanoemulsion loaded into irradiated Ca-Cas coating and irradiation at 0.5 kGy allowed a reduction of initial flora of carrots. However, nanoemulsion-loaded-Ca-Cas coating increased the weight loss of carrots. Combining coating and irradiation treatment was more effective to control carrots initial flora. Coating of pre-cut carrots with nanoemulsion-loaded-Ca-Cas allowed maintaining the carrots color and a better control of the initial flora especially when combined to an irradiation treatment at 0.5 kGy. Combining coating and irradiation treatment demonstrated a better efficiency with regards to the control of the initial total flora of carrots.

Keywords

Calcium caseinate, γ irradiation, active coating, ready-to eat vegetables

1. Introduction

Unlike most food products, fresh fruits and vegetables continue to respire after they have been harvested. This process consumes oxygen and produces carbon dioxide and water vapor which is accelerated when fresh fruits and vegetables are preprocessed (ex: cutting/ peeling/ washing). This accelerates their ripening and destroys their quality by loosing of nutrients, enhancing dehydration process, increasing water activity level, and accumulating of nutrients on the surface of these vegetables. This phenomenon stimulates bacterial growth and reduces the shelf-life of vegetables. To extend their shelf-life, and maintain their safety, several methods based on cold process could be employed. Coating of ready-to-eat (RTE) vegetables and fruits using biopolymers could be a good solution to maintain their freshness. Their efficiency to provide a selective barrier to moisture, $O_{2,}$ and $CO_{2,}$ lead to low respiration, retarding dehydration, and loss weight, improving textural quality, helping retain volatile flavor compounds and chlorophyll, and reducing microbial growth (Fan et al., 2009). Several materials could be used for coating, i.e. lipid, protein, and polysaccharides resin. Proteins provide a good barrier to gases and lipid. However, due to their predominantly hydrophilic character, protein based film remain poor water-barrier (Lacroix & Vu, 2014). It has been demonstrated that cohesion among protein polypeptide chains enhances barrier properties of protein-based films. Increasing protein-protein interaction, i.e. by means of crosslinking process, at the expense of protein-water interaction has been found to improve water-vapor barrier property as well as the mechanical properties and resistance to proteolysis of films. Such crosslinking could be obtained by means of chemical (glutaraldehyde, formaldehyde, glyceraldehyde, glyoxal), enzymatic (transglutaminase), or physical (heating, irradiation) treatment (Lacroix & Vu, 2014). Biopolymer use as edible coating provides also the possibility to entrap some bioactive ingredients in order to improve their bioactivity or solubility. The loading of nanoemulsion based essential oils into chitosane has proven its efficiency to improve the anti-listeria activity of the nanoemulsion (Severino et al., 2014)

The shelf-life of packaged minimally processed carrots is sometimes limited by a white discoloration that develops on abraded surfaces during storage, which consumers may mistake for aging. White discoloration may be due to the formation of lignin as a wound barrier and/or dehydration of abraded surfaces (Boun & Huxsoll, 1991; Howard & Griffin, 1993). Carrots could also be contaminated by several microorganisms. The mechanism of contamination of carrots remains not well known but it is generally related to hand hygiene in the field, a direct contact with wildlife feces and cross-contamination of the equipment during washing and peeling (Endley, Lu, Vega, Hume, & Pillai, 2003).

Irradiation is an effective physical, safe, environmental clean method to assure food safety. This technology could be applied to the end product and have the advantage to be applied on fresh products (Lacroix, 2014). In 1983, the Codex Alimentarius Commission accepted that foods irradiated up to 10 kGy were safe and therefore toxicological testing was no longer necessary (Codex Alimentarius Commission, 2000). Also, a joint meeting held in 1997 involving the World Health Organization (WHO, 1999), the Food and Agricultural Organization (FAO), and the International Atomic Energy Agency (IAEA) has permitted to conclude that food irradiated with an appropriate dose to achieve the intended objective was both safe to consume and nutritionally adequate (WHO, 1999). At this meeting, the United Nations also confirmed that foods could be treated at any dose without any detrimental effect on the food's wholesomeness. The group study also concluded that high-dose irradiation, conducted in accordance with good manufacturing and irradiation practices, could be applied to several types of foods to improve their hygienic quality, make them shelf stable, and produce special products (Maherani et al., 2016; Olson, 1998; Roberts, 2016). The IAEA has a Food Authorizations Database in which countries are requested to lodge their approvals. However, because there is no obligation to

lodge information, the list is not completely up to date (Roberts, 2016). Currently, food irradiation is approved in more than 60 countries (Pedreschi & Mariotti-Celis, 2020).

Irradiation (even for radurization at 0.4–10 kGy and radicidation at 40–45 kGy) does not impart heat to the food and the nutritional quality of the food is generally unaffected. However, the doses needed to reduce the pathogens to undetectable level are often higher than the doses recommended to fruits and vegetables (≤ 1 kGy) (López et al., 2005). Previous studies reported that macronutrients, carbohydrates, proteins and lipids are relatively stable when done under fresh conditions at a dose up to 10 kGy (Lima, Vieira, Santos, & de Souza, 2018). However, some vitamins are more sensitive to irradiation treatment (Fadhel et al., 2016; Lima et al., 2018).

Presently, the use of combined treatments is widely suggested as for its ability to act in synergy to reduce the dose of irradiation and the concentration of the antimicrobial compound needed to eliminate pathogens.

The aim of this study was to develop and to characterize active edible coating based on milk protein and γ-irradiation. The effect of the application on carrot surface of calcium caseinate coating incorporating an antimicrobial nanoemulsion previously developed was evaluated when applied alone or combined to gamma irradiation. The shelf-life, the physicochemical properties of pre-cut carrots during storage at 4 °C were also evaluated.

2. Material and methods

2.1. Material

Biosecur F440D (33–39%) was provided by Biosecur Lab, Inc (Mont St-Hilaire, Québec, Canada). Cranberry juice (*Vaccinium macrocarpon*) was provided by Atoka Cranberries, Inc. (Manseau, QC, Canada) and was stored at -80 °C until used. Essential oils of lemongrass, oregano and citronella were bought from Biolonreco, Inc. (Dorval, QC, Canada) and their main constituents are presented in **Table 1**. Biosecur F440D and essential oils were stored at 4 °C. Calcium caseinate 380 was kindly provided by Fonterra Cooperative Ltd., (Palmerston North, New Zealand).

Table 1. List of organic essential oils (EO) and their composition.

Common Name	Botanic Name	Part	Compositions (%) *
Cinnamon EO	*Cinnamomum verrum*	Peel	E-cinnamaldehyde (55.1), cinnamyl acetate (9.6), β-caryophyllene (4.0)
Citronella EO	*Cymbopogon winterianus*	Aerial part	Citronellal (35.4), geraniol (20.1), Citronellol (12.2), elemol (4.6), Limonene (3.0), citronellyl acetate (2.9), germacrene D (2.7), geranyl acetate de (2.5), linalool (0.6)
Lemongrass EO	*Cymbopogon flexuosus*	Herb	Geranial (39.1), neral (31.6), geraniol (6.7), geranyl acetate (3.7)
Oregano EO	*Origanum compactum*	Flower top	Carvacrol (46.1), thymol (17.6), γ-terpinene+ trans-β-ocimene (14.8), p-cymene (8.5)

* Composition was provided by Biolonreco, Inc. and was determined by CPG-SM Hewlett Packard /CPG- FID; Column: HP Innowax 60-0.5 -0.25; Carrier gas Helium: 22 psi.

2.2. 2.2. Nanoemulsion preparation

Nanoemulsion was prepared based on the antimicrobial formulation developed by Yosra Ben-Fadhel, Maherani, Aragones, and Lacroix (2019) by mixing 4 essential oils (Cinnamon, lemongrass, oregano and citronella) with Biosecur F440D citrus extract and cranberry juice with emulsifiers (sunflower lecithin and sucrose monopalmitate) and vigorously stirred. The antimicrobial agents were used at a ratio of: 0.006: 0.006:0.012: 0.012:0.2:1 of cinnamon EO: oregano EO: lemongrass EO: citronellla EO: cranberry juice Biosecur F440D (Yosra Ben-Fadhel et al., 2019; Y. Ben-Fadhel, Maherani, Manus, Salmieri, & Lacroix, 2019). The obtained coarse emulsion was then treated with sonication 10 minutes at 70 % of full power with 5 minutes “on” and 5 minutes “off” in an ice bath.

2.3. Coating preparation

Calcium caseinate (Ca-Cas) and glycerol were used as main components of protein-based film formulations. Ca-Cas (5% w/v) was solubilized in distilled water (T°= 5 °C, pH= 7, conductivity = 118 µΩ) under constant stirring and glycerol (2.5% w/v) was added into the solution. For cross-linking irradiation treatment, the mixture was degassed under vacuum to remove air bubbles and flushed with nitrous oxide for 20 min to obtain inert atmosphere and reduce the interaction with oxygen during irradiation treatment. In absence of oxygen, protein crosslinking via bi-tyrosine production is obtained (Brault, D'Aprano, & Lacroix, 1997). The solution was irradiated at room temperature with a dose rate of 9.622 kGy/h in a ^{60}Co irradiator to obtain a dose of 32 kGy, reported dose as an effective dose for cross-linking of proteins (Brault, D'Aprano, & Lacroix, 1997; Cieśla, Salmieri, & Lacroix, 2006; Vu, Hollingsworth, Salmieri, Takala, & Lacroix, 2012). A control without irradiation was studied.

To one part of each solution (non-irradiated Ca-Cas and irradiated Ca-Cas solutions) one part of the nanoemulsion was added at a ratio 1:1 (Ca-Cas: nanoemulsion). The nanoemulsion addition was drop by drop and vigorous stirring to avoid the caseinate precipitation. A Ca-Cas without nanoemulsion was studied.

2.4. Film characterization

2.4.1. Film preparation

All the resulting solutions were used to make films by casting in plastic Petri dishes (10 g/Petri dish) and left to dry for 24 h in air at room temperature to allow film formation. The non-irradiated Ca-Cas and irradiated proteins films were then separately peeled and conditioned for at least 48 h equilibration in desiccators containing a saturated NaBr solution ensuring a 60.0% relative humidity (RH) at 20 °C before further examination. Four formulations were evaluated for film characterization: (1) non irradiated Ca-Cas, (2) non-irradiated Ca-Cas + nanoemulsion, (3) irradiated Ca-Cas and (4) irradiated Ca-Cas + nanoemulsion

2.4.2. Film thickness

Thickness of the films was measured using a Mitutoyo digimatic Indicator (Type ID-110E; Mitutoyo Manufacturing Co. Ltd, Tokyo, Japan) with a resolution of 1 µm, at five random positions around the film, by slowly reducing the micrometer gap until the first indication of contact.

2.4.3. Water vapor permeability

Water vapor permeability (WVP) tests were conducted gravimetrically using an ASTM 15.09:E96 method (1983). Films were mechanically sealed onto Vapometer cells (No. 68-1, Twhing-Albert Instrument Co., West Berlin, NJ, USA) containing 30 g of anhydrous calcium chloride (0% RH). The cells were placed in a Shellab 9010L controlled humidity chamber

(Sheldon Manufacturing Inc., Cornelius, OR, USA) maintained at 25 °C and 60% RH for 24 h. The amount of water vapor transferred through the film and absorbed by the desiccant was determined from the weight gain of the cell. The assemblies were weighed initially and after 24 h for all samples and up to a maximum of 10% gain. Changes in weight of the cells were recorded to the nearest 10^{-4} g. WVP was calculated according to the combined Fick and Henry laws for gas diffusion through coatings and films, according to the following **equation 1**:

$$WVP\ (g\ mm/m^2 day\ mmHg) = x\Delta w/A\Delta P \quad (\textbf{Eq. 1})$$

where x is the film thickness (mm), Δw is the weight gain of the cell (g) after 24 h, A is the area of exposed film ($31.67 \times 10^{-4}\ m^2$), and ΔP is the differential vapor pressure of water through the film (ΔP = 32.94 mmHg at 30 °C, 60% HR).

2.4.4. Tensile strength and elongation at break

The films were equilibrated in a desiccator containing a saturated sodium bromide solution ensuring 56% RH at room temperature (21 °C) for at least 24 h. Tensile strength (TS), tensile modulus (TM) and elongation at break (Eb%) of the films were measured with a Universal Tensile Machine (Model H5KT, Tinius-Olsen Inc., Horsham, PA, USA,) equipped with a 100 N-load cell (type FBB) and 1.5 kN-specimen grips. The dimensions of the rectangular shape test specimen were 60 mm × 15 mm × 0.03 mm (**L**, **l**, **e**) with **L** the length, **l** the width and **e** the thickness as recommended by the standard ISO 14125. Measurements were carried out following an ASTM D638-699 method (1999). Film width was measured using a Traceable®Carbon Fiber Digital Caliper (resolution: 0.1 mm/0.01″; accuracy: ±0.2 mm/0.01″; Fisher Scientific, ON, Canada). UTM parameters were set up for “plastics tensile from position” test type with the following selections: 25 mm effective gauge length, flat specimen shape, 1 number of entries, minimum type. The position rate of machine control was fixed to 1 mm/s. **Y**- and **X**-axes were assigned to load (100 N-range) and position (500 mm-range) coordinates respectively. TS (maximum stress, MPa) and max elongation (%) values were collected after film break due to elongation, using Test Navigator® program.

2.4.5. Color of film

Color determination was carried out on 3 films, onto 3preselected locations on the surface of each sample using a Colormet. L* (lightness, black = 0, white = 100), a* (redness > 0, greenness 0, blue < 0), b* (blue-yellow from −300 to +299) and the total change of color (ΔE*) were quantified on each sample. The total change of color ΔE* was quantified on each sample following Equation (2).

$$\Delta E = \sqrt{[(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]} \quad (\textbf{Eq. 2})$$

2.5. Evaluation of the effect of treatment on carrots quality

2.5.1. Carrot preparation and treatment

Fresh carrots were bought at a local supermarket (IGA, Laval, Qc, Canada) and were inspected for visual damage and mold growth. The carrots were washed and cut into discs (0.7 mm thickness and 25 mm diameter), then the samples were coated with coating solutions by dipping during 30 seconds and then the coated samples were dried under a laminary hood for 30 min. All samples were distributed into Whirl-Pak™ Sterile Filter Bags (23 x 15 cm). Samples were kept in the cold room at 4 °C and 90 % RH during 24 h to allow the antimicrobial coating reacting with the initial flora. Then, samples were irradiated at a dose of 0.5 kGy in the filter bag and kept at 4 °C until analysis day. For untreated samples, carrot was put directly into filter bags without any treatments. Height different groups were prepared (1) Control: untreated carrots; (2) 0.5 kGy: irradiated carrots at 0.5 kGy, (3) I-Ca-Cas: coated carrots with irradiated calcium

caseinate, (4) I-Ca-Cas+ 0.5 kGy: coated carrots with irradiated calcium caseinate and irradiated at 0.5 kGy, (5) NE: coated carrots with nanoemulsion, (6) NE+0.5 kGy: coated carrots with nanoemulsion and irradiated at 0.5 kGy (7) I-Ca-Cas+NE: coated carrots with irradiated calcium caseinate incorporating nanoemulsion (8) I-Ca-Cas+ NE+ 0.5 kGy: coated carrots with irradiated calcium caseinate incorporating nanoemulsion and irradiated at 0.5 kGy.

2.5.2. Weight loss

Carrot slices were weighed at day 0, 5, 9, 13 and 15 of storage and weight loss (WL) was calculated from initial weight (Wi) and weights of each storage day (Wt); as detailed in **equation (3**):

$$\mathrm{WL}\ (\%)\ = \frac{(\mathrm{Wi} - \mathrm{Wt}) * 100}{\mathrm{Wi}} \qquad \textbf{(Eq. 3)}$$

Where:

W_i: initial weight, W_t: weight in day t, W_L: weight loss

Six treatments were evaluated: (1) untreated carrots; (2) irradiated carrots at 0.5 kGy, (3) coated carrots with irradiated calcium caseinate, (4) coated carrots with irradiated calcium caseinate and irradiated at 0.5 kGy, (5) coated carrots with irradiated calcium caseinate incorporating nanoemulsion (6) coated carrots with irradiated calcium caseinate incorporating nanoemulsion and irradiated at 0.5 kGy. All weight losses were expressed as WL (%).

2.5.3. Texture

The texture analysis of carrots was performed using texturometer Stevens-LFRA (model TA-1000, Texture Technologies Corp., Scarsdale, NY, USA) on day 0, 7, 14 and 28 of storage. The thickness of carrots slice (7 mm thick with a flat surface) was used for penetration force measurement. A needle probe (d = 1 mm) was used with speed and penetration depth of 2 mm s^{-1} and 4 mm, respectively. The instrument was calibrated with 500 g before starting the measurements. Six treatments were evaluated: (1) untreated carrots; (2) irradiated carrots at 0.5 kGy, (3) coated carrots with irradiated calcium caseinate, (4) coated carrots with irradiated calcium caseinate and irradiated at 0.5 kGy, (5) coated carrots with irradiated calcium caseinate incorporating nanoemulsion (6) coated carrots with irradiated calcium caseinate incorporating nanoemulsion and irradiated at 0.5 kGy. The maximum penetration force in carrots slice was recorded.

2.5.4. Color

Color determination was carried out on 3 samples, onto 2 preselected locations on the surface of each sample using a Minolta Colorimeter Color reader CR10 (Konica Minolta Sensing, Inc, Mahwah, NJ, USA). Total change of color ΔE* was quantified on each sample the **equation 2**.

Analysis was done on day 9, 17 and 23 of storage on (1) untreated carrots; (2) irradiated carrots at 0.5 kGy, (3) coated carrots with nanoemulsion, (4) coated carrots with nanoemulsion and irradiated at 0.5 kGy (5) coated carrots with irradiated calcium caseinate incorporating nanoemulsion (6) coated carrots with irradiated calcium caseinate incorporating nanoemulsion and irradiated at 0.5 kGy treatments.

2.5.5. Microbial analysis

Initial carrots total mesophilic flora (TMF) and total molds and yeasts concentration was analyzed during one week of storage at 4 °C. Treatmenrs of (1) untreated carrots; (2) irradiated carrots at 0.5 kGy, (3) coated carrots with nanoemulsion, (4) coated carrots with nanoemulsion and irradiated at 0.5 kGy (5) coated carrots with irradiated calcium caseinate incorporating nanoemulsion (6) coated carrots with irradiated calcium caseinate incorporating nanoemulsion and irradiated at 0.5 kGy were evaluated. On each day of analysis (day 0, 3 and 7), the carrots

were homogenized for 1 min in 40 g of 0.1% (w/v) peptone water (Alpha Biosciences Inc., Baltimore, MD, USA) at high speed in a Lab-blender 400 stomacher (Laboratory Equipment, London, UK), then seeded into Tryptic Soy Agar and PDA with chlomphenicol for respectively TMF and molds and yeasts and incubated for 48 h at 37 °C and 2 8°C respectively. Results were expressed as bacterial count (log CFU g^{-1}) during storage at 4 °C.

2.6. Statistical analysis

For film characterization, films were prepared in 3 replicates. For each replicate, 10 samples were analyzed (n=10). For the microbial analysis, 2 replicates were done and for each replicate, 20 g of samples were analysed. Serial dilutions were done and for each dilution, 3 petri dishes were prepared. Analysis of variances (ANOVA), Duncan's multiple range tests for equal variances and Tamhane's test for unequal variances were performed for statistical analysis using SPSS 18.0 software (SPSS Inc, USA). Differences between means were considered significant using a threshold of 5 %.

3. Results and discussion

3.1. Film characterization

3.1.1. Water vapor permeability

The effect of nanoemulsion loading and the irradiation of calcium caseinate based edible coating at 32 kGy were evaluated for their effect on the WVP of calcium caseinate based film. Depending on the formulation, the average film thickness was in the range of (45-65 µm). Results of the WVP of calcium caseinate films (Ca-Cas) are presented in **Table 2**. At 60% RH, for both irradiated and non-irradiated calcium caseinate, the loading of nanoemulsion to calcium caseinate increased significantly ($P \leq 0.05$) the WVP from 2.7 to 3.41 g mm/m^2 d mmHg for non-irradiated calcium caseinate (Ca-Cas + NE) and from 2.45 to 3 g mm/m^2 d mm Hg for irradiated calcium caseinate (I-Ca-Cas+NE) at 32 kGy. Water vapor transfer generally occurs through the hydrophilic portion of a film. Thus, WVP is dependent on the hydrophilic-hydrophobic ratio of film components (Embuscado & Huber, 2009). These results were not as expected. According to Fabra, Talens, and Chiralt (2008) and McHUGH and Krochta (1994), the addition of oily phase decrease the WVP of milk protein based films. The increase of permeability of calcium caseinate observed with the incorporation of nanoemulsion is probably due to the high hydrophilic part added including Biosecur F440D and cranberry juice (6000 ppm) as compared to 180 ppm of essential oil mix. Several ingredients induce lower WVP including hydrophobic phases and plasticizers and this due to the presence of pores and cracks in unplasticized coatings (Embuscado & Huber, 2009).

On the other hand, the irradiation treatment of calcium caseinate based edible coating produced significant reduction of WVP of Ca-Cas film showing a decrease from 2.7 to 2.45 g mm/m^2 d mmHg for calcium caseinate without nanoemulsion and from 3.41 to 3 g mm/m^2d mmHg for calcium caseinate incorporating nanoemulsion. These results suggest that an irradiation treatment of calcium caseinate at a dose of 32 kGy induces a reduction on the WVP of the obtained film. This phenomenon was previously observed Ouattara, Canh, Vachon, Mateescu, and Lacroix (2002) and was attributed to the crosslinking induction of the protein inside the films. Similar results were also obtained by using ultraviolet irradiation (Rhim, Gennadios, Fu, Weller, & Hanna, 1999).

3.1.2. Textural study

Textural properties of films are detailed in **Table 2**. Results showed that the entrapment of nanoemulsion in Ca-Cas matrix reduced the tensile strength which varies from 6.4 MPA for the control (Ca-Cas) to 3.5 MPA for Ca-Cas+NE. Similarly, Kristo, Koutsoumanis, and Biliaderis

(2008) reported an alteration of the extensibility of Sodium Caseinate films incorporating nisin and this by decreasing the Young modulus and tensile strength. This could be related to the increase of the water content of the film. On the other hand, the irradiation treatment at a dose of 32 kGy seems to increase the tensile strength to reach 8.2 MPA for irradiated calcium caseinate (I-Ca-Cas) and to 6.3 MPA for the irradiated Ca-Cas +NE (I-Ca-Cas+NE) as compared to 6.4 and 3.4 MPA respectively. Similar results were observed by Brault et al. (1997). The irradiation of calcium caseinate resulted to an increase of bityrosine suggesting more crosslinks and exhibit a greater mechanical strength (Brault et al., 1997).

Table 2. Effect of calcium caseinate irradiation and the addition of nanoemulsion on water vapor permeability (WVP), tensile strength and max elongation of calcium caseinate films

	WVP (g mm/m d mmHg)	Tensile strength (MPA)	Max elongation (%)
Ca-Cas	2.70 ±0.089^{b}	6.438±0.949^{b}	65.71±10.32^{a}
Ca-Cas + NE	3.41±0.109^{d}	3.473±0.590^{a}	88.074±14.936^{ab}
I-Ca-Cas	2.45±0.098^{a}	8.147±1.157^{c}	131.060±20.950^{c}
I-Ca-Cas+NE	3.00±0.136^{c}	6.252±0.843^{b}	108.775±14.898^{bc}

Within each column, means with the same lowercase letter are not significantly different ($P > 0.05$).

Ca-Cas: non treated calcium caseinate film

Ca-Cas + NE: calcium caseinate film incorporated nanoemulsion

I-Ca-Cas: irradiated calcium caseinate film

I-Ca-Cas + NE: irradiated calcium caseinate film incorporated nanoemulsion

3.1.3. Color

Table 3 shows the variation in the CIELAB parameters, a*, b*, L* and ΔE for green-red (-300-+299), blue-yellow (-300**-** +299), black-white (0-100) and the total color change, between non-irradiated Ca-Cas (Ca-Cas), irradiated Ca-Cas (I-Ca-Cas**)**, Ca-Cas + NE and irradiated Ca-Cas+ NE (I-Ca-Cas+ NE) films. The effects of irradiation and NE addition on film color are presented in **Table 3**. Results showed that nevertheless the treatment applied, L* values for all samples were > 83 (light level). The incorporation of nanoemulsion and the irradiation treatment of the calcium caseinate at 32 kGy decreased the L* value of films from 90.3 for the control (Ca-Cas) to 86.1 and 86.3 respectively suggesting the formation of a darker film. The a* value decreased also from -1.2 for the control (Ca-Cas) to -2.9 and -4.5 with the incorporation of NE and the irradiation at 32 kGy respectively suggesting the formation of a slight green colored film. A significant increase of b* value was also observed on films with a maximum recorded for irradiated calcium caseinate + NE (39.2) as compared to the control (1.6) suggesting the formation of a yellow colored film. All these results suggest that the treatment of film with both addition of nanoemulsion or irradiation treatment induce a more yellow-green films. Similar results were also obtained by using UV-irradiation of sodium caseinate (Rhim et al., 1999). On the other hand, Kyung Kim, Jo, Jin Park, and Woo Byun (2008) demonstrated that the yellow color of film could be obtained mainly at high irradiation doses ≥ 12 kGy.

Table 3. Color attribute of films

	L*	a*	b*	ΔE
Ca-Cas	90.3 ± 1.0^{c}	-1.2 ± 0.2 ^{c}	1.6 ± 0.6 ^{a}	-
Ca-Cas+ NE	86.1 ± 3.1^{b}	-2.9 ±0.4 ^{b}	12.3 ± 1.6 ^{b}	11.9 ± 1.1
I-Ca-Cas	86.3 ± 1.5^{b}	-4.5 ± 0.3^{a}	27.4 ± 3.6 ^{c}	26.2 ±2.4
I-Ca-Cas+ NE	83.7 ± 1.9^{a}	-3.0 ± 1.1 ^{b}	39.2 ± 6.9 ^{d}	38.5 ±3.9

Within each column, means with the same lowercase letter are not significantly different ($P > 0.05$).

Ca-Cas: Calcium caseintae film
Ca-Cas+ NE: Calcium caseintae film incorporating nanoemulsion

I-Ca-Cas : Irradiated calcium caseintae film

I-Ca-Cas+ NE : Irradiated calcium caseintae film incorporating nanoemulsion

3.2. Effect of coating on carrots quality

3.2.1. Weight loss

Whatever the treatment applied, a significant increase of weight loss (**Fig.1**) was observed during the whole storage due especially to respiration and transpiration process without having a visual effect. In this study, the rate of weight loss wasn't influenced by the applied treatments and no significant differences were observed on the weight loss of all treatments ($P > 0.05$). No significant difference of the weight loss was also observed with the irradiation treatment ($P > 0.05$). It has been noted that the addition of nanoemulsion to irradiated calcium caseinate increased significantly the weight loss of carrots from 1.08 to 1.3% on day 15 for respectively irradiated calcium caseinate (I-Ca-Cas) and irradiated calcium caseinate + NE (I-Ca-Cas+ NE).

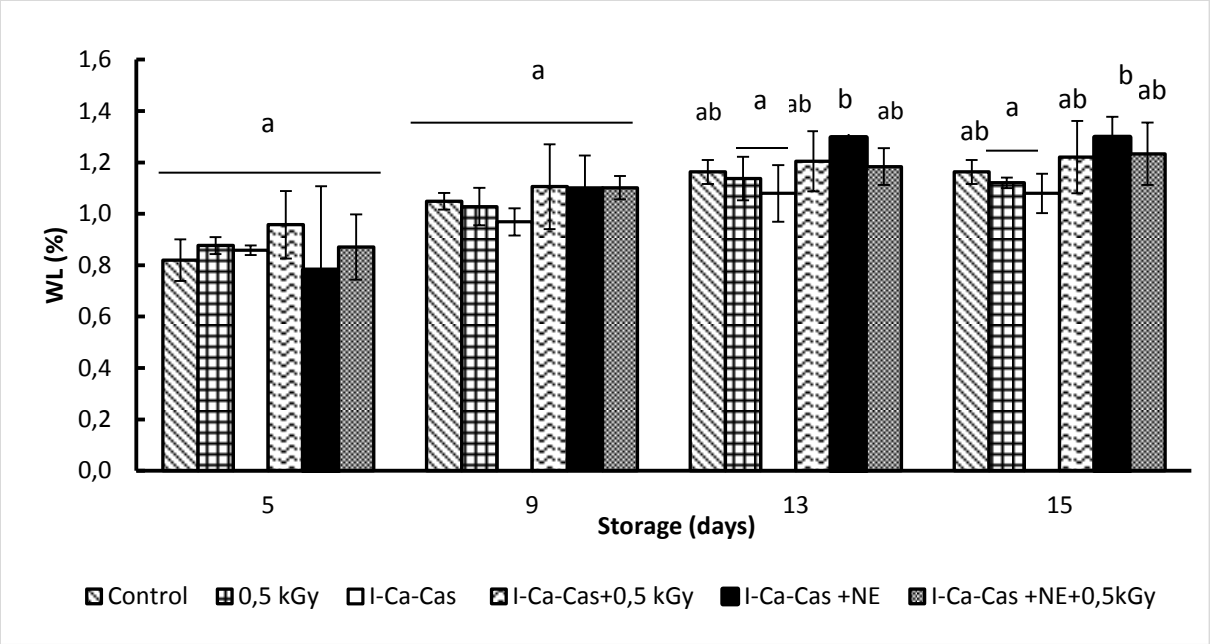


Figure 1.

0.5 kGy: coated carrots with irradiated calcium caseinate then treated with irradiation

I-Ca-Cas: coated carrots with irradiated calcium caseinate

I-Ca-Cas + 0.5 kGy: coated carrots with irradiated calcium caseinate then treated with irradiation

I-Ca-Cas+ NE: coated carrots with irradiated calcium caseinate incorporated nanoemulsion

I-Ca-Cas+ NE +0.5 kGy: coated carrots with irradiated calcium caseinate incorporated nanoemulsion then treated with irradiation

The group of irradiated carrot treated at 0.5 kGy showed that this treatment did not affect the weight loss of treated carrots and no significant difference was observed as compared to the control ($P > 0.05$). On the basis of these results, it can be noted that, combined treatments did not reduce the weight loss of carrots during storage at 4 °C.

3.2.2. Texture

Texture is a key quality factor of fresh and preprocessed carrots. **Table 4** shows the evolution of the maximum shear force as a function of storage time. Results showed whatever the applied treatment the maximum shear force increased significantly during storage at 4 °C. This could be due to the increase of the elasticity (Corrêa, Farinha, Oliveira, Campos, & Finger, 2010). As explained by Corrêa et al. (2010), the wrinkled tissues gives a more elastic behavior to the carrots, resulting in a more difficult skin to be penetrated. The increase of the elasticity is directly related to weight loss since tissue dehydration and tissue became more fibrous during storage and then more elastic which signify lower quality of carrots. On day 0, no significant effect was observed between treatments. However during storage, different behaviors were observed. Irradiation treatment of carrots at 0.5 kGy seems to not affect significantly the texture of carrots showing on day 14, a value of 1.90 N as compared to 2.04 for the non-irradiated carrots (control) and a value of 1.94 N for both irradiated and non-irradiated coated carrots with irradiated calcium caseintae + Nanoemulsion (I-Ca-Cas +NE and I-Ca-Cas +NE+ 0.5kGy) and a value of 1.87 N and 1.80 N for respectively treated or not carrots with irradiation and coated with

irradiated calcium caseinate (I-Ca-Cas and I-Ca-Cas + 0.5 kGy) was observed ($P > 0.05$). Hagenmaier and Baker (1998) reported also no effect of irradiation at doses of 0.17 and 0.45 kGy on carrots texture. Irradiation at higher doses lead to softening of tissue and this by decreasing textural properties such as hardness, cohesiveness, springiness, gumminess and chewiness of carrots (Nayak, Suguna, Narasimhamurthy, & Rastogi, 2007). The authors explained this decrease by cell damage, loss of turgor pressure and pectin degradation of carrots.

Table 4. Effect of treatments on the firmness of carrots slices stored at 4 °C.

	Max load (N)			
Treatment/ days	**0**	**7**	**14**	**28**
Control	1.72 ± 0.17^{aA}	2.00 ± 0.20^{cB}	2.04 ± 0.19^{bB}	2.00 ± 0.19^{abB}
0.5 kGy	1.62 ± 0.16^{aA}	1.88 ± 0.19^{bcB}	1.90 ± 0.19^{abB}	2.01 ± 0.20^{bB}
I-Ca-Cas +NE	1.70 ± 0.16^{aA}	1.99 ± 0.18^{cB}	1.94 ± 0.18^{abB}	1.93 ± 0.19^{abB}
I-Ca-Cas +NE+ 0.5kGy	1.70 ± 0.17^{aA}	1.82 ± 0.18^{abAB}	1.94 ± 0.18^{abB}	1.85 ± 0.17^{aB}
I-Ca-Cas	1.72 ± 0.16^{aA}	1.77 ± 0.17^{abAB}	1.87 ± 0.17^{aB}	1.84 ± 0.18^{aAB}
I-Ca-Cas + 0.5 kGy	1.63 ± 0.16^{aA}	1.71 ± 0.16^{aAB}	1.80 ± 0.17^{aBC}	1.89 ± 0.18^{abC}

Within each row, means with the same uppercase letter are not significantly different ($P > 0.05$). Within each column, means with the same lowercase letter are not significantly different ($P > 0.05$).

Control: untreated carrots

0.5 kGy: irradiated carrots at 0.5 kGy

I-Ca-Cas +NE: coated carrots with calcium caseinate incorporating nanoemulsion

I-Ca-Cas +NE+ 0.5kGy : coated carrots with calcium caseinate incorporating nanoemulsion then irradiated at 0.5 kGy

I-Ca-Cas : coated carrots with calcium caseinate

I-Ca-Cas + 0.5 kGy: coated carrots with calcium caseinate then irradiated at 0.5 kGy

It is interesting to note, that irradiated calcium caseinate (I-Ca-Cas) when it is applied on carrots surface was more efficient to reduce the maximum shear force as compared to irradiated calcium caseintae loaded nanoemulsion (I-Ca-Cas + NE) showing on day 7 and 14 respectively a values of 1.71 N and 1.87 N as compared to 2.00 N and 2.04 N for the control. These results suggest that irradiated calcium caseinate is efficient to maintain the texture of carrots. These results confirmed also the obtained results of film characterization, where the addition of nanoemulsion reduced significantly the barrier properties of films.

3.2.3. Color

The effect of applied treatments on the total color change (ΔE) is presented in **Table 5**. Results showed that applying nanoemulsion coating allowed the increase of the total color change after 23 days to reach 9.1 and 6.3 for coated carrots with nanoemulsion (NE) and coated carrots with nanoemulsion then irradiated at 0.5 kGy (NE+ 0.5 kGy) respectively as compared to the control

where 5.5 and 7.1 where observed for untreated carrots (Control) and irradiated carrots at 0.5 kGy (0.5 kGy) respectively.

Table 5. Effect of treatments on the total color change (ΔE) of carrots

	Day 9	Day 17	Day 23
Control	4.8	3.7	5.5
0.5 kGy	6.6	7.6	7.1
NE	6.3	8.0	9.1
NE + 0.5 kGy	6.9	5.9	6.3
I-Ca-Cas +NE	4.2	4.2	4.7
I-Ca-Cas +NE+ 0.5 kGy	3.6	4.1	4.7

Control : Untreated carrots

0.5 kGy: irradiated carrots at 0.5 kGy

NE : Coated carrots with nanoemulsion

NE+ 0.5 kGy: Coated carrots with nanoemulsion then irradiated at 0.5 kGy

I-Ca-Cas+ NE: Coated carrots with calcium caseinate incorporating nanoemulsion

I-Ca-Cas +NE + 0.5 kGy: Coated carrots with calcium caseinate incorporating nanoemulsion then irradiated at 0.5 kGy

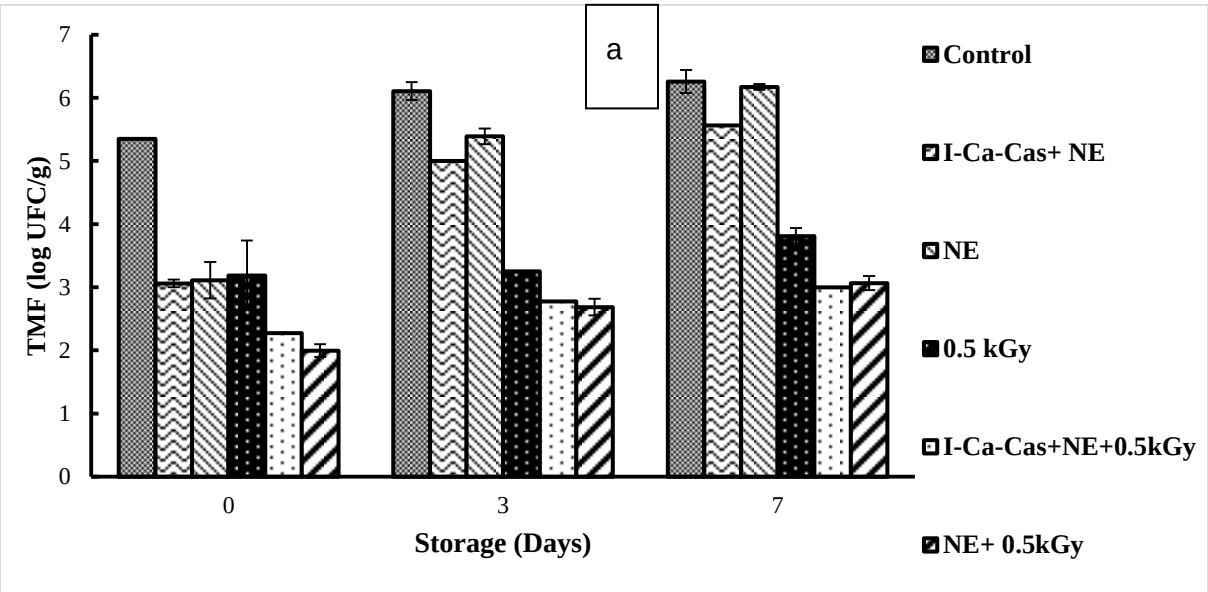
It is interesting to note that when nanoemulsion was incorporated into irradiated calcium caseinate film, the total color change of treated carrots was reduced to reach 4.7 for both irradiated (I-Ca-Cas+ NE+ 0.5 kGy) or non-irradiated carrots (I-Ca-Cas+ NE) as compared to 9.1 for coated carrots with nanoemulsion (NE) and to 6.3 for coated carrots with nanoemulsion then irradiated at 0.5 kGy (NE+ 0.5 kGy) on day 23. These results suggest that irradiated calcium caseinate film incorporating nanoemulsion allows the maintain of pre-cut carrots color during storage at 4 °C. This was related to the barrier effect of irradiated calcium caseinate which inhibits the surface browning occurred due to the oxidation of carrots phenol (Amanatidou, Slump, Gorris, & Smid, 2000). For untreated carrots, an irradiation treatment of 0.5 kGy enhanced the color change of carrots. However, when nanoemulsion was applied on carrots surface the impact of irradiation was significantly reduced. These results suggest that nanoemulsion application on carrot surface could protect the carrots from discoloration caused by irradiation treatment. Similar results were also observed with calcium caseinate film incorporating nanoemulsion where the total color change was reduced on day 23 from 7.1 for the irradiated carrots at 0.5 kGy (0.5 kGy) to 4.7 for treated carrots with I-Ca-Cas+NE+0.5 kGy.

On the basis of these results, nanoemulsion application and its entrapment on calcium caseinate film allowed the maintain of the color of carrots during 3 weeks of cold storage.

3.2.4. Microbial analysis

The effect of the application of the different treatments on TMF and molds and yeasts growth on carrots surface is detailed in respectively **Fig. 2a-b**. Results showed that on day 0, irradiation treatment at 0.5 kGy, nanoemulsion (NE) and I-Ca-Cas+ NE when applied on carrots surface reduced significantly the growth of TMF to reach 3.2, 3.1 and 3.1 log CFU/g as compared to 5.4 log CFU/g for the untreated carrots (control). This suggests a good effectiveness of the applied treatments. Nanoemulsion which is mainly composed by citrus extract and cranberry juice was

effective to control TMF similarly when its free or loaded in I-Ca-Cas coating. Indeed, the study of Boumail, Salmieri, St-Yves, Lauzon, and Lacroix (2016) showed equivalent results with the citrus extract and lemongrass essential oil on *Listeria monocytogenes* growth, this effect was explained by the presence of terpenes in essential oils. Essential oil of lemongrass, cinnamon, oregano and citronella have also previously shown an antibacterial activity against several microorganisms i.e. *Salmonella*, *Listeria* and *E. coli* and antifungal activity against *A. flavus*, *P. chrysogenum* and *C. albicans* (Yosra Ben-Fadhel et al., 2019). Their efficiency was intimately related to their main composition including cinnamaldehyde, citronellol, geraniol, geranial, neral and carvacrol. On the other hand, the study of Harich, Maherani, Salmieri, and Lacroix (2017) showed that the cranberry juice have an antimicrobial effect on *Listeria monocytogenes* and *Escherichia coli*. This behavior was related to its composition which is highly concentrated on organic acids such as malic, citric and quinic acid. Irradiation treatment at low dose of 0.5 kGy was indeed effective to control TMF showing a 2.2 log reduction on day 0. Bandekar et al. (2006) showed that D_{10} values of *Salmonella* Typhimurium and *Listeria monocytogenes* in minimally processed foods were in the range of 0.188 to 0.362 kGy, except in sprouts where, *L. monocytogenes* showed much higher D_{10} values (0.531-0.579 kGy). The same authors demonstrated that a dose of 2 kGy would be necessary for 5 log reduction of these pathogens. These results suggest that irradiation processing can ensure safety of minimally processed vegetables. When only nanoemulsion (NE) or irradiation (0.5 kGy) were applied, 2.3 and 2.2 log reduction was observed on TMF growth. Combining nanoemulsion and irradiation treatment (NE+ 0.5 kGy) allowed 3.4 log reduction. These results suggest a possible synergy between irradiation treatment and nanoemulsion application which allowed enhancing the antimicrobial effectiveness.



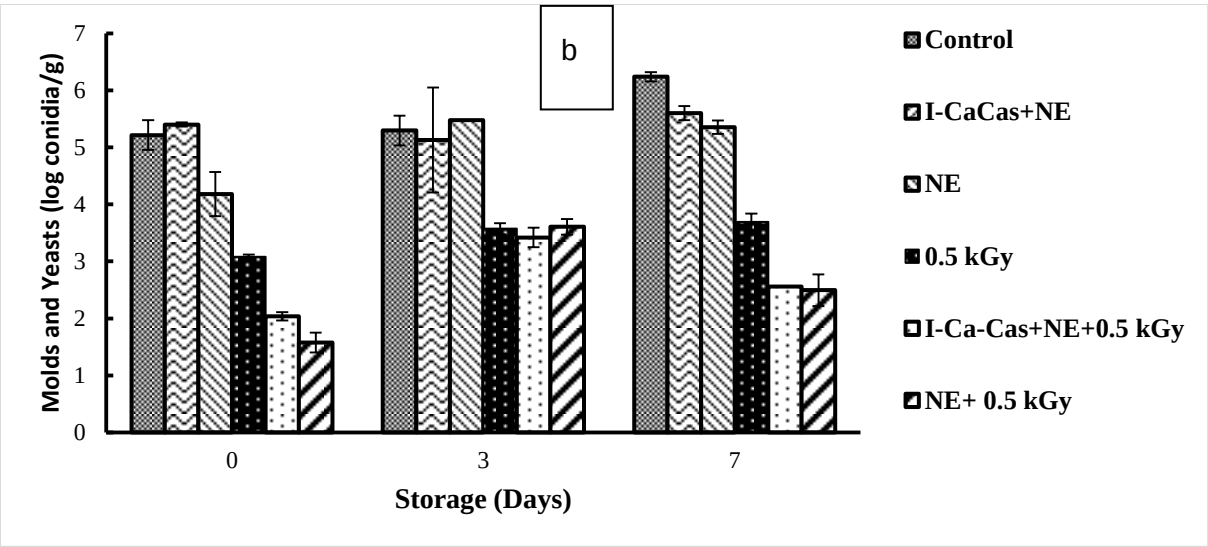


Figure 2.

Control : Untreated carrots

I-Ca-Cas+ NE: Treated carrots with irradiated calcium caseinate incorporating nanoemulsion

NE : treated carrots with nanoemulsion

0.5 kGy: irradiated carrots at 0.5 kGy

I-Ca-Cas+ NE+ 0.5 kGy : Treated carrots with irradiated calcium caseinate incorporating nanoemulsion then irradiated at 0.5 kGy

NE+ 0.5 kGy: treated carrots with nanoemulsion then irradiated at 0.5 kGy

During storage, TMF count increased and reached 6.3, 5.6 and 6.2 log CFU/g on day 7 for the control, treated carrots with I-Ca-Cas+NE and treated carrots with nanoemulsion (NE) respectively. These results suggest that the loading of nanoemulsion in irradiated calcium caseinate coating (I-Ca-Cas+ NE) allowed an improvement of the antimicrobial activity especially during storage with 0.6 log reductions as compared to treated carrots with only nanoemulsion (NE). Similar results with calcium caseinate beads were also observed in previous studies of Dehkharghanian, Lacroix, and Vijayalakshmi (2009), where green tea polyphenol extract was encapsulated and showed an improvement in the antioxidant activity. However, Pan, Chen, Davidson, and Zhong (2014), showed no efficacy on *Listeria monocytogenes*. On the other hand, no significant effect treatments ($P > 0.05$) was observed between treated carrots with NE+ 0.5 kGy and treated carrots with I-Ca-Cas+ NE+ 0.5 kGy showing 3.0 and 3.1 log CFU/g respectively, suggesting no effect of the loading of NE into calcium caseinate matrix. A slight improvement was observed when nanoemulsion was loaded into irradiated calcium caseinate coating when no irradiation was applied.

Based on molds and yeasts growth, irradiation treatment at low doses of 0.5 kGy was effective to reduce significantly the count of molds and yeasts on carrots surface by 2.1, 3.4 and 2.6 log reduction for the control, treated carrots with irradiated calcium caseinate loaded emulsion (I-Ca-Cas+ NE) and nanoemulsion (NE) respectively. Similar results were also observed by Mohácsi-Farkas, Nyirő-Fekete, Daood, Dalmadi, and Kiskó (2014) on sliced carrots stored at 5 °C. Nanoemulsion application (NE) allowed also the reduction of the growth of molds and yeasts by 1 log reduction and 0.8 log reductions on respectively day 0 and 7 and 1.5 and 1.2 log

reduction on respectively day 0 and 7 when irradiation treatment was applied (NE+ 0.5 kGy). These results suggest a synergy activity between nanoemulsion application and irradiation treatment in controlling molds and yeasts growth. However, nanoemulsion loading into calcium caseinate has no significant effect ($P > 0.05$) of the on the growth of molds and yeasts.

Microbial results showed that the combination of irradiation treatment at 0.5 kGy to active coating with nanoemulsion (NE+ 0.5 kGy) or irradiated Ca-Cas loaded nanoemulsion (I-Ca-Cas+ NE+ 0.5 kGy) was more efficient to control the total mesophilic flora of the carrots. Thus, a synergistic effect seems to be present between the evaluated coatings and the irradiation treatment. However no improvement was observed on molds and yeasts growth.

4. Conclusion

This investigation has clearly demonstrated that gamma-irradiation is responsible for the improvement of the mechanical properties of calcium caseinate films. The incorporation of nanoemulsion into calcium caseinate films reduced their mechanical properties. When the film was applied on carrots surface, an improvement of the shelf-life and of color was observed. Thus, irradiated calcium caseinate could be a good alternative for preparation of films in food packaging; as coating agent for foods and as matrix material of bioactive ingredients. Irradiation treatment and active coating could also act in synergy to maintain the quality of sliced carrots.

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