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Development of a cold pasteurization treatment (γ-irradiation) in combination with an antimicrobial formulation based on natural compounds to assure the innocuity of human milk

Par

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List of Abbreviations

ATP: Adenosine Triphosphate

CFU: Colony Forming Units

^{60}CO: Cobalt 60

Da: Dalton

EOs: Essential Oils

FDA: Food and Drug Administration

FIC: Fractional Inhibitory Concentration

g: Gram

h: Hour

HMBs: Human Milk Banks

HMW: High Molecular weight

HPLC: High Performance Liquid Chromatography

IVPD: *In vitro* Protein Digestibility

Ig: Immunoglobulin

kGy: Kilogray

Kg: Kilogram

LMW: Low Molecular weight

MMW: Medium Molecular weight

MIC: Minimum Inhibitory Concentration

MDA: Malondialdehyde

mL: Millilitre

min: Minutes

RPM: Rotations per minute

s: Seconds

TBARS: Thiobarbituric acid-reactive substances assay

µg: Microgram

v/v: Volume/Volume

WHO: World Health Organization

RÉSUMÉ

Les banques de lait humain (BML) sont une nécessité absolue car le lait maternel humain est une source de nutriments essentiels pour les nourrissons et est particulièrement recommandé pour les nouveau-nés prématurés lorsque le lait de leur propre mère n'est pas disponible. Il offre une protection contre les infections et diminue l'entérocolite nécrosante et les maladies cardiovasculaires. Néanmoins, le lait maternel peut être altéré par la contamination par des agents pathogènes, et le risque de pénurie de BML est très souvent présent. *E. sakazakii*, *E. coli* O157:H7, *S. aureus*, *L. monocytogenes*, *S.* Typhimurium et *B. cereus* sont les souches bactériennes les plus fréquentes responsables du rejet du lait maternel. Le lait maternel humain peut être contaminé à tous les stades, depuis son point de collecte jusqu'à son stockage et sa livraison. La pasteurisation thermique est la technique couramment utilisée pour la conservation du lait dans les BML mais elle n'est pas efficace pour éliminer les spores de *B. cereus* et affecte la qualité nutritionnelle par des réactions de Maillard. L'utilisation de la pasteurisation à froid telle que l'irradiation γ est une approche alternative efficace pour la conservation du lait humain.

Le but de cette étude est de développer une technique de pasteurisation à froid par irradiation γ dans des formulations antibactériennes combinées à base d'huiles essentielles (origan, formulation méditerranéenne, citronnelle, clou de girofle, cannelle), d'acides et de bases organiques (acide citrique, acide lactique et carbonate de sodium) et d'extrait d'agrumes.

L'activité antibactérienne de l'origan, de la formulation méditerranéenne, de la citronnelle, du clou de girofle, de la cannelle, de l'acide citrique, de l'acide lactique, du carbonate de sodium et de l'extrait d'agrumes a été évaluée pour sa capacité à inhiber la croissance de pathogènes alimentaires sélectionnés en termes de concentration minimale inhibitrice (CMI). D'après les résultats, la valeur de la CMI varie de 312,5 à 5000 ppm contre toutes les souches bactériennes testées, l'extrait d'agrumes présentant l'activité antibactérienne la plus élevée (312,5 ppm). L'interaction entre les combinaisons binaires, trinaires et quaternaires d'agents antibactériens sélectionnés a été testée. Notamment, la combinaison quaternaire de composés : Formulation 1 (Origan/formulation méditerranéenne/extrait d'agrumes/acide lactique), Formulation 2 (Cannelle/Lemongrass/extrait d'agrumes/acide citrique), Formulation 3 (Origan/formulation méditerranéenne/extrait d'agrumes/acide citrique), Formulation 4 (Cannelle/Lemongrass/extrait d'agrumes/acide lactique) a montré un effet synergique contre tous les pathogènes testés. Le traitement combiné de l'irradiation γ et de quatre formulations antibactériennes quaternaires a augmenté efficacement la

radiosensibilité de 1,34 à 3,99 dépendamment des agents pathogènes testés dans le lait maternel congelé. Les formulations 3 et 4 ont induit une plus grande radiosensibilité des souches bactériennes, y compris *B. cereus* sporulé (1,90 et 1,89 respectivement), par rapport aux formulations 1 et 2. Toutes les souches bactériennes du lait maternel congelé ont été éliminées à 5 kGy en présence des formulations 3 et 4. De même, le traitement combiné de l'irradiation γ à 5 kGy avec les formulations 3 et 4 n'a pas modifié de manière significative la teneur en lactose et en immunoglobulines et le potentiel lipidique du lait maternel en condition congelée. La digestibilité *in vitro* des protéines a augmenté dans le lait maternel congelé soumis au traitement combiné et l'hydrolyse des grosses protéines en peptides plus petits a été démontrée. Le traitement combiné a eu un changement minime sur la couleur et la viscosité du lait maternel humain. Par conséquent, le traitement combiné de l'irradiation γ à 5 kGy avec des formulations antibactériennes quaternaires a été efficace pour préserver la qualité du lait maternel congelé.

ABSTRACT

Human milk banks (HMBs) are an absolute necessity as human breast milk is a source of essential nutrients for infants and is particularly recommended for preterm neonates when their own mother's milk is not available. It provides protection against infections and decreases necrotizing enterocolitis and cardiovascular diseases. Nevertheless, mother's milk spoilage can occur due to contamination by pathogens, and the risk of a shortage of human milk is very often present. *E. sakazakii*, *E. coli* O157:H7, *S. aureus*, *L. monocytogenes*, *S.* Typhimurium and *B. cereus* are the most frequent bacterial strains responsible for mother's milk being discarded. Human breast milk in HMB can be contaminated at all stages, from its collection point to the storage and delivery. Thermal pasteurization is the commonly used technique for milk preservation in HMBs but it's not effective in eliminating the *B. cereus* spores and affects the nutritional quality by Maillard reactions. The use of cold pasteurization such as γ-irradiation is an effective alternative approach for the preservation of human milk. The aim of this study to develop a cold pasteurization technique of γ-irradiation in combination with antibacterial formulations based on essential oils (oregano, mediterranean formulation, lemongrass, clove, cinnamon), organic acids and bases (citric acid, lactic acid and sodium carbonate) and citrus extract.

The antibacterial activity of oregano, mediterranean formulation, lemongrass, clove, cinnamon, citric acid, lactic acid, sodium carbonate and citrus extract was evaluated for their ability to inhibit the growth of selected foodborne pathogens in terms of minimum inhibitory concentration (MIC). Based on the results, MIC value ranges from 312.5 to 5000 ppm against all tested bacterial strains, with citrus extract showing the highest antibacterial activity (312.5 ppm). The interactions between the binary, trinary and quaternary combinations of selected antibacterial agents have been tested. Notably, the quaternary combination of compounds: Formulation 1 (Oregano/Mediterranean formulation/Citrus extract/Lactic acid), Formulation 2 (Cinnamon/Lemongrass/Citrus extract/Citric acid), Formulation 3 (Oregano/Mediterranean formulation/Citrus extract/Citric acid), Formulation 4 (Cinnamon/Lemongrass/Citrus extract/Lactic acid) showed a synergistic effect against all tested pathogens. The combined treatment of γ-irradiation and four quaternary antibacterial formulation effectively increased the radiosensitivity (1.34 - 3.99) of the tested pathogens in frozen mother's milk. Formulation 3 and 4 induced greater radiosensitivity in bacterial strains including sporulated *B. cereus* (1.90 and 1.89 respectively) compared to formulation 1 and 2. All bacterial strains in frozen mother's milk were eliminated at 5 kGy in the presence of formulation 3 and 4. Also, the combined

treatment of γ-irradiation at 5 kGy along with formulation 3 and 4 did not significantly alter the lactose and immunoglobulin content, lipid potential of mother's milk under frozen condition. The *in vitro* protein digestibility was increased in frozen mother's milk when subjected to the combined treatment and hydrolysis of larger proteins to smaller peptides was demonstrated. Combined treatment had a minimal change in color and viscosity of human breast milk. Hence, combined treatment of γ-irradiation at 5 kGy along with quaternary antibacterial formulations was effective in preserving of the quality of frozen mother's milk.

Synopsis

1. Introduction générale

Le lait maternel agit comme le principal apport nutritif, aide à renforcer le système immunitaire et fournit également des facteurs bioactifs non nutritifs qui favorisent la survie et le développement sain des nourrissons **(Ballard et al., 2013)**. Il contient des glucides, des protéines, des lipides, des vitamines, des minéraux, des enzymes digestives et des hormones. En plus de ces nutriments, il est riche en cellules immunitaires, dont des macrophages, des cellules souches et de nombreuses autres molécules bioactives. Certaines de ces molécules bioactives sont dérivées de protéines et dérivées de lipides, tandis que d'autres sont dérivées de protéines et non digestibles, comme les oligosaccharides. Les oligosaccharides du lait maternel (HMO) possèdent des propriétés anti-infectieuses contre les agents pathogènes dans le tractus gastro-intestinal du nourrisson **(Gura, 2014)**. Les oligosaccharides jouent également un rôle vital dans le développement des réponses immunitaires innées et adaptatives essentielles **(Walker, 2013)**.

Les banques de lait de donneuses aident à préserver le lait maternel humain recueilli auprès de diverses donneuses en bonne santé et jouent un rôle majeur dans l'alimentation des prématurés, car le lait produit lors d'une naissance prématurée contient une quantité inappropriée de macro et micro nutriments. Et il y a des circonstances où le lait n'est pas disponible auprès de la propre mère du nourrisson et ce déficit nutritionnel devrait être comblé par du lait de donneuses **(Haiden et al., 2016)**. Le principal problème auquel sont confrontées les banques de lait humain est la détérioration du lait. La principale source de contamination est la bactérie. Lorsque les nouveau-nés sont nourris avec du lait contaminé, cela provoque de graves maladies de la santé comme l'entérocolite nécrosante (NEC) et la septicémie **(Arnold, 2006)**. De nombreuses recherches ont mis en évidence les souches bactériennes responsables de la contamination des préparations pour nourrissons et du lait maternel.

Les banques de lait et les industries alimentaires utilisent de nombreuses techniques de stérilisation afin d'éliminer les contaminations bactériennes. La pasteurisation thermique est très utilisée pour la conservation du lait mais elle ne peut pas éliminer les bactéries sporulées, en particulier Bacillus cereus **(Stadhouders, 1982)**, l'utilisation de composants chimiques et d'antibiotiques affecterait gravement la santé des nourrissons **(Irkin et al., 2015)** et elle est

fortement interdite dans les aliments pour nourrissons. Cela exige l'utilisation de produits naturels ayant des propriétés antibactériennes pour la conservation.

Les huiles essentielles sont des composés volatils et aromatiques qui sont extraits de différentes parties des plantes (feuilles, racines, écorces, fleurs, fruits, graines, etc). Elles sont obtenues par des procédures d'extraction et des distillations à la vapeur **(Burt, 2004; Hyldgaard et al., 2012)**. En raison de la présence de métabolites secondaires comme les tanins, les phénols, les alcaloïdes, les flavonoïdes, de nombreux extraits naturels et huiles essentielles ont une activité antibactérienne élevée **(Hyldgaard et al., 2012; Akash et al., 2018)**. Le Codex Alimentarius a autorisé l'utilisation de certains additifs alimentaires tels que l'acide lactique, l'acide citrique, le carbonate de sodium, le citrate de sodium, le carbonate de potassium et l'acide malique dans les préparations pour nourrissons **(Codex alimentarius, 1981)**. L'irradiation est une méthode non thermique de pasteurisation, qui peut être utilisée comme alternative au traitement thermique. Les rayonnements ionisants utilisés sont les rayons gamma, Rayons X et faisceaux d'électrons **(Morehouse et al., 2004)**. Conformément aux réglementations approuvées par la Food and Drug Administration (FDA), une dose d'irradiation allant jusqu'à 45 kGy est autorisée pour la stérilisation d'échantillons d'aliments surgelés **(Maherani et al., 2016)**. La teneur en macronutriments des produits alimentaires tels que les glucides, les protéines et les graisses n'est pas modifiée. Cependant, la matrice nutritionnelle est préservée en fonction des conditions environnementales, de la composition des aliments et du dosage d'irradiation (kGy). L'oxydation des lipides et quelques micronutriments ont tendance à être affectés par l'irradiation de manière dose-dépendante, comme indiqué dans des études de recherche antérieures **(Dionísio, Gomes et Oetterer, 2009; Robichaud et al., 2020)**.

Cette thèse porte sur l'évaluation de la radiosensibilité des agents pathogènes d'origine alimentaire ciblés en combinaison avec des formulations antibactériennes naturelles ainsi que sur l'effet de l'irradiation γ sur le lactose, la digestion des protéines, le profil peptidique, l'oxydation des lipides, la couleur et la texture du lait maternel.

2. Problématique, Objectif, Hypothèse & Méthodologie

2.1. Problématique

Le lait maternel est une source majeure de nutriments pour aider à renforcer le système immunitaire et fournit également des facteurs bioactifs non nutritifs qui favorisent la survie et le développement sain des nourrissons. Même s'il est naturellement disponible pour les

nourrissons, le rôle des lactariums est très important car les laits de donneuses sont la meilleure source alternative de nutriments pour les nourrissons en cas d'absence de lait maternel. Mais le lactarium rencontre des difficultés dans la conservation du lait humain, car le risque de contamination bactérienne est élevé. Le traitement de pasteurisation thermique, qui est actuellement suivi par les banques de lait pour la conservation, peut provoquer des réactions de Maillard qui entraînent une perte nutritionnelle car le lait contient une grande quantité de protéines et de sucres, qui à haute température font réagir le sucre réduit et les acides aminés libres pour produire des mélanoïdines qui affectent la qualité du lait, un autre problème rencontré est que les bactéries formant des spores, en particulier *B. cereus* et les bactéries comme *S. aureus* qui produisent des entérotoxines qui sont des protéines toxiques provoquant une infection intestinale, ne peuvent pas être éliminées car elles résistent à la chaleur. Les principales espèces *bactériennes responsables de la détérioration du lait maternel sont L. monocytogenes*, *E. coli*, *B. cereus*, *S. Typhimurium*, *S. aureus* et *E. sakazakii*. Les contaminations causées par ces souches bactériennes entraînent des maladies néonatales graves telles que l'entérocolite nécrosante (ENC) et la septicémie.

2.1. Objectif

Le but de cette étude est de démontrer l'effet deTraitement par irradiation γ (pasteurisation à froid) en combinaison avec des formulations antibactériennes naturelles pour l'inactivation microbienne, les propriétés nutritionnelles et bioactives, la digestibilité in vitro des protéines, le profil peptidique et les propriétés physicochimiques du lait maternel humain à l'état congelé.

2.2. Hypothèse

- Les huiles essentielles, l'extrait d'agrumes et les acides organiques montreront une activité antibactérienne élevée contre les souches bactériennes testées.
- La formulation quaternaire développée à partir d'huiles essentielles, d'extraits d'agrumes et d'acides organiques montrera un effet synergique.
- Le traitement par irradiation γ en combinaison avec une formulation antibactérienne peut agir en synergie pour augmenter la radiosensibilité bactérienne et permettre d'éliminer tous les agents pathogènes, y compris les bactéries sporulées, à une dose d'irradiation plus faible dans le lait maternel congelé.
- Le traitement combiné de l'irradiation γ et de la formulation antibactérienne ne modifiera pas les qualités nutritionnelles, la digestibilité des protéines in vitro, le profil peptidique et les qualités physicochimiques du lait maternel à l'état congelé.

2.3. Objectifs

Objectif 1: Pour déterminer la concentration minimale inhibitrice (CMI) d'huiles essentielles sélectionnées, d'extraits d'agrumes et d'acides organiques.

Objectif 2: Pour déterminer l'interaction entre différents composés antibactériens à l'aide de l'indice de concentration inhibitrice fractionnaire (FIC).

Objectif 3: Développer des formulations antibactériennes quaternaires basées sur l'indice FIC.

Objectif 4: Pour évaluer le D_{10} et la radiosensibilité du traitement combiné desIrradiation γ et formulation antibactérienne dans le lait maternel congelé.

Objectif 5: Évaluer l'efficacité des traitements combinés (formulation antimicrobienne naturelle avec irradiation γ à 5 kGy) sur l'élimination des agents pathogènes et évaluer l'effet du traitement combiné sur les protéines nutritionnelles, immunologiques, la digestibilité des protéines, le profil peptidique et les qualités physicochimiques des surgelés. lait maternel sous traitement combiné d'irradiation à 5 kGy et de formulation antibactérienne.

2.4. Méthodologie

Objectif 1: Les concentrations minimales inhibitrices (CMI) des composés antibactériens sélectionnés seront déterminées à l'aide de la méthode des microplaques à 96 puits décrite par **Turgis et al. (2012)** et **Hossain et al. (2016)**. La densité optique sera lue à une longueur d'onde de 595 nm à l'aide d'un spectrophotomètre pour évaluer la CMI.

Objectif 2: L'indice de concentration inhibitrice fractionnelle (FIC) des différentes combinaisons de composés antibactériens contre les agents pathogènes cibles sera déterminé par un test en damier à l'aide de microplaques à 96 puits selon la procédure expliquée par Gutierrez et al. (2008). Sur la base des valeurs FIC, l'interaction entre les composés est classée comme a) FIC < 0,5 a été indiqué comme effet synergique (S), b) 0,5 < FIC ≤ 1 a été interprété comme effet additif (AD), c) 1 < FIC ≤ 4 a été interprété comme aucun effet interactif (NI), d) FIC > 4 a été représenté comme effet antagoniste (A) **(Turgis et al., 2012; Ayari et al., 2020)**.

Objectif 3: Le développement des formulations antibactériennes quaternaires sera réalisé en sélectionnant les composés antimicrobiens les plus efficaces contre les bactéries étudiées, leur effet synergique et aussi les plus compatibles avec le lait maternel **(Ayari et al., 2020)**.

Objectif 4: Le D10 et la radiosensibilité de pathogènes sélectionnés dans le cadre du traitement combiné deγ-l'irradiation et la formulation antibactérienne seront faites selon le protocole de **Robichaud et al. (2021)**. L'irradiation du lait maternel se fera dans un irradiateur gamma utilisant une source Cobalt 60 (Nordion Inc., Kanata, ON, Canada) à une dose de 0,5, 0,75, 1, 1,5, 2, 2,5, 3, 4, 5 kGy et un débit de 6,318 kGy/h au Centre canadien de rayonnement.

Objectif 5: L'effet du traitement combiné sur le lactose, la composition immunologique, les peptides de distribution du poids moléculaire sera déterminé à l'aide du système HPLC Agilent série 1260selon le protocole expliqué par **Robichaud et al. (2020)**. Le potentiel d'oxydation des lipides du lait maternel sous traitement combinéLe dosage des substances réactives à l'acide thiobarbiturique (TBARS) sera utilisé sur la base du protocole décrit par **Criado et al. (2020)**. La digestibilité in vitro des protéines (IVPD) sous traitement combiné d'irradiation dans le lait maternel sera déterminée par digestion séquentielle à l'aide de pepsine et de trypsine selon la méthode mentionnée par **Wang et al. (2008)**.Le pourcentage de libération d'azote au cours du processus de digestion sera calculé à partir de la méthode Kjeldahl selon le protocole expliqué par **Manus et al. (2021)**. La mesure des paramètres de couleur du lait maternel soumis au traitement combiné se fera en mesurant les paramètres L* (clarté), a* (vert à rouge), b* (bleu à jaune) et ΔE (différence de couleur) avec un colorimètre CR10-Plus **(Ben-Fadhel et al., 2021)**. La viscosité du lait maternel soumis au traitement combiné sera mesurée à l'aide d'un viscosimètre DV-II+ (Okyere & Odamtten, 2014).

3. Flux de travail global du projet

3.1. Préparation du stock bactérien, des échantillons, des composés antimicrobiens naturels et des procédures d'inoculation

E. sakazakii (ATCC 29004), *E. coli* O157:H7 (ATCC 43895), *B. cereus* (ATCC 14579), *L. monocytogenes* (HPB 2812 sérovar 1/2a), *S. aureus* (ATCC 29213) et *S. Typhimurium* (ATCC SL1344) ont été cultivées à l'aide de TSB et pour un stockage prolongé ont été maintenues à -80 ˚C dans un mélange TSB-glycérol (10 % v/v). Un millilitre de culture a été incubé à travers deux incubations successives de 24 h à 37 ˚C dans du TSB pour obtenir environ 108 UFC/mL. Les cultures ont été centrifugées à 1300 tr/min pendant 15 min, lavées et stérilisées avec 0,85 % (p/v) de solution saline stérile pour obtenir des cultures de travail fraîches.

Pour les expériences, le lait maternel lyophilisé a été reconstitué sous forme liquide en ajoutant de l'eau stérile dans des conditions stérilisées jusqu'à ce qu'il atteigne sa forme

originale (environ 71,3 g d'eau ont été ajoutés à 5 g de lait lyophilisé). Ensuite, le lait reconstitué a été stocké à -20 ˚C pour réaliser les expériences à l'état congelé. Les composés antimicrobiens naturels : les HE (formulation méditerranéenne, origan, citronnelle, clou de girofle et cannelle) et l'extrait d'agrumes ont été stockés à 4 ˚C avant utilisation. L'émulsion d'huiles essentielles a été préparée en mélangeant chaque HE (2,5 % v/v) avec du Tween 80 (Laboratoire Mat, QC, Canada) dans un rapport de 1:1. Ces mélanges ont été homogénéisés pendant 5 min avec un homogénéisateur Ultra-Turrax (modèle TP18/1059, Allemagne) à 15000 rpm pour obtenir la suspension colloïdale finale **(Ayari et al., 2020)**. La solution mère (2. 5% v/v) d'acides et de bases organiques (acide citrique, acide lactique et carbonate de sodium) ont été préparés par dilution à partir de sa concentration initiale. Toutes les solutions antimicrobiennes ont été filtrées avec un filtre stérile de 0,2 µm puis utilisées pour les expériences.

3.2. Détermination de la concentration minimale inhibitrice (CMI) de composés sélectionnés à l'aide d'un test de microdilution en bouillon double

La valeur de la CMI de chaque composé (HE, acides organiques et bases) a été déterminée par un test de microdilution en série à l'aide de microplaques à 96 puits stérilisées. En bref, une suspension de dilution en série double de 10 ppm à 10 000 ppm dans une microplaque à 96 puits de chaque composé a été réalisée à l'aide de MHB. Chaque puits contient 100 µL d'aliquot de la suspension. Ensuite, chaque puits a été inoculé avec 100 µL de 10^6 UFC/mL de culture bactérienne et incubé à 37 ˚C pendant 24 h. Pour le contrôle négatif, 2 rangées de microplaques ont été utilisées et 100 µL de solution saline ont été ajoutés à la place de la culture bactérienne. Le témoin positif était une colonne de microplaque (sans agent antimicrobien) composée de 100 µL de culture bactérienne et 100 µL de MHB. Il convient de noter que le Tween 80 stérile (2,5 % v/v) utilisé pour l'émulsion n'a montré aucune activité antimicrobienne. Et l'expérience a été faite en triple. Enfin, la croissance bactérienne a été évaluée par Ultra Microplate Reader (Biotek Instruments, Winooski, VT, USA). La densité optique a été mesurée à 595 nm. La CMI est la concentration minimale requise pour éliminer complètement la souche bactérienne et montrant l'absorbance en blanc **(Turgis et al., 2012; Ayari et al., 2020)**.

3.3. Détermination de l'interaction entre les composés antimicrobiens à l'aide du test Checkerboard

L'indice de concentration inhibitrice fractionnelle (FIC) des diverses combinaisons de composés antibactériens contre les agents pathogènes cibles a été calculé par dosage en damier

à l'aide de microplaques à 96 puits selon la procédure expliquée par **Gutierrez et al (2008)** et l'expérience a été réalisée en triple.

L'interaction entre deux composés a été déterminée selon les formules suivantes

$FICa = CMI_a$ combiné/CMI_a seul

$FICb = CMI_b$ combinée/CMI_b seule

$FIC = FIC_A + FIC_b$

Ici, FIC_a et FIC_b représentaient les valeurs FIC du composé antibactérien a et du composé antibactérien b. C'est le rapport de la concentration minimale du composé « a » et du mélange du composé « a » et « b » respectivement qui était responsable de l'inhibition de la croissance bactérienne lorsqu'il était utilisé dans un traitement combiné. La somme des deux indique l'indice FIC.

Le FIC est la concentration d'un composé antibactérien responsable de l'inhibition de la croissance bactérienne en combinaison avec un autre composé divisée par la concentration requise pour inhiber la croissance bactérienne lorsqu'il est utilisé seul. Sur la base des valeurs FIC, l'interaction entre les composés est classée comme a) FIC < 0,5 a été indiqué comme effet synergique (S), b) 0,5 < FIC ≤ 1 a été interprété comme effet additif (AD), c) 1 < FIC ≤ 4 a été interprété comme aucun effet interactif (NI), d) FIC > 4 a été représenté comme effet antagoniste (A) **(Turgis et al., 2012; Ayari et al., 2020)**.

3.4. Développement de nouvelles formulations antibactériennes naturelles à base d'HE, d'acides organiques et d'extraits d'agrumes

La meilleure combinaison de composés antimicrobiens a été sélectionnée sur la base de l'effet synergique et 4 nouvelles formulations antimicrobiennes naturelles ont été créées. Ces formulations sont composées d'huiles essentielles et d'acides organiques. Les formulations sont répertoriées dans le **tableau 3.1**.

La composition de chaque formulation et le rapport de chaque composé ajouté à la formulation ont été effectués sur la base de l'indice FIC et le mélange résultant a été homogénéisé dans un ultra-turrax pour obtenir une suspension colloïdale. La formulation antimicrobienne de travail a été préparée après filtration à travers un filtre stérile à pores de 0,2 µm **(Turgis et al., 2012; Ayari et al., 2020; Ji et al., 2021)**.

3.5. Effet combiné de l'irradiation γ et de nouvelles formulations antimicrobiennes

Pour le traitement par irradiation, le lait maternel reconstitué a été inoculé avec une culture bactérienne sélectionnée (10^6 UFC/mL) en dilution 1:10. Ensuite, la formulation antimicrobienne naturelle développée (0,025 % v/v) a été ajoutée à des échantillons de lait inoculés par des bactéries. Le volume de culture bactérienne et de formulation antimicrobienne ajouté était le même (rapport 1:1). L'échantillon de lait inoculé avec 10^6 UFC/mL de culture bactérienne (sans formulation antibactérienne) a été utilisé comme témoin. L'irradiation γ a été effectuée par le dispositif d'irradiation UC-15A avec une source ^{60}Cobalt (Nordion Inc., Laval, QC, Canada) et elle a été certifiée par l'Institut national des normes et de la technologie (Gaithersburg, Md.). Le débit de dose d'irradiation de l'appareil était de 6,318 kGy/h et les échantillons ont été irradiés à l'état congelé (-20 ˚C). Les échantillons ont été soumis à différentes doses d'irradiation comme suit: 0, 0,5, 0,75, 1, 1,5, 2, 2,5, 3, 4, 5 kGy. Au total, quatre formulations antibactériennes ont été testées contre six souches bactériennes pour cette expérience. Lors de l'irradiation, 100 µL d'échantillon ont été inoculés à une gélose spécifique dans des boîtes de Pétri en utilisant la technique de la plaque étalée. Les boîtes inoculées ont été incubées à 37 ˚C pendant 24 à 48h selon la souche bactérienne. Le lendemain, un comptage des colonies a été effectué pour déterminer le D_{10} et la radiosensibilité (R_s). L'expérience d'irradiation gamma a été réalisée en triple. La valeur D_{10} est définie comme la dose nécessaire pour réduire la population de bactéries de 1 Log **(Lacroix, 2010)**. Et il a été calculé en traçant le graphique (régression linéaire) entre la dose d'irradiation (kGy) sur l'axe des x et la population bactérienne (Log UFC/mL) sur l'axe des y. L'inverse de la pente a donné la valeur D_{10} **(Maherani et al., 2019, Ghabraie et al., 2016)**. R_s a été calculé par l'équation suivante à partir des valeurs D_{10} de l'échantillon et du témoin.

Radiosensibilité (Rs) = D_{10} (kGy, contrôle) / D_{10} (kGy, échantillon)

Après calcul du D_{10} et de la radiosensibilité, l'expérience d'irradiation a été réalisée dans deux types d'échantillons, l'un était du lait maternel inoculé avec une culture bactérienne (106 UFC/mL) et une formulation antibactérienne dans un rapport de 9:1:1; l'autre était exempt de formulations antimicrobiennes et a été considéré comme témoin. Le traitement utilisé pour les expériences était de 0 kGy et 5 kGy respectivement. Il s'agissait principalement de trouver une dose d'irradiation unique (kGy) capable d'éliminer tous les pathogènes testés.

3.6. Détermination du lactose

La concentration de lactose dans le traitement combiné d'irradiation (5 kGy) et de formulation antibactérienne (3 et 4) dans le lait maternel et le lait maternel non irradié a été mesurée et comparée à l'aide du système HPLC Agilent série 1260 (Agilent Technologies, Palo Alto, CA, USA) avec une colonne de glucides ZORBAX (4,6*250 mm, taille des pores 70 Å, 5taille des particules µm). Les échantillons ont été préparés en ajoutant une quantité égale d'acide trichloroacétique (TCA) à 12 % (p/v) dans le lait maternel irradié et non irradié. Ensuite, les échantillons ont été soumis à une centrifugation (Sorvall® Instrument, Du Pond, USA) à 10 000 g pendant 20 min à 4 ◦C pour éliminer les matières grasses et la caséine. Le surnageant a été filtré avec 0,2filtre seringue µm et stocké au congélateur à -20˚C avant utilisation. La séparation du lactose par HPLC a été effectuée dans des conditions isocratiques. L'eau Mili-Q a été utilisée comme phase mobile à un débit de 1,5 mL/min. Le volume d'échantillon injecté dans la colonne était de 20µL. Le chromatogramme a été analysé par Chemstation v.2.0 (Agilent). La courbe standard a été préparée en utilisant une concentration de lactose de 0 à 50 mg/mL. Le détecteur d'indice de réfraction a été utilisé comme détecteur à la température 50 ˚C et la colonne a été fixée à 80 ˚C **(Robichaud et al., 2020)**.

3.7. Digestion in vitro des protéines et détermination de la libération d'azote

La digestibilité in vitro des protéines (IVPD) du traitement combiné de l'irradiation (5 kGy) et de la formulation antibactérienne (3 et 4) dans le lait maternel et le lait maternel non irradié a été déterminée par digestion séquentielle à l'aide de pepsine et de trypsine selon la méthode mentionnée par **Manus et al. (2021)**. Selon le protocole, une aliquote contenant au moins 0,5 g de protéines doit être utilisée pour l'expérience. Concrètement, 40 g de protéines de lait maternel (100 g de lait maternel contiennent 1,25 g de protéines) ont été mis en suspension dans 9,5 mL de HCl (0,1 M). Lors de l'addition de HCl, le pH est passé à 2, puis 0,5 ml de pepsine (11 mg/ml dans HCl 0,1 M) a été ajouté à la solution. Le rapport du substrat protéique à l'enzyme était de 1:50 p/p. Le mélange a été incubé à 37 ˚C pendant 120 min à 100 g. La solution digérée a été ajustée à pH 8 en utilisant NaOH (1,0 M) et 10 mg de trypsine ont été ajoutés (le rapport entre l'enzyme et la protéine initiale était de 1:50 p/p). Après incubation dans les mêmes conditions, des échantillons de 1 ml ont été prélevés à différents temps de digestion et soumis à la chaleur pendant 5 min à 95 ˚C dans un bain-marie pour arrêter la réaction enzymatique. Après cela, il a été centrifugé à 10, Filtre seringue µm pour déterminer

la distribution du poids moléculaire des peptides par SEC-HPLC sur une colonne Biosep-SEC 2000.

Le pourcentage de relargage d'azote au cours du processus de digestion a été calculé à partir du surnageant obtenu après précipitation à l'acide trichloroacétique (TCA) selon le protocole expliqué par **Manus et al. (2021)**. La digestibilité est proportionnelle à l'hydrolyse des peptides de haut poids moléculaire (HMW) en peptides de poids moléculaire moyen (MMW) et en peptides de bas poids moléculaire (SMW). Plus l'hydrolyse des protéines est grande, plus la digestibilité est grande. En bref, 6 ml de l'échantillon d'hydrolysat de protéines digéré par une enzyme ont été mélangés avec un volume égal de TCA (20 % p/p), suivi d'une centrifugation à 10 000 g pendant 20 minutes. Ensuite, le surnageant a été soumis à la méthode Kjeldahl (N * 6,25) selon la méthode mentionnée par AOAC International (2000). La formule utilisée pour la concentration en pourcentage de libération d'azote était :

% N libéré = (Azote dans le surnageant / Azote dans l'échantillon) * 100

3.8. Analyse peptidique

La distribution du poids moléculaire des peptides et la teneur en immunoglobulines du traitement combiné de l'irradiation (5 kGy) et de la formulation antibactérienne (3 et 4) dans le lait maternel et le lait maternel non irradié ont été déterminées selon le protocole décrit par **Manus et al. (2021)**. La CLHP d'exclusion stérique a été utilisée pour l'analyse avec une colonne Biosep-SEC 2000 (300 * 7,8 mm, taille des pores 145 UNE, granulométrie de 5 mm) de Phenomenex (Torrance, CA, USA). Le système HPLC utilisé pour l'expérience était le système Agilent 1260 HPLC infinity (Agilent Technologies, Palo Alto, Californie, États-Unis). En bref, les échantillons de lait maternel avant et pendant la digestion des protéines in vitro ont été centrifuges à 10 000 g pendant 20 min et le surnageant a été filtré à l'aide de 0,2Filtre seringue µm. Ensuite, 10 µL du surnageant filtré ont été injectés dans la colonne. La phase mobile utilisée ici était une solution tampon de phosphate de sodium 100 mM (pH 6,8). L'échantillon a été injecté au débit de 1 ml/min pendant 20 min. Un détecteur à barrette de diodes à 280 nm a été utilisé pour la détection, et les normes utilisées pour l'analyse étaient la thyroglobuline bovine (670 KDa), la gamma globuline (300 KDa), l'IgG (150 KDa), l'ovalbumine (44 KDa), la myoglobine (17 KDa) et l'uridine (244 Da).

3.9. Dosage des substances réactives à l'acide thiobarbiturique (TBARS)

Le potentiel d'oxydation des lipides du lait maternel sous traitement combiné d'irradiation (5 kGy) et de formulation antibactérienne (3 et 4) a été évalué par le dosage des substances réactives à l'acide thiobarbiturique (TBARS) selon le protocole décrit par Criado et al. (2020). Une portion de 10 ml d'échantillon de lait maternel a été mélangée avec 10 ml d'acide trichloroacétique à 15 %. Ensuite, le mélange a été centrifugé à 5900 g pendant 5 min et le surnageant a été filtré à travers 0,45Filtre seringue µm. Après cela, 2 ml d'acide thiobarbiturique 0,06 M (TBA) dissous dans de l'acide acétique à 10% ont été ajoutés à 8 ml du surnageant filtré, bien vortexés et placés dans un bain-marie à 80°C pendant 90 min et immédiatement refroidi dans de la glace. Enfin, l'absorbance a été lue à 520 nm à l'aide du spectrophotomètre Cary 1 (Agilent Technologies inc., Mississauga, ON, Canada) et la concentration de malondialdéhyde (MDA) a été déterminée. Des dilutions en série d'une solution de 1,1,3,3 tétraméthoxypropane (97 % v/v) ont été utilisées comme standard.

3.10. Analyse de la couleur et de la viscosité

La variation de couleur des échantillons de lait maternel (irradiés et non irradiés) a été mesurée à l'aide du lecteur de couleurs CR10-Plus (Konica Minolta, Ramsey, NJ, USA). Paramètres de couleur tels que L* (clarté, blanc = 0, blanc = 100), a* (rouge > 0, vert = 0, bleu < 0), b* (bleu-jaune de -300 à +299) et le global différence de couleur (ΔE*) ont été déterminées (Ben-Fadhel et al., 2021). La différence de couleur totale ΔE* a été calculée à l'aide de la formule suivante:

$$\Delta E^* = \sqrt{[(\Delta L^*)2 + (\Delta a^*)2 + (\Delta b^*)2]}$$

La viscosité du traitement combiné d'irradiation (5 kGy) et de formulation antibactérienne (3 et 4) dans le lait maternel et le lait non irradié (témoin) a été mesurée à l'aide d'un viscosimètre DV-II+ (Brookfield engineering Laboratories, Middleboro, MA, USA). La broche YULA-15 a été utilisée pour l'expérience. Toutes les mesures ont été calculées à 100 g et 23,5 ◦C±0,1 ◦C (Okyere & Odamtten, 2014).

4. Résultats

4.1. Concentration minimale inhibitrice d'agents antibactériens sélectionnés

La concentration minimale inhibitrice (CMI) des acides organiques (acide lactique, acide citrique), sels (carbonate de sodium), HE (origan, formulation méditerranéenne, cannelle, citronnelle, clou de girofle), extrait d'agrumes (F440D) contre les souches bactériennes *E.*

sakazakii (ATCC 29004), *E. coli* O157:H7 (ATCC 43895), *B. cereus* (ATCC 14579), *L. monocytogenes* (HPB 2812 sérotype 1/2a), *S. aureus* (ATCC 29213) et *S. Typhimurium* (ATCC SL1344) sont présentés dans le **tableau 3.2**. Les résultats ont montré que l'extrait d'agrumes a une plus grande efficacité antibactérienne contre toutes les souches bactériennes présentant une CMI de 312,5 ppm, suivi de l'origan avec une valeur de CMI de 625 ppm contre toutes les souches sauf *B. cereus*, pour laquelle une CMI valeur de 1250 ppm a été enregistrée. La formulation méditerranéenne a montré une activité antibactérienne notable avec une valeur CMI de 1250 ppm contre la plupart des souches testées à l'exception de *B. cereus*, dont la valeur MIC s'est produite à une concentration élevée de 2500 ppm. D'autres HE comme le clou de girofle présentaient une valeur CMI de 2500 ppm contre toutes les souches bactériennes et la citronnelle avec une valeur CMI de 2500 ppm contre *E. coli* O157:H7, L. monocytogenes, *S. aureus* et 5000 ppm contre *B. cereus, S. Typhimurium, E. sakazakii*. La cannelle a présenté une valeur CMI de 1250 ppm contre *E. coli* O157:H7 et *S. aureus* et celle de 2500 ppm contre le reste des souches bactériennes. D'autre part, les acides organiques et les sels ont montré comparativement une activité antibactérienne plus faible. La valeur MIC de l'acide lactique s'est produite à 2500 ppm contre *E. coli* O157:H7, *L. monocytogenes, S. Typhimurium, S. aureus* et celle de 5000 ppm contre *B. cereus, E. sakazakii*, acide citrique avec une valeur CMI de 5000 ppm contre *E. coli* O157:H7, *S. aureus, L. monocytogenes* et > 5000 ppm contre les autres souches bactériennes se montrant moins efficaces contre les pathogènes. Parmi tous les composés testés, le carbonate de sodium avait l'activité antibactérienne la plus faible avec une valeur CMI> 5000 ppm contre toutes les souches bactériennes. La CMI la plus basse enregistrée était de 312,5 ppm pour l'extrait d'agrumes contre toutes les souches bactériennes, parmi les HE, l'origan a montré une plus grande activité antibactérienne à 625 ppm et parmi les acides organiques, l'acide lactique a montré un effet bactéricide comparativement plus élevé avec une valeur de CMI de 2500 ppm.

4.2. Indice FIC des HE et acides organiques sélectionnés

Les résultats du test en damier mettant en évidence l'indice FIC des interactions binaires d'interaction des HE et entre les acides organiques sont présentés dans le **tableau 3.3a**. Dans les HE, la formulation méditerranéenne et la combinaison d'origan ont montré un effet synergique contre *E. coli* O157:H7 et *S. aureus*, et un effet additif contre d'autres souches bactériennes. D'autres combinaisons d'HE cannelle et citronnelle, clou de girofle et citronnelle ont montré un effet additif contre toutes les souches bactériennes testées. D'autre part, la

combinaison d'acide lactique et d'acide citrique a montré un effet non interactif contre toutes les souches bactériennes.

4.3. Indice FIC de triple combinaison d'HE avec d'autres HE, extrait d'agrumes et acides organiques

Les résultats de l'indice FIC correspondant aux triples combinaisons d'HE avec d'autres HE, extrait d'agrumes, acides organiques et sels sont présentés dans le **tableau 3.3b**. La combinaison d'HE d'origan et de formulation méditerranéenne, de cannelle et de citronnelle ainsi que d'extrait d'agrumes a montré un effet synergique contre toutes les souches bactériennes sauf *E. sakazakii*, montrant un effet additif. L'autre combinaison d'origan et de formulation méditerranéenne avec de la cannelle a montré un effet additif contre tous les agents pathogènes testés. Pendant ce temps, les triples combinaisons cannelle/citronnelle/acide citrique et origan/formulation méditerranéenne/acide lactique ont montré un effet non interactif contre toutes les souches testées.

4.4. Indice FIC des formulations quadruples antibactériennes

Les résultats de l'indice FIC des formulations quaternaires développées avec de l'origan, du thym, de l'extrait d'agrumes et de la citronnelle, de la cannelle, de l'extrait d'agrumes ainsi que de l'acide citrique et de l'acide lactique sont présentés dans le **tableau 3.3c**. Toutes les quatre formulations : origan/ formulation méditerranéenne/ extrait d'agrumes/ acide lactique ; origan/ formule méditerranéenne/ extrait d'agrumes/ acide citrique; cannelle/citronnelle/extrait d'agrumes/acide lactique et cannelle/citronnelle/extrait d'agrumes/acide citrique ont montré un effet synergique contre toutes les souches bactériennes testées à l'origine de la contamination du lait maternel.

4.5. Inactivation de bactéries dans des échantillons de lait congelé par irradiation γ combinée à des formulations antibactériennes synthétisées

Les valeurs D_{10} (kGy) du traitement combiné de l'irradiation γ avec la formulation antibactérienne et le contrôle (irradiation seule) et l'effet des quatre formulations antibactériennes développées en combinaison avec l'irradiation γ sur la radiosensibilité des souches bactériennes sont présentés dans le **tableau 3.4a**. La dose d'irradiation (kGy) requise pour réduire la population bactérienne de 6 log dans le lait maternel par un traitement par irradiation seul (témoin) et en combinaison avec des formulations antibactériennes est présentée dans le **tableau 3.4b**. Pour E. coli O157:H7, une valeur D_{10} comprise entre 0,130 et

0,132 kGy avec une radiosensibilité allant de 2,57 à 2,61 a été observée dans les traitements combinés, tandis que la valeur D_{10} pour le contrôle était de 0,340 kGy. Le D_{10} le plus bas enregistré contre *E. coli* O157:H7 était de 0,130 kGy pour le traitement combiné respectif à la formulation 3 montrant une radiosensibilité de 2. 61 (**Tableau 3.4a**). La dose nécessaire pour réduire la limite de détection d' *E. coli* O157:H7 (6-Log) pour le contrôle était de 2,04 kGy et elle a été réduite à 0,78 kGy avec le traitement combiné respectif à la formulation 3 qui était la plus faible par rapport à l'autre formulations 1, 2 et 4 (**Tableau 3.4b**).

Pour *S. aureus*, les formulations 1 et 2 présentaient une valeur D_{10} similaire et une radiosensibilité de 0,175 kGy et 2,48. Ces valeurs sont passées à 0,170 kGy et 2,55 pour les formulations 3 et 4 respectivement (**Tableau 3.4a**). Les valeurs D_{10} du traitement combiné avec les quatre formulations contre *S. aureus* sont significativement différentes ($p < 0,05$) de ce qui a été enregistré pour le contrôle 0,433 kGy (**Fig. 3.1d**). La dose la plus faible requise pour la réduction de 6 log de *S. aureus* était de 1,02 kGy pour le traitement combiné respectif pour les formulations 3 et 4. Pour le contrôle, la dose requise pour la réduction de 6 log était de 2,59 kGy (**Tableau 3.4b**).

Pour B. cereus, une radiosensibilité plus élevée a été observée avec les valeurs de 2,03 et 2,06 pour les formulations 3 et 4, par rapport aux valeurs respectives pour les formulations 1 et 2 (1,38 et 1,4). Les changements de radiosensibilité étaient parallèles à ceux du D_{10} car les formulations 3 et 4 nécessitaient moins de dose (0,176 et 0,174 kGy) par rapport aux formulations 1 et 2 (0,261 et 0,256 kGy) (**Tableau 3.4a**). La valeur D_{10} pour le contrôle était de 0,358 kGy contre *B. cereus*, ce qui est plus élevé et significativement différent ($p < 0,05$) par rapport au traitement combiné. La dose la plus faible requise pour la réduction de 6 log de B. cereus était de 1,04 kGy pour le traitement combiné respectif à la formulation 4. Pour le contrôle, la dose requise pour la réduction de 6 log était de 2,14 kGy (**Tableau 3.4b**).

Pour *E. sakazakii* (**Fig. 3.1b**), une valeur D_{10} de 0,372 kGy a été enregistrée pour le contrôle. Pour le traitement combiné concernant les formulations 1 et 2, des valeurs D_{10} de 0,277 et 0,270 kGy ont été enregistrées respectivement, qui sont significativement plus élevées par rapport aux valeurs D_{10} respectives des formulations 3 et 4 (0,176 kGy) (**Tableau 3.4a**). Une radiosensibilité de 1,34 et 1,38 a été enregistrée pour les formulations 1 et 2 respectivement, qui sont inférieures par rapport aux formulations 3 et 4 (2.11). La dose la plus faible requise pour la réduction de 6-log d' *E. sakazakii* était de 1,06 kGy pour le traitement

combiné par rapport aux formulations 3 et 4. Pour le contrôle, la dose requise pour la réduction de 6 log était de 2,23 kGy (**Tableau 3.4b**).

Pour *L. monocytogenes*, la valeur D_{10} du contrôle était de 0,538 kGy et pour le traitement combiné avec les formulations 3 et 4, les valeurs étaient respectivement de 0,298 et 0,314 kGy, qui sont significativement inférieures ($p < 0,05$) par rapport aux valeurs D_{10} respectives de 0,380 et 0,381 kGy pour les formulations 1 et 2 (**Tableau 3.4a**). La radiosensibilité la plus élevée de 1,81 contre *L. monocytogenes* a été obtenue pour la formulation 3, suivie de 1,71 pour la formulation 4. Les formulations 1 et 2 ont adopté la radiosensibilité la plus faible de 1,41 contre *L. monocytogenes* (**Fig. 3.1a**). La dose nécessaire pour réduire la limite de détection de *L. monocytogenes* (6-Log) pour le contrôle était de 3,23 kGy, alors qu'elle a diminué à 1,78 kGy avec pour le traitement combiné respectif à la formulation 3 qui était la plus faible par rapport aux autres formulations 1, 2 et 4 (**Tableau 3.4b**).

Pour *S. Typhimurium* (**Fig. 3.1c**), la radiosensibilité la plus élevée de 3,99 parmi toutes les souches bactériennes testées a été trouvée avec la formulation 3. La valeur D_{10} du contrôle était de 0,528 kGy diminuant à 0,270, 0,268, 0,132 et 0,133 kGy pour les traitements combinés concernant formules 1, 2, 3 et 4 respectivement. Les valeurs de D_{10} des traitements combinés étaient significativement différentes ($p < 0,05$) par rapport au témoin (**Tableau 3.4a**). Comme d'autres souches bactériennes, les formulations 3 et 4 (3,99 et 3,97) étaient plus efficaces pour augmenter la radiosensibilité de *S. Typhimurium* par rapport aux formulations 1 et 2 (2,0 et 2,35). La dose la plus faible requise pour la réduction de 6 log (UFC/mL) de *S. Typhimurium* était de 0,792 kGy pour le traitement combiné respectif à la formulation 3 montrant une perte notable par rapport à celle du contrôle (3,17 kGy) (**Tableau 3.4b**).

Concernant la forme sporulée de *B. cereus*, une valeur D10 de 1,513 kGy a été observée pour le témoin. L'introduction des formulations de 1, 2, 3 et 4 a diminué les valeurs de D10 à 0,986, 0,984, 0,796 et 0,801 kGy respectivement (**Tableau 3.4a**). La radiosensibilité des formulations 1 et 2 (1,53 et 1,54) était inférieure à celle des formulations 3 et 4 (1,90 et 1,89). La forme sporulée de B. cereus est la bactérie la plus résistante et la dose requise pour sa réduction de 6 log sous condition de spores (9,08 kGy) est réduite à 4,78 kGy en appliquant la formulation 3 (**Tableau 3.4b**).

4.6. Inactivation des bactéries à l'aide d'un traitement combiné d'irradiation γ et d'une nouvelle formulation antimicrobienne

L'effet du traitement combiné des formulations antimicrobiennes et de l'irradiation γ à 5 kGy contre les agents pathogènes d'origine alimentaire sélectionnés est présenté dans **(Tableau 4.2)**. Les résultats ont montré que lale traitement combiné des formulations 3 et 4 avec irradiation γ à 5 kGy a éliminé toutes les souches bactériennes testées (6 log) même B. cereus sporulé dans le lait maternel **(Tableau 4.2)** qui est l'espèce la plus résistante à l'irradiation. Alors que le traitement combiné des formulations 1 et 2 avec irradiation γ à 5 kGy a limité la détection de 6 log de tous les agents pathogènes d'origine alimentaire, à l'exception de la forme sporulée de B. cereus. La teneur initiale en spores de B. cereus dans le lait maternel était 6,25 ± 0,03log UFC/mL. Après le traitement par irradiation à 5 kGy sans formulation antimicrobienne (contrôle) une valeur de 3,64 ± 0,01 log UFC/mL deB. cereus sporulé a été enregistré (tableau 2). L'incorporation des formulations 1 et 2 dans le lait maternel a encore réduit *B. cereus* par 1. 25 et 1.19 log UFC/mL respectivement. En présence des formulations 3 et 4 la teneur en spores de B. cereus était nulle **(Tableau 4.2)**. D'après les résultats, on peut observer que le traitement combiné d'irradiation γ avec les formulations 3 et 4 était significativement plus efficace ($p > 0,05$) pour éliminer tous les agents pathogènes testés (souches sensibles et résistantes) par rapport aux formulations 1 et 2. Pour pour cette raison, les formulations 3 et 4 ont été sélectionnées comme les mieux adaptées pour les expériences suivantes.

4.7. Teneur en lactose et oxydation des lipides dans le lait maternel irradié et non irradié

Dans la présente étude, la teneur en lactose du lait maternel congelé n'a pas été modifiée après le traitement combiné de l'irradiation et formulations antibactériennes (3 et 4) par rapport au contrôle (0 kGy) (**Fig. 4.1a**). La teneur en lactose dans l'échantillon non irradié (contrôle) était de 7,7 ± 0,16 g/100 mL et il n'y avait aucune altération tangible dans les échantillons irradiés avec les formulations 3 et 4 qui présentaient respectivement 7,9 ± 0,29 g/100 mL et 7,4 ± 0,16 g/ 100 ml.

Comme pour la teneur en lactose, il n'y avait aucune différence dans la teneur en malondialdéhyde (MDA) du lait maternel congelé avant et après le traitement combiné (**Fig. 4.1b**). La teneur en MDA de l'échantillon témoin (0 kGy) était de 2,16 ± 0,11 µg/g passant à 2,26 ± 0,13 µg/g et 2,21 ± 0,08 µg/g pour l'échantillon contenu avec les formulations 3 et 4 et exposé à 5 kGy.

4.8. Profil peptidique et teneur en immunoglobulines

La distribution du poids moléculaire des peptides de l'échantillon témoin (lait maternel non irradié) et le traitement combiné d'irradiation (5 kGy) et antibactérienformulations (3 et 4) dansle lait maternel estprésenté dans le **Tableau 4.3**. La digestion enzymatique avec la pepsine et la trypsine décompose les protéines et les peptides plus gros en peptides plus petits et en acides aminés libres. Les peptides ont été divisés en trois groupes différents; Ceux de poids moléculaire > 2700 Da (polypeptides) sont considérés comme de haut poids moléculaire (HMW); entre 2700 et 200 Da sont dits de poids moléculaire moyen (MMW) (oligopeptides et tripeptides) et <200 Da sont du groupe de bas poids moléculaire (LMW) (dipeptides et acides aminés libres). La proportion totale de peptides HMW et MMW a diminué au cours de la digestion pour les échantillons irradiés et non irradiés, tandis que la proportion de LMW a augmenté. Comparer la différence d'efficacité d'hydrolyse des protéines lors de la digestion enzymatique d'échantillons combinés traités et non irradiés, il a montré que le traitement combiné de l'irradiation γ à 5 kGy et de la formulation antimicrobienne 3 a entraîné une réduction des peptides HMW dans le lait maternel de 7 %, ce qui est significativement supérieur ($p \leq 0,05$) par rapport à celui de l'échantillon non irradié. De même, le traitement combiné de l'irradiation γ et de la formulation antimicrobienne 4 a conduit à une réduction de HMW de 10 %. La proportion deLes peptides MMW ont été réduits de 15 % et 21 % dans les échantillons irradiés contenus avec les formulations 3 et 4, ce qui est significativement plus élevé ($p \leq 0,05$) que celui des échantillons non irradiésconduisant à l'augmentation de la surface totale sous la courbe pour LMW. Ces résultats ont démontré que le traitement combiné de l'irradiation γ et des formulations antibactériennes de la mèrele lait à 5 kGy a augmenté l'hydrolyse des protéines pendant la digestion enzymatique entraînant la libération de peptides LMW ($p \leq 0,05$) par rapport aux échantillons non irradiés.

Le contenu en immunoglobulines (IA, IgG, IgM) du traitement combiné d'irradiation etformulation antibactérienne (3 et 4) dansle lait maternel et le lait maternel non irradié ont été comparés à l'aide de l'analyse HPLC. La comparaison de la concentration en immunoglobulines est présentée dans le **Tableau 4.4**. La teneur en immunoglobulines dans le lait maternel des traitements combinés n'a pas été significativement modifiée ($p > 0,05$) par rapport à l'échantillon témoin.

4.9. In vitrodigestibilité des protéines (IVPD)

L'IVPD du traitement combiné de l'irradiation (5 kGy) et de la formulation antibactérienne (3 et 4) dansLe lait maternel et le lait maternel non irradié ont été determines par la libération d'azote au cours de la digestion in vitro à l'aide de pepsine et de trypsine. Les résultats ont montré que les échantillons témoins (0 kGy) et irradiés (5 kGy) ont le même schéma de libération d'azote lors de la digestion in vitro. Le pourcentage de libération d'azote après digestion à la pepsine (0 à 120 min) était de 73,31 % et a augmenté à 94,73 % après digestion à la trypsine (120 à 240 min) pour le témoin. Les deux traitements combinés à 5 kGy ont également montré un schéma similaire de libération d'azote dans le lait maternel après digestion par la pepsine et la trypsine. Pour que le relargage total d'azote après 240 min (dige pepsine + trypsinestion) pour les échantillons irradiés en combinaison avec les formulations 3 et 4 était de 95,86 % et 95,23 %, respectivement.

4.10. Couleur et viscosité du lait maternel irradié et non irradié

Les paramètres de couleur (L*, a*, b* et ΔE) du traitement combiné de l'irradiation (5 kGy) et de la formulation antibactérienne (3 et 4) dans le lait maternel et le lait maternel non irradié sous condition congelée ont été présentés dans le tableau 5. Il n'y avait pas d'effet significatif sur L* (luminosité), a* (rougeur) du lait maternel soumis au traitement combiné par rapport au témoin. Mais, b* (jaunissement) des deux échantillons soumis à un traitement combiné a montrémoins de valeurs par rapport à celle du contrôle. La valeur ΔE était de 1,39 ± 0,07, ce qui montre une très petite différence de couleur.

D'après le **Tableau 4.5**, on peut également observer que le traitement combiné d'irradiation (5 kGy) et de formulation antibactérienne (3 et 4) a montré une légère augmentation de la viscosité du lait maternel par rapport à l'échantillon non irradié. La viscosité de l'échantillon non irradié était3,2 ± 0,06 mPa.s et pour les échantillons irradiés en combinaison avec les formulations 3 et 4 était de 3,02 ± 0,08 et 3,05 ± 0,07, respectivement.

5. Discussion générale

5.1.Formulation antimicrobienne naturelle

La présente étude a confirmé l'effet inhibiteur accru de quatre formulations antibactériennes quaternaires contre les pathogènes d'origine alimentaire. Toutes les quatre formulations antibactériennes: origan/ formulation méditerranéenne/ extrait d'agrumes/ acide lactique ;

origan/ formule méditerranéenne/ extrait d'agrumes/ acide citrique; cannelle/citronnelle/extrait d'agrumes/acide lactique et cannelle/citronnelle/extrait d'agrumes/acide citrique ont montré un effet synergique contre toutes les souches bactériennes testées (*E. sakazakii*, *E. coli* O157:H7, *B. cereus*, *L. monocytogenes*, *S. aureus* et *S. Typhimurium*). **Sadekuzzaman et al. (2018)** ont constaté une réduction significative des *E. coli, Salmonella* et *Listeria monocytogenes* par l'application d'HE de thym/arbre à thé. Dans la présente étude, l'HE de cannelle seule a présenté la meilleure activité antibactérienne contre *P. amylolyticus*, mais ce composé était non interactif dans la plupart des combinaisons. En revanche, l'HE de cannelle seule n'était pas efficace contre B. cereus mais une inhibition synergique a été obtenue lorsqu'elle était associée à l'HE de thym. Une autre étude a révélé que l'origan/nisine, le thym/nisine et l'OE/pédiocine de S. montagna ont montré un effet additif contre *E. coli* O157:H7, *L. monocytogenes* et *B. cereus* (**Turgis et al., 2012**). **Gavaric et al. (2015)** ont également prouvé que le thymol et le carvacrol en association avec l'eugénol avaient un effet synergique contre *E. coli*, ce qui peut être attribué à la désintégration de la membrane cellulaire provoquée par le carvacrol et le thymol facilitant la pénétration de l'eugénol dans le cytoplasme cellulaire présentant des propriétés antibactériennes. cereus mais une inhibition synergique a été obtenue lorsqu'elle a été associée à l'HE de Thym.

5.2. Effet combiné de l'irradiation et de la formulation antibactérienne

Des études antérieures ont rapporté que le traitement par irradiation γ en combinaison avec des formulations antimicrobiennes naturelles est capable d'augmenter la radiosensibilité des pathogènes potentiels d'origine alimentaire. **Robichaud et al. (2020)** ont montré que 14,2 kGy sont nécessaires pour atteindre une réduction de 6 log des spores de *B. cereus*, tandis que dans nos études, l'irradiation à 5 kGy en combinaison avec les formulations 3 et 4 était efficace sur tous les pathogènes testés, y compris les *B. cereus* sporulés suggérant que le traitement combiné de l'irradiation avec une formulation antibactérienne est plus efficace par rapport à l'irradiation seule. **Turgis et al. (2008)** ont prouvé que l'effet combiné des HE de cannelle de Chine et d'origan d'Espagne avec γ-irradiation à 0,3, 0,4, 0,5, 0,58 et 0,6 kGya augmenté la radiosensibilité (une mesure montrant son efficacité par rapport au témoin) de *E. coli* O157:H7 et *S. Typhimurium* de 3,57 et 3,26 dans le bœuf haché. **Maherani et al. (2019)** ont montré que l'ajout d'une formulation antimicrobienne naturelle contenant de l'extrait d'agrumes dans du jus d'orange avec γ-irradiation à 1 kGy augmentéela radiosensibilité des espèces fongiques A. niger, *S. cerevisiae* et *P. chrysogenum* de 1,54, 2,10 et 2,32 respectivement. **Robichaud et al. (2021)** ont rapporté que l'incorporation d'acide citrique dans les préparations pour nourrissons

en poudre et congelées avait une radiosensibilité de 2,1 et 1, respectivement, contre *B. cereus*. Ces valeurs sont passées à 4,1 et 2,1 pour le carbonate de sodium dans les préparations en poudre et congelées pour nourrissons, respectivement. **Chiasson et al. (2005)** ont également étudié l'effet combiné de l'irradiation γ avec le carvacrol et le pyrophosphate tétrasodique dans le bœuf réduisant la dose de rayonnement nécessaire pour limiter la détection de *S. Typhimurium* de 1,55 kGy à 0,25 kGy. Cette valeur a été réduite de 0,65 kGy à 0,55 kGy en présence de carvacrol et de pyrophosphate tétrasodique pour *E. coli*. Les souches bactériennes sporulées nécessitent une dose d'irradiation plus élevée pour limiter leur détection dans les produits alimentaires. Les rayonnements ionisants inactivent les micro-organismes en perturbant le double standard de l'ADN (rupture de la liaison sucre-phosphate) empêchant ainsi la réplication des micro-organismes (**Lacroix, 2012**). **Caillet & Lacroix (2006)** ont rapporté que le traitement simultané des HE et de l'irradiation γ à 1,2 kGy avait un effet synergique affectant négativement l'intégrité de la membrane bactérienne et de la muréine ainsi que des dommages à l'ADN double standard entraînant un dysfonctionnement cellulaire et la libération de constituants cellulaires de les cellules bactériennes modifiant ainsi le pH interne et l'ATP.

5.3. Teneur en lactose, oxydation des lipides et immunoglobuline

Il est bien connu que dans des conditions opérationnelles appropriées (débit de dose, température, présence d'oxygène et type d'aliment), les valeurs nutritionnelles ne sont pas affectées par un traitement par irradiation jusqu'à 45 kGy (Maherani et al., 2016). **Robichaud et al. (2021)** ont prouvé que l'irradiation γ jusqu'à 10 kGy n'avait aucun effet significatif sur la teneur en lactose, en protéines et en MDA des préparations liquides pour nourrissons, ce qui coïncidait avec les données obtenues dans nos études. De même, **Ham et al. (2009)** ont également prouvé que la teneur en lactose du yaourt nature n'était pas altérée par l'irradiation γ à différentes doses (1, 3, 5 et 10 kGy). L'utilisation de doses plus élevées, par exemple à 25 kGy, a montré un effet croissant sur le MDA des préparations en poudre pour nourrissons (**Tesfai et al., 2014**) confirmant les résultats que nous avions obtenus sur la concentration de MDA en utilisant le test TBARS. L'augmentation de la formation d'oxydes lipidiques est due au changement des conditions environnementales du traitement par irradiation en produisant des radicaux oxygénés (**Stefanova, Vasilev, & Sapassov, 2010**). Aucun changement significatif n'a été observé dans la concentration d'immunoglobuline après le traitement combiné. Les résultats obtenus sont en parallèle avec ceux de **Tran et al. (2004)**. Ils ont rapporté que l'activité structurelle et fonctionnelle de la molécule d'IgG n'était pas endommagée par l'irradiation γ jusqu'à 50 kGy. De même, **Smeltzer et al. (2015)** ont également prouvé que

le niveau d'IgG n'était pas altéré et que l'intégrité de sa chaîne polypeptidique était complètement protégée à une dose d'irradiation de 50 kGy à -80 ◦C. Il indique que l'intégrité conformationnelle des immunoglobulines est restée intacte et n'a pas été affectée par l'irradiation γ. Les résultats obtenus sont en parallèle avec ceux de **Tran et al. (2004)**. Ils ont rapporté que l'activité structurelle et fonctionnelle de la molécule d'IgG n'était pas endommagée par l'irradiation γ jusqu'à 50 kGy. De même, **Smeltzer et al. (2015)** ont également prouvé que le niveau d'IgG n'était pas altéré et que l'intégrité de sa chaîne polypeptidique était complètement protégée à une dose d'irradiation de 50 kGy à -80 ◦C. Il indique que l'intégrité conformationnelle des immunoglobulines est restée intacte et n'a pas été affectée par l'irradiation γ. Les résultats obtenus sont en parallèle avec ceux de **Tran et al. (2004)**. Ils ont rapporté que l'activité structurelle et fonctionnelle de la molécule d'IgG n'était pas endommagée par l'irradiation γ jusqu'à 50 kGy. De même, **Smeltzer et al. (2015)** ont également prouvé que le niveau d'IgG n'était pas altéré et que l'intégrité de sa chaîne polypeptidique était complètement protégée à une dose d'irradiation de 50 kGy à -80 ◦C. Il indique que l'intégrité conformationnelle des immunoglobulines est restée intacte et n'a pas été affectée par l'irradiation γ.

5.4. Digestibilité in vitro des protéines et profil peptidique

Les résultats ont montré que l'irradiation diminuait les peptides HMW par rapport à l'échantillon non irradié (tableau 3). Une diminution de 7 % et 10 % de la HMW a été observée dans les échantillons soumis au traitement combiné avec les formulations 3 et 4 après la digestion avec la pepsine et la trypsine par rapport au témoin traité. **Robichaud et al. (2020)** ont prouvé que les préparations pour nourrissons en poudre et liquides irradiées à 10 kGy réduisaient la proportion de peptides > 500 kDa de 18 % par rapport aux échantillons non irradiés. L'irradiation en générant des radicaux libres libérés par la radiolyse de l'eau perturbe les liaisons disulfure des peptides HMW et par conséquent l'affaiblissement structurel. Un dépliement partiel le rend sensible aux attaques protéolytiques **(Hassan et al., 2018)**. L'irradiation peut également induire une désamination qui peut en outre conduire au dépliement des protéines **(Dogbevi et al., 2000)**. Après le déploiement des protéines, les résidus hydrophobes enfouis à l'intérieur seront exposés et facilement affectés par les enzymes pepsine et trypsine (**Murray et., 2009**). Selon **Fombang et al. (2005)**, les liaisons peptidiques sont exposées aux enzymes protéolytiques en raison de la modification des structures secondaires et tertiaires des protéines causée par l'irradiation γ.

Malgré une plus grande teneur en peptides LMW dans les échantillons irradiés par rapport au contrôle,nos résultats n'ont montré aucune différence significative dans la digestibilité des protéines in vitro par rapport à l'échantillon témoin. Au contraire, certaines études ont rapporté une augmentation de l'IVPD après hydrolyse des protéines (**Manus et al., 2021; Dridi et al., 2021**). Le dépliement induit de la protéine expose les groupes non polaires et augmente l'hydrophobicité de la protéine. D'où la possibilité d'une plus grande présence de peptides hydrophobes dans les échantillons sous l'effet des traitements combinés. En considérant le fait que la taille et l'hydrophobicité des peptides affectent leur solubilité dans le TCA, les résultats similaires pour l'azote soluble impliquent la même solubilité totale pour les peptides accumulés dans le surnageant après précipitation au TCA.

5.5. Analyse de la couleur et de la texture du lait maternel

Les différences de couleur peuvent être analytiquement classées comme très distinctes ($\Delta E>3$), distinct ($1,5<\Delta E<3$) et petite différence ($1.5>\Delta E$) **(Lin et al., 2020)**. **Park & Ha (2019)** ont montré qu'une irradiation à 0,8 kGy n'altérait pas significativement les valeurs L* (clarté), a* (rougeur), b* (jaunissement) du fromage en tranches. Les résultats ont montré que la viscosité du lait maternel après un traitement combiné d'irradiation (5 kGy) et de formulation antimicrobienne (3 et 4) était légèrement réduite par rapport au témoin (tableau 5). **Okyere & Odamtten (2014)** ont montré que l'augmentation de la dose d'irradiation de 0 à 10 kGy diminuait d'autant la viscosité du lait de souchet. La réduction de la viscosité de l'échantillon irradié a été causée par la formation de radicaux libres par irradiation **(Sokhey & Hanna, 1993)**. Les radicaux anions hydroxyle et superoxyde générés par l'irradiation γ pourraient modifier la structure primaire des protéines, entraînant des distorsions des structures secondaires et tertiaires. De plus, l'exposition des protéines aux radicaux oxygène entraîne une fragmentation non aléatoire et aléatoire qui peut être la raison possible de la réduction de la viscosité (Lee et al., 2003).

6. Conclusion générale

Dans la présente étude, l'inactivation des agents pathogènes d'origine alimentaire dans le lait maternel congelé à l'aide d'un traitement combiné d'irradiation γ et de formulations antimicrobiennes a été démontrée. L'interaction entre les HE, les acides organiques et l'extrait d'agrumes contre toutes les souches bactériennes testées a également été démontrée. Quatre formulations antibactériennes développées ont montré un effet synergique contre les agents pathogènes testés. Le traitement combiné de l'irradiation γ et des formulations antibactériennes

nouvellement développées a augmenté la radiosensibilité des souches bactériennes et l'activité antibactérienne et deux formulations (3 et 4) contenant la plus grande quantité d'extrait d'agrumes étaient particulièrement plus efficaces. La teneur en lactose, en immunoglobulines et l'oxydation des lipides dans le lait maternel congelé n'ont pas été affectées parle traitement combiné. L'irradiation γ à 5 kGy a contribué à l'hydrolyse de grosses molécules protéiques en peptides plus petits,mais aucun effet sur la digestibilité n'a été observé in vitro. La différence de couleur (ΔE) et la différence de viscosité entre le lait maternel irradié et non irradié étaient minimes. Cette recherche a démontré que le traitement combiné de l'irradiation γ avec des formulations antimicrobiennes est efficace pour réduire la dose d'irradiation pour l'élimination des bactéries et assure l'innocuité du lait maternel congelé.

Chapter 1: General Introduction

Mother's milk acts as the major nutrient supply, helps to build the immune system and also provides non-nutritive bioactive factors that promote survival and healthy development in infants **(Ballard *et al*., 2013)**. It contains carbohydrates, protein, fat, vitamins, minerals, digestive enzymes and hormones. In addition to these nutrients, it is rich in immune cells, including macrophages, stem cells, and numerous other bioactive molecules. Some of these bioactive molecules are protein-derived and lipid-derived, while others are protein-derived and indigestible, such as oligosaccharides. Human milk oligosaccharides (HMOs) possess anti-infective properties against pathogens in the infant gastrointestinal tract **(Gura, 2014)**. Oligosaccharides also play a vital role in the development of essential innate and adaptive immune responses **(Walker, 2013)**.

Donor milk banks helps to preserve human breast milk collected from various healthy donors and they play a major role in feeding premature infants because the milk produced during preterm birth contains inappropriate amount of macro and micro nutrients. And there are circumstances where milk is not available from the infant's own mother and this nutritional gap should be filled by donor milk **(Haiden *et al*., 2016)**. The major problem faced by Human milk banks is milk spoilage. The main source of contamination is bacteria. When new-born infants are fed with contaminated milk it causes serious heath diseases like necrotizing enterocolitis (NEC) and sepsis **(Arnold, 2006)**. Many research studies have demonstrated the bacterial strains that are responsible for contamination in infant formula and mother's milk. The major bacterial strains are *Listeria monocytogenes*, *Escherichia coli*, *Bacillus cereus*, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Enterobacter sakazakii* **(Al-Nabulsi *et al*., 2015; Robichaud *et al*., 2020)**.

Milk banks and food industries follow numerous sterilization techniques in order to overcome bacterial contaminations. Thermal pasteurization is highly used for milk preservation but it cannot eliminate spore forming bacteria especially *Bacillus cereus* **(Stadhouders, 1982)**, the use of chemical constituents and antibiotics would severely affect infant health **(Irkin *et al*., 2015)** and it is highly prohibited in infant foods. This demands the use of natural products with antibacterial properties for preservation.

Essential oils are volatile and aromatic compounds which are extracted from various parts of plants (leaves, roots, barks, flowers, fruits, seeds, etc). And they are obtained through extraction procedures and steam distillations **(Burt, 2004; Hyldgaard *et al.*, 2012)**. Due to the presence of secondary metabolites like tannins, phenols, alkaloids, flavonoids, many natural extracts and essential oils have high antibacterial activity **(Hyldgaard *et al.*, 2012; Akash *et al.*, 2018)**. Codex Alimentarius has permitted the use of some food additives like lactic acid, citric acid, sodium carbonate, sodium citrate, potassium carbonate and malic acid in infant formula **(Codex alimentarius, 1981)**. Irradiation is a non-thermal method of pasteurization, which can be used as an alternative to the heat treatment. The ionizing radiations that are used are gamma rays, X rays and electron beams **(Morehouse *et al.*, 2004)**. As per the regulations approved by Food and Drug Administration (FDA) up to 45 kGy of irradiation dosage is allowed to be used for sterilization of frozen food samples **(Maherani *et al.*, 2016)**. The macronutrient content of the food products such as carbohydrates, proteins and fats are not altered. However, the nutritional matrix is preserved depending on the environmental condition, food composition and irradiation dosage (kGy). Lipid oxidation and few micronutrients tend to be affected by the irradiation in a dose dependent manner as reported in previous research studies **(Dionísio, Gomes, & Oetterer, 2009; Robichaud *et al.*, 2020)**.

This dissertation focuses on the evaluation of the radiosensitivity of the targeted foodborne pathogens in combination with natural antibacterial formulations as well as the effect of γ-irradiation on the lactose, protein digestion, peptide profile, lipid oxidation, color and texture of mother's milk.

Chapter 2: Literature review

2.1. Human Milk Banks

2.1.1. Need for milk bank

It is probably not widely appreciated that human milk banking is an absolute necessity if all infants are to enjoy the benefits of human milk. This is so because a substantial number of infants, especially premature infants, are unable to receive adequate amounts of their mothers' milk for a variety of reasons. Were it not for milk banks, these infants could not be fed human milk and would suffer the consequences. Premature infants derive very important protections from human milk. Unfortunately, there are circumstances where milk from the infant's own mother is not available. Milk donated by other women (donor milk) must then fill the gap. Premature infants constitute the largest and most important group of infants where milk from other women is needed because their own mothers' milk is not available or is not available in sufficient quantity. Human milk banks collect, screen, pasteurize, and distribute donated breast milk to hospitals or outpatient recipients. Usually the collection, storage, and processing in a human milk bank follows established guidelines. Milk banks are by far the most important providers of donor milk. The mother's milk contains high amount of protein, zinc, sodium and calcium than normal human milk, which also leads to hazardous conditions in infants **(Tudehope, 2013)**. Usage of donor milk is the best option when compared to artificial infant milk formulas because infant formula has high risk of developing necrotizing enterocolitis (NEC) and sepsis, these two diseases have high mortality rate in infants **(Arnold, L.D., 2006)**. The main purposes for human milk bank are to preserve and provide milk for **(Haiden *et al*., 2016)**.

a) Premature infants
b) Infants with gastrointestinal abnormalities
c) For supporting mothers temporarily unable to provide milk
d) Infants with metabolic disorders
e) Weaning from parenteral nutrition
f) First few days of birth before the mother's milk comes in adequately

2.1.2. Process of preservation of human milk in milk banks

Milk banks always follow standardized procedures for the collection and handling of donated milk **(Bharadva *et al*., 2014)**. The general steps involved in the storage of human milk are **(Haiden *et al*., 2016)**.

- Screening of Donor
- Collection of breast milk
- Stored in freezer
- Transported to Milk bank
- Pasteurized (62.5 ˚C for 30 minutes)
- Microbiological testing in milk
- Stored in Bank freezer
- Delivered to the recipient

2.1.2.1. Screening of donor

The donor population is formed by healthy lactating mothers with healthy babies, who are voluntarily willing to give their extra breastmilk for other babies without compromising the nutritional needs of their own baby. The donors can include mothers attending well baby clinics, mothers whose babies are in neonatal intensive care units, those who have lost their babies but are willing to donate their milk, or lactating working staff in the hospital, and motivated mothers from the community **(Haiden *et al*., 2016)**. Donors are not paid for their donations.

Who can donate?

A lactating woman who:

- is in good health, good health-related behaviour, and not regularly on medications or herbal supplements (with the exception of prenatal vitamins, human insulin, thyroid replacement hormones, nasal sprays, asthma inhalers, topical treatments, eye drops, progestin-only or low dose oestrogen birth control products);
- is willing to undergo blood testing for screening of infections; and
- has enough milk after feeding her baby satisfactorily and baby is thriving nicely.

Who cannot donate?

A donor is disqualified who:

- uses illegal drugs, tobacco products or nicotine replacement therapy; or regularly takes more than two ounces of alcohol or its equivalent or three caffeinated drinks per day;
- has a positive blood test result for HIV, HTLV, Hepatitis B or C or syphilis;
- is herself or has a sexual partner suffering from HBV, HIV, HCV and venereal diseases or either one has high risk behaviour for contracting them in last 12 months;
- has received organ or tissue transplant, any blood transfusion/blood product within the prior 12 months.
- is taking radioactive or other drugs or has chemical environmental exposure or over the counter prescriptions or mega doses of vitamins, which are known to be toxic to the neonate and excreted in breastmilk;
- has mastitis or fungal infection of the nipple or areola, active herpes simplex or varicella zoster infections in the mammary or thoracic region **(Bharadva *et al*., 2014)**.

2.1.2.2. Collection of breast milk

After proper counselling, checking suitability for donation, getting written informed consent, history taking, physical examination and sampling for laboratory tests, the donor is sent to designated breastmilk collection area in the milk bank or in the milk collection centre. Breastmilk is collected by trained staff with hygienic precautions, after method of breastmilk expression is chosen by the donor. Home collection of breastmilk is better avoided at present in our country due of the risk of contamination. Washing the breast with water before expression is as good as washing with disinfectant **(Thompson *et al*., 1997)**. The breastmilk may be expressed manually (hand expression) or with breast pumps. Manual expression is a low cost and effective method of expression, and associated with less risk of contamination. Simultaneous breast expression in breastfeeding women is more efficacious than sequential breast expression **(Prime *et al*., 2012)**. Milk should be collected in properly labelled sterile container and transported to HMB under cold storage condition.

2.1.2.3. Storage and transport to milk banks

All batches of collected raw breastmilk should be refrigerated immediately till the serological report comes negative. Fresh raw milk should not be added to the frozen milk since this can result in defreezing with hydrolysis of triglycerides **(Pons *et al*., 1998)**. While mixing

fresh raw breastmilk to frozen raw breastmilk previously collected from same donor, it should be chilled before adding to frozen milk **(Handling, C.P., 2013)**. Temperature control containers are used for transportation of mother's milk from hospital to milk banks.

2.1.2.4. Pasteurization and microbiological screening of donor milk

Donor milks were pasteurized at 62.5 ºC for a period of 30 min using Pretoria Holder pasteurization method. Microbiological screening of donor milk is done before (if there is no cost constraint), and as soon as possible after pasteurization. Pre-pasteurization microbiology can result in wastage of milk to the tune of about 30% in some cases **(Simmer & Hartmann, 2009)**. Even after pasteurization, the endotoxins of organisms are still present in the milk in some cases but they have not been found to have any clinical effect on the baby. A bacterial count of 10^5 CFU/ mL or more in raw breastmilk can be considered as an indicator of the poor quality of milk. Based on this and on the theoretical concern that heavily contaminated milk with specific bacteria (e.g., *S. aureus*, *E. coli*) may contain enterotoxins and thermostable enzymes even after pasteurization, expert panel selected 10^5 CFU/mL for total bacterial count, 10^4 CFU/mL for Enterobacteriaceae and S. aureus as threshold values, which are in consonance with milk banks operating in other parts of the world **(Centre for Clinical Practice at NICE, 2010**; **Hartmann *et al.*, 2007)**. No growth is acceptable in post-pasteurization microbiology cultures. Whole batch of culture positive container of pasteurized milk should be discarded.

2.1.2.5. Storage after pasteurization

Pasteurized milk awaiting culture report should be kept in dedicated freezer/freezer area taking precaution not to disburse it till the culture is negative. Storage should be done in the same container that is used for pasteurization. It is advisable not to transfer processed milk in other containers as it has risk of contamination. Culture negative processed milk should be kept at -20°C in tightly sealed container with clear mention of expiry date and other relevant data on the label. It can be preserved for 3 to 6 months. Random cultures of preserved milk before disbursal can aid quality assurance **(Bharadva *et al.*, 2014)**.

2.1.2.6. Delivered to recipients

Pasteurized donor milk should be disbursed at physician's requisition from NICU physician after informed consent from the parents of the recipient. It should be done on First-in-first-out basis from the storage. Transport of PDHM should be done under cold storage in the same pasteurized container till its use. Frozen milk should be thawed by either defrosting

the milk rapidly in a water bath at a temperature not exceeding 370C, or under running lukewarm water taking care that the cap of the container does not come in contact with the water as it is likely to get contaminated **(Protocol, A.B.M., 2010)**. It should never be thawed in a microwave as this results in reduction in the IgA content of the milk and there is a risk of burns if the milk is used too soon **(Hartmann *et al*., 2007)**. Milk should not be refrozen after being thawed as this increases the hydrolysis of the triglycerides in the milk. While bringing to room temperature, it should be gently agitated to make a homogenous mixture before use and should be used preferably within 3 hours to prevent contamination.

2.2. Nutritional composition of breast milk

Human milk is the complex mixture of 87% water, 7% lactose, 1.0% protein, and 3.8% fat **(Martin *et al*., 2016)**. More than 50% total energy of milk is provided by fat and lactose **(Guo, 2014)**. The first fluid produced by mothers after delivery is colostrum, which is distinct in volume, appearance, and composition. Colostrum is rich in immunologic components such as secretory immunoglobulin, lactoferrin, leukocytes, and developmental factors such as epidermal growth factor. It contains lower amount of lactose, potassium and calcium but contains high level of sodium, chloride and magnesium. This typically occurs from day 5 to 2 weeks. By 4 to 6 weeks postpartum, human milk is considered completely matured **(Ballard *et al*., 2013)**. Human milk composition is dynamic and changes over time according to the needs of the infant's growth. During the initial session it is called foremilk which is thinner with high lactose content which satisfies baby's thirst and following the foremilk, hindmilk, is creamier with a much higher content of fat. Proteins, fats, lactose are considered to be the macro nutrients in human milk.

Table 2.1. Nutritional composition of mother's milk

References	Protein (g/dL) ±2 SD	Fat (g/dL) ±2 SD	Lactose (g/dL) ±2 SD	Energy (Kcal/dL) ±2 SD
Wojcik *et al*., 2009 (for matured milk)	1.2 (0.7, 1.7)	3.2 (1.2, 5.2)	7.8 (6.0, 9.6)	65 (43, 87)
Landers *et al*., 2013 (for pre-term milk)	1.4 (0.8, 1.9)	4.2 (2.4, 5.9)	6.7 (5.5, 7.9)	70 (53, 87)

2.2.1. Protein content

Breast milk contains 2 types of proteins, one is casein and the other is whey protein. The amino acid profiles of casein and whey proteins are different, the overall amino acid profile

of human milk varies depending on the stage of lactation. The ratio of casein and whey in human milk oscillates between 70/30 and 80/20 (whey/casein) in early stage of lactation and it reduces to 50/50 in mature stage **(Guo, 2014)**. Glutamine, the most abundant free amino acid, is nearly 20 times higher in mature milk than in colostrum **(Zhang *et al*., 2013)**. The main whey proteins that are present in human milk are alpha-lactalbumin, lactaferrin and secretory IgA. Other proteins include lysozyme, folate-binding protein, bifidus factor, casein, lipase and amylase, alpha1-antitrypsin and antichymotrypsin, and haptocorrin. These proteins have bioactive functions and non-nutritive functions **(Guo, 2014)**. Casein assists to form masses with calcium and phosphorus. Lactoferrin and lysozyme act against potentially pathogenic bacteria preventing illness in infants. The IgA antibody destroys bacteria and protects the mucosal surface of the gut.

2.2.2. Fat content

Fats constitutes of 4 % of total nutrient content in human milk, providing energy and help in developing the central nervous system. The main lipid fraction are triglycerides, which account for about 95% of total lipids. Nearly 50% of fatty acids in milk are saturated fatty acids, especially with 23% of palmitic acid (C16:0) among overall fatty acids. Oleic acid (18:1w9) is a monounsaturated fatty acid, which is 36% and it is the highest of all. Human milk also contains 2 essential fatty acids linoleic acid (C18:2w6) (15%) and α-linolenic acid (C18:3w3) (0.35%) **(Guo, 2014)**.

2.2.3. Micronutrients

Apart from proteins and fats, human milk contains varied amounts of micronutrients including vitamins A, B1, B2, B6, B12 and iodine and have very low traces or does not contain vitamin D and K, which are given as supplements to infants to prevent from vitamin deficiencies (Martin *et al*., 2016; Ballard *et al*., 2013). Vitamin A stimulates growth and differentiation of cells and maintains the integrity of respiratory epithelium, vitamin E has antioxidant activity, preventing peroxidation of polyunsaturated fatty acids, vitamin C helps in antioxidant activity and vitamin B (thiamine and riboflavin) plays major role in metabolic reactions especially in Krebs' cycle. Minerals also contributes to a variety of physiological functions and helps to form essential parts of enzymes. The major minerals that are present in breast milk are calcium, magnesium, iron, zinc, copper, sodium and potassium. **Dewey *et al*., 1984; Lammi-Keefe *et al*., 1984; Ford *et al*., 1983 and Leaf *et al*., 2004** determined the micronutrient content of mother's milk which is shown in Table 2.

Table 2.2. Micronutrient composition of human milk.

Minerals in fully matured milk	Concentration (µg/ml)
Calcium	236 ± 29
Magnesium	31.7 ± 4.8
Iron	0.19 ± 0.10
Zinc	0.40 ± 0.22
Copper	0.17 ± 0.07
Sodium	96 ± 42
Potassium	391 ± 77
Vitamin A	0.40 ± 0.12
Vitamin E	1.80 ± 0.45
Vitamin C	38 ± 3.2
Riboflavin	0.310 ± 0.15
Thiamine	0.183 ± 0.09
Pantothenic acid	2.61 ± 0.22
Nicotinic acid	1.82 ± 0.18
Vitamin B6	0.107 ± 0.09
Biotin	0.0053 ± 0.001
Vitamin B12	0.00023 ± 0.00
Folic acid	0.0423 ± 0.002

2.2.4. Bioactive components in mother's milk

Bioactive compounds are defined as the element that affect biological processes or substrates and hence have an impact on body function or condition and ultimately health **(Schrezenmeir *et al*., 2000)**. Bioactive components in human milk come from a various source; some are produced and secreted by the mammary epithelium, some are produced by cells carried within the milk, while others are drawn from maternal serum and carried across the mammary epithelium by receptor-mediated transport **(Garofalo, 2010)**. The major bioactive compounds are displayed in **Table 3 (Ballard *et al*., 2013)**.

Table 2.3. Bioactive composition of mother's milk.

Type	Bioactive Components
Cells	Macrophages, stem cells
Bioactive proteins	Immunoglobulins, cytokines, chemokines, mucins, lactoferrin, lactadherin, hormones, growth factors
Non-nutritive carbohydrates	Oligosaccharides, Glycans

2.3. Foodborne pathogens in stored human milk

2.3.1. Major bacterial strains in human milk

Breast milk may contain harmful bacterial pathogens. The major bacterial strains that are present in human milk are *Escherichia coli*, *Bacillus cereus*, *Staphylococcus* aureus, *Salmonella* Typhimurium, *Enterobacter sakazakii* and *Listeria monocytogenes* so to avoid contamination during preservation the human milk is freeze dried and then stored **(Castro-Albarrán *et al*., 2016)**. Sporulated bacteria are a big challenge to eliminate in food products because they are highly resistant and require high radiation and antibacterial doses **(Dauphin *et al*., 2008)**. But when reconstituted to the original form before feeding it to the infants, these bacteria have the potential to multiply in hazardous levels and that leads to diarrhoea, meningitis, brain damage and even death in babies **(Arroyo *et al*., 2017)**. Foodborne illnesses due to immature digestive and immune systems but some clinical cases have been associated with poor manufacturing procedures and sterilisation process carried out by the industries.

2.3.1.1. *Enterobacter sakazakii*

E. sakazakii is a gram-negative, rod-shaped bacterium and a potential pathogen that may cause sepsis, meningitis, bacteraemia and necrotizing enterocolitis, especially in preterm and low birth weight infants, and immunocompromised infants. *E. sakazakii* has the ability to adapt several environmental stresses including chilling, heating, drying and osmotic stresses and natural antibiotics. This characteristic enables the organism to survive in foods and environments to cause foodborne illnesses **(Lee *et al*., 2008)**. Although *E. sakazakii* has been isolated from several food sources including dairy products, meat products, vegetable origin food, water and other foods **(Friedemann, 2007)**, the *E. sakazakii* infection has been linked epidemiologically to the consumption of contaminated powdered infant milk formula. *E. sakazakii* can be inactivated during milk pasteurization; however, post-processing contamination of powdered infant milk formula is mainly responsible for the presence of *E. sakazakii* in these products **(Nazarowec-White *et al*., 1997)**. Furthermore, *E. sakazakii* has the ability to colonize on the surfaces of rehydrated infant milk formula preparation equipment and utensils **(Al-Nabulsi *et al*., 2012)**.

2.3.1.2. *Bacillus cereus*

B. cereus is a gram-positive, round shaped foodborne pathogen which involves in food spoilage and causes foodborne illness especially potential cause of neonatal bacteraemia. It is

a common contaminant of milk and dairy products **(Wong *et al*., 1988)**. Infant foods based on milk and milk products are of especial importance because new-borns have a high degree of susceptibility and, therefore, are at a higher health risk **(Becker *et al*., 1994)**. Generally, dried milk products will be contaminated with *B. cereus* via raw milk that contains the organism frequently in low numbers. It is a spore forming bacteria and they are capable of surviving. There are 2 types of spores slow and fast germinating spores **(Stadhouders, 1982)**. The initial heat treatment step applied in the production of dried milk is very important for the activation and germination of *B. cereus* spores. *B. cereus* is the most frequent bacteria found in human milk following pasteurization, and it is responsible for high rate of bacteriologic rejection in human milk banks **(Lewin *et al*., 2019)**. *B. cereus* strains isolated from milk and milk products showed cytotoxicity after incubation in laboratory media and, therefore, have to be considered potentially enterotoxigenic **(Chopra *et al*., 1980)**.

2.3.1.3. *Listeria monocytogenes*

L. monocytogenes is a gram-positive bacterium and one of the major bacterial pathogens for new-born infants which causes neonatal listeriosis **(Bortolussi, 1999; Doganay, 2003)** and other gastrointestinal infections in immunocompromised infants. The prevalence of *L. monocytogenes* in human milk (21%) lead to neglection of human milk in banks which has been reported by **Togo *et al*, 2020**. This organism is ubiquitous in the environment and can be found in soil, vegetation and animals **(Day *et al*., 2015)**. It is a non-spore forming bacteria. *L. monocytogenes* is widely present in plant, soil, and surface water samples. The virulence of *L. monocytogenes* influenced by six genes on the chromosome in the Prf A-dependent virulence gene cluster and by other important virulence genes located outside the gene cluster **(Tompkin, 2002)**.

2.3.1.4. *Escherichia coli*

E. coli is a gram-negative bacterium that causes meningitis, septicaemia and intestinal sickness. The pathotypes in *E. coli* are characterized by their ability to produce certain virulence factors, toxins and adhesins. They include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAgEC), Shigatoxigenic *E. coli* (STEC) (verotoxigenic *E. coli* (VTEC)), enteroinvasive *E. coli* (EIEC), and diffuse adherent *E. coli* (DAEC). It is present in the vagina of women and that the acquisition of these *E. coli* by babies is related to the length of time that the birth takes **(Bettelheim *et al*.,**

2015). It is highly present in unpasteurized milk, infant milk formula and other food products that do not undergo proper sterilization procedure **(Robichaud *et al*., 2020)**.

2.3.1.5. *Staphylococcus aureus*

It is a gram-positive, round shaped bacterium. Dry infant foods could be potentially contaminated by *S. aureus*. It is an important foodborne pathogen and a major cause of food poisoning cases and outbreaks worldwide. It produces numerous exotoxins, such as enterotoxins (SEs), exfoliative toxins A and B (ETA and ETB), toxic shock syndrome toxin-1 (TSST-1), and Panton–Valentine leukocidin (PVL), that are implicated in the pathogenesis of *S. aureus* infections **(Dinges *et al*., 2000)**. It was shown that about 95% of staphylococcal food poisoning outbreaks were caused by the classical SE (SEA through SEE), and the remaining 5% of outbreaks were associated with other identified SEs **(Kokan *et al*., 1987)**. The TSST-1 toxin is a distantly related protein that causes toxic shock syndrome **(Schlievert *et al*., 1981)**. In 2005, an outbreak associated with *Salmonella*-contaminated powdered infant formula in France affecting more than 141 children **(Angulo *et al*., 2008; Wang *et al*., 2012)**.

2.3.1.6. *Salmonella* Typhimurium

S. Typhimurium is a gram-negative pathogenic bacterium that is mainly responsible for foodborne illness especially gastroenteritis in infants **(Tsen *et al*., 2000; Correa-Matos *et al*., 2003)**. Its toxicity is due to an outer membrane largely of lipopolysaccharides. They are found in dairy products, fish, eggs and other food products. It is also major cause of typhoid fever but the food borne diseases are caused by non typhoidal *Salmonella* **(Gomez *et al*., 1997)**. Breast-fed infants have fewer episodes of acute diarrhoea and intestinal infections than formula-fed infants **(Howie *et al*., 1990)**. Heat treatment is one of the major procedures carried out by the food industries to get rid of *Salmonella* infections **(Robichaud *et al*., 2020)**.

2.4. Natural antimicrobial compounds used in food preservation

2.4.1. Plant derived antimicrobial agents

Antimicrobials are compounds that inhibits the growth of microorganisms. They are mainly derived from plant sources and microbial fermentation, the antimicrobial properties of plants and products of microbial fermentation have been studied for centuries **(Smid *et al*., 1999)**. Natural antimicrobials like phytoalexins, essential oils, organic acids, plant extracts and their phytochemicals are used as food preservatives **(Irkin *et al*., 2015; Smid *et al*., 1999)**. These natural antimicrobials of plant origin help in the defense mechanism of plants against

invading pathogens. Natural antimicrobials are effective in its natural form and also at very low concentration; it should be of low cost, it should not cause any sensory changes in the food product, it should not be toxic to the consumers and it should be wide spectrum **(Davidson *et al*., 2015)**.

2.4.2. Organic acids in food preservation

Organic acids are weak acids, that are obtained from plant and microbial sources. Carboxylic acid is the most common group of organic acids and is characterized by the presence of COOH. Organic acids like citric acid, malic acid and tartaric acid are commonly present in fruits and vegetables. Lactic acid and propionic acid are produced through microbial fermentation as they are not present in plants. They act as antimicrobials, antioxidants, emulsifiers and acidity regulators **(Codex Alimentarius, 1981; Hinton *et al*., 2006; Pundir *et al*., 2011; Liato *et al*., 2016; Paneva *et al*., 2011)**. Organic acids have been used in several researches with the aim of reducing microbial flora in food, in order to ensure healthy products for the consumer, but their use is made in well-defined concentrations. The mechanism of action is that disrupt cell membrane (due to low pH conditions), they cause damage to metabolic enzymes, affects translation process and genetic materials **(Smid *et al*., 1999).** In 1981, Codex Alimentarius released the standard and approved chemical constituents that can be added to food products under certain limitations. Sodium hydroxide, Sodium carbonate, Potassium hydroxide, Potassium carbonate, Calcium hydroxide, Lactic acid, Citric acid, Potassium citrate, Ascorbyl palmitate, Sodium dihydrogen citrate, Trisodium citrate, Sodium dihydrogen phosphate, disodium hydrogen phosphate, Potassium dihydrogen phosphate and Tocopherol.

Table 2.4. Antibacterial activity of compounds approved by Codex Alimentarius.

Organic compounds & Chemical compounds	**Growth inhibitory concentration of bacteria**	**References**
Lactic acid	*E. coli* (0.78 mg/ml) *L. monocytogenes* (0.39 mg/ml) *S. aureus* (0.5% v/v) *C. sakazakii* (0.3% v/v) *S. typhimurium* (0.78 mg/ml) *B. cereus* (0.39 mg/ml)	**Chotigarpa *et al.* 2018;** **Al-Holy *et al.* 2010.**
Citric acid	*E. coli* (1500 µg/ml) *L. monocytogenes* (0.39 mg/ml)	**Adamczak *et al.* 2020**

	S. aureus (900 µg/ml) *S. typhimurium* (0.78 mg/ml) *B. cereus* (0.39 mg/ml)	
Sodium citrate	*S. aureus* (3.2 mg/ml) *E. coli* (250 mg/ml)	**Nagaoka *et al.* 2010; Lee *et al.* 2002**
Potassium hydroxide	*E. coli* (0.1%) *L. monocytogenes* (0.1%) *S. aureus* (0.1%) *S. typhimurium* (0.1%)	**Hinton *et al.* 2006**
Potassium citrate	*E. coli* (0.1%)	**Liato *et al.* 2016**
α-Tocopherol	*E. coli* (≥ 1024 µg/ml) *S. aureus* (≥ 1024 µg/ml)	**Andrade *et al.* 2014**

2.4.3. Essential oils and plant extracts

Essential oils are aromatic and volatile liquids extracted from plant material, such as flowers, roots, bark, leaves, seeds, peel, fruits, wood, and whole plant **(Hyldgaard *et al*., 2012)**. The compounds such as tannins, terpenoids, alkaloids, phenols and flavonoids that are present in essential oils are secondary metabolites, which play a major role in plant defence as they possess antimicrobial properties **(Hyldgaard *et al*., 2012; Aisha *et al*., 2018)**. Natural plant extracts are mostly not toxic to mammalian cells when compared to chemical constituents **(Zhao *et al*., 1998; Karthik *et al*., 2019)**. The interest in essential oils and their application in food preservation has been amplified in recent years by an increasing negative consumer perception of synthetic preservatives and it is highly effective and safe when compared to artificial compounds. Moreover, food-borne diseases are a growing public health problem worldwide, calling for more effective preservation strategies. The antibacterial properties of essential oils and their constituents have been documented extensively and mainly focuses on use of essential oils in human milk as a natural antibacterial agent. The mode of action of natural antimicrobials is generally based on a morphological alteration and disruption of the bacterial wall; or may affect the extracellular ATP and leakage of potassium ions. The constituents and activities of various essential oils has been displayed in **Table 5**.

Table 2.5. Properties of essential oils and plant extracts

Essential oils	Major components	Activities	Mechanism of action	References
Oregano	Carvacrol, Thymol, γ-Terpinene, p-Cymene	Antibacterial, Antifungal, Antioxidant	Dissipated potassium gradient; permeabilized membranes; inhibited cell respiration; affected cell structure: coagulated cytoplasmic material. Increased extra cellular ATP; released cellular content; reduced intra cellular pH; affected membrane integrity.	**Govaris *et al*., 2011**
Thyme	Thymol, Carvacrol, p-Cymene, γ-Terpinene	Antibacterial, Antifungal, Antiviral, Antioxidant	Damaged cell envelope; Permeabilized membrane; caused changes in outer membrane protein profile of Erwinia strains	**Nzeako *et al*., 2006**
Cinnamon	Trans-cinnamaldehyde, Eugenol	Antibacterial, Antifungal	Inhibited histidine decarboxylase; *P. aeruginosa*: depolarized and permeabilized membranes; leakage and coagulation of cytoplasmic content; inhibited respiration activity *S. aureus*: entered a viable but non-cultivable state, and lost membrane integrity	**Zhang *et al*., 2016**
Clove	Eugenol, Acetyl eugenol	Antimicrobial, anticarcinogenic, insecticidal	Inhibits histidine decarboxylase **(***E. aerogenes***)**	**Ayoola *et al*., 2008**
Lemongrass	Myrcene, Geraniol	Antioxidant, Antimicrobial, Anti-cancer	Permeabilizes membrane	**Thorat *et al*., 2017**
Citrus extract	Polyphenols and Flavonoids	Antibacterial, Antifungal, Antioxidant	Intracellular damages to cells; Affects membrane integrity, released cell contents, decreased intracellular ATP and pH.	**Ben-Fadhel *et al*., 2020**

Among these years, various scientific investigations from all parts of the world have been done on essential oils to study the various characteristics apart from using them as flavouring agents for food products **(Zotti *et al*., 2013)**. And these investigations proved that

many essential oils like Oregano, Thyme, Peppermint, Cinnamon, Lemongrass, Clove, Savory, Rosemary, Mustard, Garlic provides various health benefits **(Djilani *et al*., 2012)**.

2.4.3.1. Oregano essential oil

Ground oregano (*Oreganum vulgare*), which is an herb of the Labiatae family have long been used as flavouring agents in various food products, including feta cheese. Oregano EO possess considerable antibacterial properties due primarily to their carvacrol and thymol content **(Burt 2004)**. It also contains camphene, αpinene. The antibacterial activity of the EO of oregano against food-borne pathogens has been examined extensively in many in vitro studies and also in milk products **(Govaris *et al*., 2011)**. It also shows antifungal and antioxidant properties.

2.4.3.2. Thyme essential oil

Garden Thyme (*Thymus vulgaris*) a member of the family Lamiaceae, essential oil from thyme have been pointed out to possess better antibacterial potential and used in food packaging, which could be ascribed to the presence of phenolic compounds such as thymol and carvacrol. The essential oil from thyme also contains β-pinene, camphene, myrcene, α-pinene the most important monoterpenes hydrocarbons **(Imelouane *et al*., 2009)**. In specific, the most abundant compound group of the oil are oxygenated monoterpenes (54.82%). Recent studies have shown that Thymus species have strong antibacterial, antifungal, antiviral, antiparasitic, spasmolytic and antioxidant activities **(Nzeako *et al*., 2006)**.

2.4.3.3. Cinnamon essential oil

Cinnamon (*Cinnamomum verum*), it belongs to Lauraceae family. The essential oil from cinnamon is commonly used in the food industry for its quintessential aroma in addition to its medicinal properties. In recent years, some studies have reported that cinnamon oil had a broad range of antimicrobial activities against gram-positive and gram-negative bacteria **(Cui *et al*., 2016)**. These study results provided a possibility for the application of cinnamon oil in the food preservation, especially they are very useful in persevering dairy products. The main components present in cinnamon oil are eugenol, trans-cinnamaldehyde and eugenyl acetate **(Zhang *et al*., 2016)**.

2.4.3.4. Lemongrass essential oil

Lemongrass (*Cymbopogon citratus*), it comes under family Poaceae. It is a perennial herb and it has been cultivated for its medicinal purposes in different countries. Lemongrass is vastly used as remedy for malaria, coughs, elephantiasis, ophthalmia, consumption, pneumonia and vascular disorders. Researchers have found that lemongrass holds antidepressant, antioxidant, antiseptic, astringent, bactericidal, fungicidal, nervine and sedative properties **(Naik *et al*., 2010)**. And many workers had reported about the antimicrobial activity of lemongrass oil against a diverse range of organisms comprising gram-positive and gram-negative organism, yeast and fungi **(Tiziana *et al*., 1998; Aiemsaard *et al*., 2011)**. Lemongrass found to contain phytochemicals like alkaloids, Saponins, tannins, steroids, phenols, flavonoids which plays the role such as anti-cancer, anti-diabetes, anti-inflammation agent in human body **(Thorat *et al*., 2017)**.

2.4.3.5. Clove essential oil

Clove (*Syzygium aromaticum*), it belongs to Myrtaceae. It is commonly used as a spice and is one of the most important herbs in traditional medicine, having a wide spectrum of biological activity. Phytoconstituents profile of clove comprises vast variety of chemical compounds such as monoterpenes, phenolics, sesquiterpenes and hydrocarbon compounds. The major phytochemicals found in clove oil is mainly eugenol, eugenyl acetate, vanillin and β-caryophyllene. Apart from using clove oil as flavouring agent for foods worldwide, it has also been employed for centuries as a topical analgesic in dentistry. Their derivatives result in biological benefits such as antibacterial, antifungal, insecticidal, antioxidant, anticarcinogenic capacities **(Ayoola *et al*., 2008)**. The antibacterial activity of the EO of clove against food-borne pathogens has been examined extensively in milk products **(Cava *et al*., 2007)**. Sweet basil (*Ocimum basilicum*) belongs to Lamiaceae family and basil extracts are used for flavouring, traditional medicines, dentistry and oral treatment. The major chemical constituents are linalool, methyl chavicol, methyl cinnamate, camphor **(Simon *et al*., 1990)**. They are proven to have antimicrobial properties against food pathogens **(Moghaddam *et al*., 2011; Phanthong *et al*., 2013)**.

2.4.3.6. Other notable essential oils

Cuminum cyminum (Cumin) belongs to Umbelliferae family, it is used as flavouring agent, antifungal and antibacterial agent. *p*-Cymene, γ-Terpinene, β-Pinene, Cuminal are abundant chemical constituents in cumin **(Li *et al*., 2004)**. Coriander (*Coriandrum sativum*)

comes under Umbellifers family, it mainly consists of linalool, α-Pinene, camphor, geraniol **(Gil *et al*., 2002)**. Cumin is widely known for its antibacterial properties **(Bag *et al*., 2015)**. Garlic (*Allium sativum*) belongs to Amaryllidaceae family, pharmacological studies confirms that it possesses antioxidant, antimicrobial, anti-inflammatory, antimutagenic and other medicinal properties. It is composed of eugenol, elemicin, α-guaiene, α-caryophyllene **(Dziri *et al*., 2014)**.

Mustard (*Sinapis alba*) is part of Cruciferae family. It is highly rich in Allyl isothiocyanate which is mainly responsible for its antimicrobial property **(Kirk *et al*., 1964; Bag *et al*., 2015)**. Rosemary (*Rosmarinus officinalis*) belongs to Lamiaceae family, it is a woody, perennial herb and it is known for aromatic and food flavouring properties. Major chemical constituents that are present in mustard are carvacrol, thymol, linalool, γ-Terpinene, α-Pinene which is responsible for its antibacterial activity **(Napoli *et al*., 2010; Chraibi *et al*., 2020; Esmael *et al*., 2020)**. *Satureja montana* (winter savory) is a perennial shrub which comes under Labiatae family and the major chemical constituents that responsible for its antimicrobial properties are carvacrol, *p*-Cymene, linalool, γ-Terpinene **(Piccaglia *et al.*, 1991)**. And apart from this, some essential oil and its phytochemical constituents are also tested for antibacterial activity in milk samples.

In a research study, Cinnamon oil showed high bactericidal effect against *Cronobacter sakazakii* in reconstituted infant milk formula **(Al-Nabulsi *et al.*, 2015)**. Since there are no standards available for the use of essential oils in mother's milk, this research review tries to promote the use of highly effective essential oils in milk preservation. The essential oils having high antibacterial activity against pathogens that contaminating mother's milk is displayed in the **Table 2.6**.

Table 2.6. **Antibacterial activity of essential oils**

Essential oils	**Bacterial strains (MIC)**	**References**
Cinnamon	*E. coli* (0.4 mg/ml) *L. monocytogenes* (0.51 mg/ml) *S. aureus* (0.1 mg/ml) *C. sakazakii* (0.5 mg/ml) *S. typhimurium* (0.8 mg/ml) *B. cereus* (0.1 mg/ml)	**Frankova *et al.* 2014; Liu *et al.* 2015; Fei *et al.* 2011**

Thyme	*E. coli* (0.8 mg/ml) *L. monocytogenes* (0.8 mg/ml) *S. aureus* (0.4 mg/ml) *S. typhimurium* (0.25 mg/ml) *B. cereus* (0.2 mg/ml)	**Liu *et al.* 2015; Fei *et al.* 2011**
Clove	*E. coli* (0.8 mg/ml) *L. monocytogenes* (0.5 mg/ml) *S. aureus* (0.8 mg/ml) *C. sakazakii* (0.5 mg/ml) *S. typhimurium* (0.8 mg/ml) *B. cereus* (0.4 mg/ml)	**Fei *et al.* 2011; Cui *et al.* 2018**
Lemongrass	*E. coli* (0.6 mg/ml) *L. monocytogenes* (0.4 mg/ml) *S. aureus* (0.3 mg/ml) *C. sakazakii* (0.5 mg/ml) *S. typhimurium* (0.5 mg/ml) *B. cereus* (0.3 mg/ml)	**Frankova *et al.* 2014; Naik *et al.* 2010; Shin 2005; Oussalah *et al.* 2007**
Oregano	*E. coli* (0.25 mg/ml) *L. monocytogenes* (0.25 mg/ml) *S. aureus* (0.25 mg/ml) *C. sakazakii* (0.2 mg/ml) *S. typhimurium* (0.05 mg/ml) *B. cereus* (0.032 mg/ml)	**Frankova *et al.* 2014; Ozkalp *et al.* 2010; Oussalah *et al.* 2007**
Basil	*E. coli* (0.018 mg/ml) *S. aureus* (0.018 mg/ml) *S. typhimurium* (0.05 mg/ml) *B. cereus* (0.036 mg/ml)	**Moghaddam *et al.* 2011; Phanthong *et al.* 2013**
Coriander	*E. coli* (0.14 mg/ml) *L. monocytogenes* (0.20 mg/ml) *S. aureus* (0.10 mg/ml) *S. typhimurium* (0.19 mg/ml) *B. cereus* (0.05 mg/ml)	**Bag *et al.* 2015**
Cumin	*E. coli* (0.30 mg/ml) *L. monocytogenes* (0.31 mg/ml) *S. aureus* (0.13 mg/ml) *S. typhimurium* (0.38 mg/ml) *B. cereus* (0.11 mg/ml)	**Bag *et al.* 2015**
Garlic	*E. coli* (0.68 mg/ml) *L. monocytogenes* (0.02 mg/ml)	**Ross *et al.* 2001; Kim *et al.* 2004; Shim *et al.* 1999**

	S. aureus (0.1 mg/ml) *S. typhimurium* (0.34 mg/ml) *B. cereus* (0.08 mg/ml)	
Mustard	*E. coli* (0.4 mg/ml) *L. monocytogenes* (0.33 mg/ml) *S. aureus* (0.10 mg/ml) *S. typhimurium* (0.45 mg/ml) *B. cereus* (0.15 mg/ml)	**Bag *et al.* 2015**
Rosemary	*E. coli* (0.25 %v/v) *L. monocytogenes* (0.5%) *S. aureus* (0.156 mg/ml) *C. sakazakii* (0.4 mg/ml) *S. typhimurium* (1250 ppm) *B. cereus* (0.07 mg/ml)	**Chraibi *et al.* 2020; Esmael *et al.* 2020; Fu *et al.* 2007; Campo *et al.* 2000; Raeisi *et al.* 2017**
Savory	*E. coli* (0.78 mg/ml) *L. monocytogenes* (0.39 mg/ml) *S. aureus* (0.78 mg/ml) *S. typhimurium* (0.78 mg/ml) *B. cereus* (0.39 mg/ml)	**Miladi *et al.* 2013**

2.4.4. Combined effect of antimicrobial agents

The mixture of several antimicrobial treatments allows a more effective reduction of spoilage microorganisms while reducing the impact on the sensory quality of food. Ouattara *et al*. **(2002)** found that the combination of irradiation (dose up to 3 kGy) and the spraying of an antimicrobial formulation based on soy protein and essential oil reduced the development of *Pseudomonas* and the count of aerobic bacteria showing the combined effect of the irradiation and the antimicrobial formulation without affecting the sensory quality of the sensory quality of the tested foods. In other studies, **Lin *et al* (2004; 2005)** reported that an antimicrobial effect was observed after the addition of lactic acid (at pH 6) to a mixture of oregano essential oil and cranberry extract. In addition, the study conducted by **Naveena *et al* (2006)** showed that the mixture of clove oil and lactic acid significantly increased the shelf life of buffalo meat at 4°C. **Tawema *et al* (2014)** also reported that two natural formulations F2 and F6 based on a mixture of organic acid (lactic acid) and essential oil (oregano and citrus extract) exhibited high antibacterial activity. Indeed, these two formulations reduced *E. coli* O157:H7, *S.* Typhimurium, total bacterial count and yeasts and molds below the detection limit after an

initial inoculation of 4.5 log. It is important to note that these formulations did not affect the organoleptic quality of cauliflower at a spray volume of 5 mL per 100 g of vegetables.

2.5. Ionizing radiations

2.5.1. γ-Irradiation

The cold pasteurization techniques such as gamma irradiation made it possible to bring new possibilities for the decontamination of the fresh products in particular the ready-to-eat foods. Approved since 1981 by the United Nations as a conservation treatment, irradiation is experiencing a considerable growth. It is a promising non-thermal approach that is used to provide stability and safety. Radiation for the treatment of food is done by applying gamma rays (with Cobalt-60 or Cesium-137 radioisotope), electron beams (high energy up to 10 MeV), X rays (high energy up to 5 MeV) and UV rays (at least 400 J/m^2) **(Morehouse *et al.* 2004; Lopez-Malo *et al.* 2005)**. Various research experiments proved that ɣ-irradiation treatment was highly effective in eliminating food pathogens. The radiation sensitivity of microorganisms depends on a variety of intrinsic and extrinsic factors such as the temperature during irradiation, stage of growth, oxygen availability, water activity, and the medium chemical composition **(Lacroix 2012)**.

The principle of irradiation is when the gamma, electron beams or X-rays comes in contact with the matter, it makes the atom reach an excited state and releasing ions by freeing electron from their atomic bond, a process called ionization **(Morehouse *et al.* 2004)**. This ionization causes the disruption of DNA in microbes which leads to genetic instability and inhibits microbial growth **(Selzer *et al.* 2012)** and it also causes indirect damage by water radiolysis. This radiation can be emitted by a radioactive substance or can be generated electrically.

The approval of this technology by the Food and Drug Administration for the treatment of food has significantly increased the interest of industry. Bacterial radiosensitization through combined treatments would allow the development of reliable technologies, provide tools for decision-making in the control of foodborne infectious diseases, and significantly reduce national health expenditures and increase the well-being of Canadians. This new alternative has the advantage of preserving the food under mild conditions that do not affect the sensory and nutritional qualities of the food. The advantage of irradiation is that it can be used on fresh food. However, studies have shown that, as with heating, some pathogens can be reduced to an

undetectable level after treatment and reappear during storage **(Stadhouders, 1982; Lewin *et al.*, 2019)**.

2.5.2. Mechanisms of Radiation - Bacterial inactivation

As mentioned earlier, when the atoms and molecules are exposed to radiation, they absorb energy and this causes release of electrons from the atom. The electron which is released is highly energized and it removes electron from other atoms and convert them to ions. This process of energization and ionization affects the general characteristics of biological system (**Morehouse *et al.* 2004)**. Radiation effects on biological substances are classified as direct and indirect. The direct damage is caused by the removal of electrons as a result of energy deposition by radiation on target molecules, such as DNA **(Selzer *et al.* 2012)**. The indirect damage happens when the reactive diffusible free radical formed due to the radiolysis of water, such as hydroxyl radical, hydrated electron, H atom, hydrogen peroxide, and hydrogen radical. H radical is a strong reducing agent, while H_2O_2 and OH^- are strong oxidizing agents. The H^+ and OH^- radicals are highly reactive, and cause oxidation and reduction on materials as well as the breakdown of carbon–carbon bonds, bonds of other molecules, and single and double strands in DNA at the sugar–phosphate bonds. In addition, the radicals can change the bases, such as thymine to dihydroxydihydrothymine (**Erkem *et al.* 2016)**.

The principal targets of irradiation are nucleic acids and cell membrane lipids. Ionizing radiation disrupts the cell membrane and other structures (sublethal injury). Alteration in membrane lipids, particularly polyunsaturated lipids, leads to disturbance in membranes and effects on various membrane functions like a state of perturbation, and causes permeability. The activity of enzymes in membranes can also be affected. Bacterial chromosomes are very sensitive to free radicals which leads to lethal damage. Ionization radiations change the DNA structure by disrupting certain bonds, which prevents replication of DNA and certain functions. The ability of bacterial strains to repair cell damage provides resistance to radiations. This ability of bacteria varies considerably. Irradiation can cause mutations in some microbial cells leading to either a possible increase in pathogenicity by producing toxins or loss of some metabolic abilities. The death rate of microorganisms by irradiation follows a similar straight line as the thermal destruction curve **(Selzer *et al.* 2012; Erkem *et al.* 2016)**.

2.5.3. Radiosensitivity of food pathogens

The irradiation effect on bacterial strains depends on various environmental factors such as media used, water content, temperature during treatment, stage of growth, oxygen availability and bacteria's form whether it is spore forming or vegetative form **(Sun, 2011)**. The radio sensitivity also differs within the same bacterial families. It is reported that 18 serotypes of *Salmonella* had narrow range of D10 values from 0.52 to 0.77 kGy **(Comer *et al.* 1963)**. In some cases, the radiation resistance is unusual due to the presence of exceptional DNA excision and DNA recombination. For example, the D10 value is reported as 10 kGy for *Micrococcus radiodurans* because of DNA repair mechanism **(Peter *et al.* 2013)**. And apart from this spore forming bacteria also needs higher dosage of radiation than vegetative cells as spores can survive at extreme conditions like heat, chemicals and other environmental stress. For example, it requires 8 kGy for complete elimination of *Bacillus cereus* **(Hong *et al.* 2008)**. Another study reported that the D10 value of *B. cereus* (vegetative cells) is 0.17 kGy and *B. cereus* (spores) is 1.6 kGy **(Farkas 2007)**. The D10 values against various bacterial strains are displayed in Table 5 **(Farkas 2007; Osaili *et al.* 2007; Robichaud *et al.* 2020)**.

Table 2.7. D_{10} values of food pathogens

Bacterial species in fresh samples	**D10 value (kGy)**
Escherichia coli	0.23 -0.35
Bacillus cereus	0.17 – 5.72
Listeria monocytogenes	0.27 – 1.0
Salmonella Typhimurium	0.3 – 0.8
Staphylococcus aureus	0.26 – 0.6
Cronobacter sakazakii	0.21 – 1.71

2.6. Problematic, Aim, Hypothesis, Objective & Methodology

2.6.1. Problematic

Mother's milk is a major nutrient source to helps build the immune system and also provides non-nutritive bio-active factors that promote survival and healthy development in infants. Even if it is a naturally available for infants, the role of milk banks is highly significant because donor milks are best alternative source of nutrient for infants in case of absence of mother's milk. But the milk bank faces difficulties in human milk preservation, as there is a high risk of bacterial contamination. Heat pasteurization treatment, which is currently followed by the milk banks for preservation may cause Maillard reactions which cause nutritional loss

as milk contains large amount of protein and sugars, which at high temperature make the reduced sugar and free amino acids reacts to produce melanoidins which affect the milk quality, another issue faced, is spore forming bacteria especially *B. cereus* and bacteria like *S. aureus* that produce enterotoxins which are toxic proteins causing intestinal infection, cannot be eliminated as they are heat resistant. The major bacterial species responsible for human milk spoilage are *L. monocytogenes, E. coli, B. cereus, S.* Typhimurium, *S. aureus* and *E. sakazakii.* The contaminations caused due to these bacteria strains leads to serious neonatal diseases like necrotizing enterocolitis (NEC) and sepsis.

2.6.2. Aim

The aim of this study is to demonstrate the effect of γ-irradiation (cold pasteurization) treatment in combination with natural antibacterial formulations for microbial inactivation, nutritional and bioactive properties, *in vitro* protein digestibility, peptide profile and physicochemical properties of human breast milk in frozen condition.

2.6.3. Hypothesis

- Essential oils, citrus extract and organic acids will show high antibacterial activity against tested bacterial strains.
- Quaternary formulation developed from essential oils, citrus extract and organic acids will show synergistic effect.
- γ-Irradiation treatment in combination with antibacterial formulation can act in synergy to increase the bacterial radiosensitivity and permit to eliminate all pathogens including sporulated bacteria at lower irradiation dose in frozen mother's milk.
- Combined treatment of γ-irradiation and antibacterial formulation will not modify the nutritional, *in vitro* protein digestibility, peptide profile and physicochemical qualities of mother's milk in frozen condition.

2.6.4. Objectives

Objective 1: To determine the minimal inhibitory concentration (MIC) of selected essential oils, citrus extract and organic acids.

Objective 2: To determine the interaction between different antibacterial compounds using fractional inhibitory concentration (FIC) index.

Objective 3: To develop quaternary antibacterial formulations based on FIC index.

Objective 4: To evaluate the D10 and radiosensitivity of the combined treatment of γ-irradiation and antibacterial formulation in frozen mother's milk.

Objective 5: To evaluate the effectiveness of the combined treatments (natural antimicrobial formulation with γ-irradiation at 5 kGy) on pathogen elimination and to evaluate the effect of the combined treatment on the nutritional, immunologic proteins, protein digestibility, peptide profile and physicochemical qualities in frozen mother's milk under combined treatment of irradiation at 5 kGy and antibacterial formulation.

2.6.5. Methodology

Objective 1: The minimum inhibitory concentrations (MICs) of the selected antibacterial compounds will be determined using 96-well microplate method described by Turgis *et al.* **(2012)** and Hossain *et al.* **(2016)**. The optical density optical density will be read at a wavelength of 595 nm using spectrophotometer to evaluate MIC.

Objective 2: The Fractional inhibitory concentration (FIC) index of the various antibacterial compound's combinations against target pathogens will be determined by checkerboard assay using 96-well microplates as per the procedure explained by Gutierrez *et al.* **(2008)**. Based on the FIC values, the interaction between the compounds is classified as a) FIC < 0.5 was indicated as synergistic effect (S), b) 0.5 < FIC ≤ 1 was interpreted as additive effect (AD), c) 1 < FIC ≤ 4 was interpreted as no interactive effect (NI), d) FIC > 4 was represented as antagonistic effect (A) **(Turgis *et al.*, 2012; Ayari *et al.*, 2020)**.

Objective 3: The development of the quaternary antibacterial formulations will be achieved by selecting the most effective antimicrobial compounds against the bacteria studied, their synergistic effect and also the most compatible with mother's milk **(Ayari *et al.*, 2020)**.

Objective 4: The D10 and radiosensitivity of selected pathogens under the combined treatment of γ-irradiation and antibacterial formulation will be done as per the protocol by Robichaud *et al.* **(2021)**. Irradiation of mother's milk will be done in a gamma irradiator using a Cobalt 60 source (Nordion Inc., Kanata, ON, Canada) at a dose of 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5 kGy and a flow rate of 6.318 kGy/h at the Canadian Radiation Centre.

Objective 5: The effect of combined treatment on lactose, immunological composition, molecular weight distribution peptides will be determined using Agilent 1260 series HPLC

system as per the protocol explained by Robichaud *et al.* **(2020)**. The lipid oxidation potential of mother's milk under combined treatment Thiobarbituric acid reactive substances (TBARS) assay will be used based on the protocol described by Criado *et al.* **(2020)**. The *in vitro* protein digestibility (IVPD) under combined treatment of irradiation in mother's milk will be determined by sequential digestion using pepsin and trypsin as per the method mentioned by Wang *et al.* **(2008)**. The percentage of nitrogen release during the digestion process will be calculated from using the Kjeldahl method as per the protocol explained by Manus *et al.* **(2021)**. The measurement of the color parameters of mother's milk subjected to combined treatment will be done by measuring the parameters L* (lightness), a* (green to red), b* (blue to yellow) and ΔE (color difference) with a colorimeter CR10-Plus **(Ben-Fadhel *et al.*, 2021)**. The viscosity of mother's milk subjected to combined treatment will be measured using DV-II+ viscometer **(Okyere & Odamtten, 2014)**.

Chapter 3

Combined effect of γ-irradiation and antibacterial formulations based on natural compounds for preservation of mother's milk.

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Contribution of the authors

Prof Monique Lacroix is responsible for the project. She coordinated of the research activities, she has done the corrections of the paper, participated to the scientific discussion. I have performed the research, statistical analysis and written the article, Dr. Zahra Allahdad helped me with figure illustrations and article correction.

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Combined effect of γ-irradiation and antibacterial formulations based on natural compounds for preservation of mother's milk

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Abstract:	The antibacterial potential of citrus extract, EOs (mediterranean formulation, oregano, lemongrass, cinnamon and clove), organic acids and salts (citric acid, lactic acid and sodium carbonate) was evaluated against E. sakazakii , E. coli O157:H7, S. aureus , L. monocytogenes , S. Typhimurium , B. cereus by determining the minimal inhibitory concentration (MIC). Based on the results, MIC value ranges from 312.5 to 5000 ppm against tested bacterial strains, with citrus extract showing the highest antibacterial activity (312.5 ppm). Four antimicrobial formulations based on quaternary combination of compounds were developed based on the fractional inhibitory concentration (FIC) index. Formulation 1 (Oregano/Mediterranean formulation/Citrus extract/Lactic acid), Formulation 2 (Cinnamon/Lemongrass/Citrus extract/Citric acid), Formulation 3 (Oregano/Mediterranean formulation/Citrus extract/Citric acid), Formulation 4 (Cinnamon/Lemongrass/Citrus extract/Lactic acid) showed a synergistic effect against all tested pathogens. Results demonstrated that bacterial radiosensitivity (1.34 - 3.99) was increased by the combined treatment of γ-irradiation with developed formulations in frozen mother's milk. Formulation 3 and 4 induced greater radiosensitivity in bacterial strains including sporulated B. cereus (1.90 and 1.89 respectively) compared to formulation 1 and 2. Therefore, the combined treatment can be an effective method to reduce the γ-irradiation dose (kGy) required to eliminate the pathogens and assures the safety of frozen mother's milk.
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Résumé

Le potentiel antibactérien d'un extrait d'agrumes, des huiles essentielles (HE) (formulation méditerranéenne, origan, citronnelle, cannelle et clou de girofle), des acides organiques et des sels (acide citrique, acide lactique et carbonate de sodium) a été évalué contre *E. sakazakii*, *E. coli* O157:H7, *S. aureus*, *L. monocytogenes*, *S.* Typhimurium, *B. cereus* en déterminant la concentration minimale inhibitrice (CMI). D'après les résultats, la valeur de la CMI varie de 312,5 à 5000 ppm contre les souches bactériennes testées, l'extrait d'agrumes présentant l'activité antibactérienne la plus élevée (312,5 ppm). Quatre formulations antimicrobiennes basées sur une combinaison quaternaire de composés ont été développées sur la base de l'indice de concentration inhibitrice fractionnelle (CIF). La formulation 1 (formulation origan/méditerranée/extrait d'agrumes/acide lactique), la formulation 2 (cannelle/citronnelle/extrait d'agrumes/acide citrique), la formulation 3 (formulation origan/méditerranée/extrait d'agrumes/acide citrique), la formulation 4 (cannelle/ citronnelle /extrait d'agrumes/acide lactique) ont montré un effet synergique contre tous les pathogènes testés. Les résultats ont démontré que la radiosensibilité bactérienne (1,34 - 3,99) était augmentée par le traitement combiné de l'irradiation γ avec les formulations développées dans le lait maternel congelé. Les formulations 3 et 4 ont induit une plus grande radiosensibilité dans les souches bactériennes, y compris *B. cereus* sporulé (1,90 et 1,89 respectivement) par rapport aux formulations 1 et 2. Par conséquent, le traitement combiné peut être une méthode efficace pour réduire la dose d'irradiation γ (kGy) nécessaire pour éliminer les agents pathogènes et assurer la sécurité du lait maternel congelé.

Mots-clés : Lait maternel; Pathogènes d'origine alimentaire; γ-Irradiation; Formulation antimicrobienne; Synergie; Radiosensibilité.

Abstract:

The antibacterial potential of citrus extract, essential oils (EOs) (mediterranean formulation, oregano, lemongrass, cinnamon and clove), organic acids and salts (citric acid, lactic acid and sodium carbonate) was evaluated against *E. sakazakii*, *E. coli* O157:H7, *S. aureus*, *L. monocytogenes*, *S.* Typhimurium, *B. cereus* by determining the minimal inhibitory concentration (MIC). Based on the results, MIC value ranges from 312.5 to 5000 ppm against tested bacterial strains, with citrus extract showing the highest antibacterial activity (312.5 ppm). Four antimicrobial formulations based on quaternary combination of compounds were developed based on the fractional inhibitory concentration (FIC) index. Formulation 1 (Oregano/Mediterranean formulation/Citrus extract/Lactic acid), Formulation 2 (Cinnamon/Lemongrass/Citrus extract/Citric acid), Formulation 3 (Oregano/Mediterranean formulation/Citrus extract/Citric acid), Formulation 4 (Cinnamon/Lemongrass/Citrus extract/Lactic acid) showed a synergistic effect against all tested pathogens. Results demonstrated that bacterial radiosensitivity (1.34 - 3.99) was increased by the combined treatment of γ-irradiation with developed formulations in frozen mother's milk. Formulation 3 and 4 induced greater radiosensitivity in bacterial strains including sporulated *B. cereus* (1.90 and 1.89 respectively) compared to formulation 1 and 2. Therefore, the combined treatment can be an effective method to reduce the γ-irradiation dose (kGy) required to eliminate the pathogens and ensure the safety of frozen mother's milk.

Keywords: Mother's milk; Foodborne pathogens; γ-Irradiation; Antimicrobial formulation; Synergy; Radiosensitivity.

1. Introduction

The major and best source of nutrients for new-borns and infants is breast milk. It helps in their growth and also to develop their immune system by providing bioactive compounds elevating the healthy development and survival of infants **(Ballard & Morrow, 2013)**. Breast milk is a complex mixture which consists of carbohydrates (lactose 7%), protein (0.6 to 1.6 %), fat (3 %) and water (87 %) **(Martin *et al*., 2016)**. 50% of total energy is provided by lactose and fat content of mother's milk **(Guo, 2014)**. It also contains micronutrients and other compounds responsible for the growth of infants like vitamins, minerals, digestive enzymes and hormones **(Ballard & Morrow, 2013; Dewey *et al*., 1984)**. For the nourishment of immune system, it provides bioactive compounds like macrophages, stem cells, immunoglobulins (IgA, IgG, IgM), cytokines, antimicrobials (Lactoferrin, Lactadherin), oligosaccharides and various other compounds **(Ballard & Morrow, 2013; Adamkin, 2012)**.

Human milk banks are absolute necessity to preserve and provide milk for the infants whenever needed, so that all infants will be benefited to maintain the proper level of macro and micro nutrients. Mother's milk from various donors is collected, pasteurized (62.5 ˚C for 30 min) and stored in human milk banks in order to provide milk for preterm infants and in the situation when a mother is unable to provide milk for her new-borns **(Haiden & Ziegler, 2016)**. But the main obstacle faced by the donor milk banks in the preservation are milk spoilage due to bacterial contamination. The contaminated milk fed to new-born leads to serious heath complications and affects the infant with severe diseases like Necrotizing enterocolitis (NEC) and Sepsis **(Arnold, 2006)**. Previous research studies revealed that bacterial strains like *Enterobacter sakazakii*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *Salmonella* Typhimurium are the most important pathogens responsible for breast milk rejection in milk banks **(Al-Nabulsi *et al*.,2008; Robichaud *et al*., 2021)**.

The thermal pasteurization technique applied by human milk bank does not completely eliminate the bacterial species **(Arnold, 2006; Keim *et al*., 2013)**. The spore forming bacteria like *B. cereus* and the endotoxins that are produced by *S. aureus* are highly resistant to heat (**Keim *et al*., 2013; Forsythe, 2005)**. This prompted the researchers to find an alternative and effective cold pasteurization techniques for the preservation of food products. Food industries are moving towards cold pasteurization techniques which are interesting as they are non-thermal and more effective in inactivation of pathogens, extending the shelf-life of products while maintaining their nutrient matrix **(Lacroix, 2010)**. As per the regulations approved by

Food and Drug Administration (FDA) up to 45 kGy of irradiation dosage is allowed to be used for sterilization of frozen food samples **(Maherani *et al*., 2016)**.

In recent years natural preservative agents are becoming the need of food industries. It has been proven that plant extracts and essential oils (EOs) have greater antimicrobial and antioxidant properties among various plant extracts and are used as a safe food preservative in food products. Numerous scientific studies have demonstrated significant bactericidal capacities of citrus extract, oregano, mediterranean formulation, cinnamon, clove, lemongrass, peppermint, basil **(Oussalah *et al*., 2006; Burt, 2004; Turgis *et al*., 2012; Maherani *et al*., 2019)**. It should be noted that the concentration of the EOs applied is limited because of their taste and odor. To reduce the concentration the use of compounds acting in synergy is necessary **(Maherani *et al*., 2019, Codex alimentarius, 1981)**.

Codex Alimentarius has permitted the use some of the food additives like lactic acid, citric acid, sodium carbonate, sodium citrate, potassium carbonate and malic acid **(Codex alimentarius, 1981)** in infant formula. They are accepted to be used as antimicrobials, additives, acid regulators and stabilizers as per approved concentrations **(Robichaud *et al*., 2021; Codex alimentarius, 1981)**. The synergistic effect of EOs, organic acids and salts have also been reported in various studies with more bactericidal effect at low concentration was emerged when compared to antimicrobial effect of individual compound **(Codex alimentarius, 1981; Ghabraie *et al*., 2016; Ayari *et al*., 2020)**. However, only few studies were done until now on the use of antimicrobial formulation based on natural extracts in combination with γ-irradiation demonstrating the improved inhibition of food pathogens through radiosensitivity increment while reducing the irradiation dosage required to eliminate pathogens and reduce the total normal flora in food products **(Robichaud *et al*., 2021; Lacroix, 2010; Shankar *et al*; 2019)**.

Previous studies have also shown that frozen samples require higher irradiation dosage (kGy) to eliminate bacteria compared to normal samples **(Robichaud *et al*., 2021; Cugia *et al*., 2011)**. Therefore, the major objective of this research was to develop antimicrobial formulations using EOs, citrus extract, organic acids and determine the combined effect of antimicrobial formulations along with γ irradiation in order to enhance the radiosensitivity of the bacterial strains (*E. sakazakii*, *E. coli O157:H7*, *S. aureus*, *L. monocytogenes*, *S.* Typhimurium, *B. cereus*) by reducing the γ irradiation dosage in frozen mother's milk since

frozen samples require higher irradiation dosage (kGy) to eliminate bacteria compared to normal samples.

2. Materials and Methods

2.1. Materials

Mother's milk was provided by the HEMA Quebec in frozen condition, it was then freeze-dried under sterile conditions and stored in 4 ˚C for further use. The essential oils were purchased from Zayat Aroma (Bromont, QC, Canada), mediterranean formulation were bought from BSA (Montreal, QC, Canada), citrus extract from Kerry int. (Woodstock, ON, Canada), organic acids and all other chemicals were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). The culture media for bacterial growth like Tryptic soy broth (TSB), Mueller Hinton Broth (MHB), Tryptic soy agar (TSA), PALCAM agar, B. cereus agar, Mannitol agar, MacConkey agar, DCLS agar, S. aureus agar and peptone water were purchased from Alpha Biosciences Inc. (Baltimore, MD, USA) and Thermo Fisher Scientific (Nepean, ON, Canada).

2.2. Preparation of bacterial stock, samples, natural antimicrobial compounds and inoculation procedures

E. sakazakii (ATCC 29004), *E. coli* O157:H7 (ATCC 43895), *B. cereus* (ATCC 14579), *L. monocytogenes* (HPB 2812 serovar 1/2a), *S. aureus* (ATCC 29213) and *S.* Typhimurium (ATCC SL1344) were cultured using TSB and for prolonged storage were maintained at -80 ˚C in TSB-glycerol mixture (10% v/v). One millilitre of culture was incubated through two successive incubations of 24 h at 37 ˚C in TSB to obtain approximately 10^8 CFU/mL. The cultures were centrifuged at 1300 rpm for 15 min, washed and sterilized with 0.85% (w/v) of sterile saline solution to obtain fresh working cultures.

For experiments, the freeze-dried mother's milk was reconstituted to liquid form by adding sterile water under sterilized conditions until its original form was reached (approximately 71.3 g of water was added to 5 g of freeze-dried milk). Then reconstituted milk was stored in -20 ˚C to carry out the experiments in frozen condition. The natural antimicrobial compounds: EOs (mediterranean formulation, oregano, lemongrass, clove and cinnamon) and citrus extract were stored at 4 ˚C prior to use. Essential oil emulsion was prepared by mixing each EOs (2.5% v/v) with Tween 80 (Laboratoire Mat, QC, Canada) at the ratio of 1:1. These mixtures were homogenized for 5 min with an Ultra-Turrax homogenizer (model TP18/1059,

Germany) at 15000 rpm to obtain the final colloidal suspension **(Ayari *et al*., 2020)**. The stock solution (2.5% v/v) of organic acids and bases (citric acid, lactic acid and sodium carbonate) were prepared by diluting from its initial concentration. All the antimicrobial solutions were filtered with 0.2 µm sterile filter and then used for the experiments.

2.3. Determination of minimum inhibitory concentration (MIC) of selected compounds using two-fold broth microdilution assay

The MIC value of each compound (EOs, organic acids and bases) was determined by serial microdilution assay using sterilized 96-well microplates. Briefly, two-fold serial dilution suspension from 10 ppm up to 10000 ppm in 96-well microplate of each compound was done using MHB. Each well contains 100 µL aliquot of the suspension. Then, each well was inoculated with 100 µL of 10^6 CFU/mL of bacterial culture and incubated at 37 °C for 24 h. For negative control 2 rows of microplate were used and 100 µL of saline solution was added instead of bacterial culture. The positive control was one column of microplate (without antimicrobial agent) consisted of 100 µL of bacterial culture and 100 µL of MHB. It should be noted that the sterile Tween 80 (2.5% v/v) used for emulsion did not show any antimicrobial activity. And the experiment was done in triplicates. Finally, the bacterial growth was evaluated by Ultra Microplate Reader (Biotek Instruments, Winooski, VT, USA). The optical density was measured at 595 nm. The MIC is the minimum concentration required to completely eliminate the bacterial strain and showing absorbance as blank **(Turgis *et al*., 2012; Ayari *et al*., 2020)**.

2.4. Determination of interaction between antimicrobial compounds using Checkerboard assay

The Fractional inhibitory concentration (FIC) index of the various antibacterial compound's combinations against target pathogens was calculated by checkerboard assay using 96-well microplates as per the procedure explained by Gutierrez *et al* **(2008)** and the experiment was carried out in triplicates.

The interaction between two compounds was determined according to the following formulae

$FIC_a = MIC_a$ combined/MIC_a alone

$FIC_b = MIC_b$ combined/MIC_b alone

$FIC = FIC_a + FIC_b$

Here, FIC_a and FIC_b represented the FIC values of antibacterial compound a and antibacterial compound b. It is the ratio of the minimum concentration of compound 'a' and mixture of compound 'a' and 'b' respectively that was responsible for inhibition of bacterial growth when used in combined treatment. The sum of both indicated the FIC index.

FIC is the concentration of an antibacterial compound responsible for the inhibition of bacterial growth in combination with another compound divided by the concentration required to inhibit bacterial growth when used alone. Based on the FIC values, the interaction between the compounds is classified as a) FIC < 0.5 was indicated as synergistic effect (S), b) 0.5 < FIC ≤ 1 was interpreted as additive effect (AD), c) 1 < FIC ≤ 4 was interpreted as no interactive effect (NI), d) FIC > 4 was represented as antagonistic effect (A) **(Turgis *et al*., 2012; Ayari *et al*., 2020)**.

2.5. Development of novel natural antibacterial formulations using EOs, organic acids and citrus extract

The best combination of antimicrobial compounds was selected based on the synergistic effect and 4 new natural antimicrobial formulations were made. These formulations are made up of essential oils and organic acids. The formulations are listed in **Table 3.1**.

Table 3.1. Composition of antimicrobial formulation used in this study

Formulation	Composition
1	Oregano + Mediterranean formulation + Citrus extract + Lactic acid
2	Cinnamon + Lemongrass + Citrus extract + Citric acid
3	Cinnamon + Lemongrass + Citrus extract + Lactic acid
4	Oregano + Mediterranean formulation + Citrus extract + Citric acid

The composition of each formulation and the ratio of each compound added to the formulation was done based on the FIC index and the resulting mixture was homogenized in ultra-turrax to obtain a colloidal suspension. The working antimicrobial formulation was prepared following filtration through 0.2 µm pore size sterile filter **(Turgis *et al*., 2012; Ayari *et al*., 2020; Ji *et al*., 2021)**.

2.6. Combined effect of γ-irradiation and novel antimicrobial formulations

For irradiation treatment, the reconstituted mother's milk was inoculated with selected bacterial culture (10^6 CFU/mL) in 1:10 dilution. Then, the developed natural antimicrobial formulation (0.025% v/v) was added to bacteria inoculated milk samples. The volume of bacterial culture and antimicrobial formulation added was the same (1:1 ratio). The milk sample inoculated with 10^6 CFU/mL bacterial culture (without antibacterial formulation) was used as control. The γ-irradiation was done by the UC-15A irradiator device with a ^{60}Cobalt source (Nordion Inc., Laval, QC, Canada) and it was certified by national Institute of Standards and Technology (Gaithersburg, Md.). The irradiation dose rate of the device was 6.318 kGy/h and the samples were irradiated under frozen condition (-20 ˚C). The samples were subjected to various irradiation dosages as follows: 0, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5 kGy. Totally four antibacterial formulations were tested against six bacterial strains for this experiment. Upon irradiation, 100 µL of sample was inoculated to specific agar in petri plates using spread plate technique. Inoculated plates were incubated at 37 ˚C for 24 to 48h depending on the bacterial strain. The next day colony counting was done to determine the D10 and Radiosensitivity (R_s). The γ-irradiation experiment was carried out in triplicates. D10-value is defined as the dose required to reduce the population of bacteria by 1 Log **(Lacroix, 2010)**. And it was calculated by plotting the graph (linear regression) between irradiation dosage (kGy) on x-axis and bacterial population (Log CFU/mL) on y-axis. The reciprocal of slope gave the D10-value **(Maherani *et al*., 2019, Ghabraie *et al*., 2016)**. R_s was calculated by the following equation from the D10-values of sample and control.

Radiosensitivity (R_s) = D10 (kGy, control) / D10 (kGy, sample)

2.7 Statistical analysis

All the experiments were done in triplicates (each replicate included three samples) and analysed to check the significant difference by One way ANOVA (Analysis of variance) using Duncan test with alpha = 0.05 in SPSS v23 software (SPSS, Chicago, USA).

3. Results

3.1. Minimum inhibitory concentration of selected antibacterial agents

The minimum inhibitory concentration (MIC) of organic acids (lactic acid, citric acid), salts (sodium carbonate), EOs (oregano, Mediterranean formulation, cinnamon, lemongrass, clove), citrus extract (F440D) against bacterial strains *E. sakazakii* (ATCC 29004), *E. coli*

O157:H7 (ATCC 43895), *B. cereus* (ATCC 14579), *L. monocytogenes* (HPB 2812 serovar 1/2a), *S. aureus* (ATCC 29213) and *S.* Typhimurium (ATCC SL1344) are displayed in **Table 3.2**. The results showed that citrus extract has greater antibacterial efficacy against all the bacterial strains showing a MIC of 312.5 ppm, followed by oregano with MIC value of 625 ppm against all the strains except *B. cereus*, for which a MIC value of 1250 ppm has been recorded. Mediterranean formulation showed notable antibacterial activity with the MIC value of 1250 ppm against most of the tested strains except *B. cereus*, whose MIC value occurred at high concentration of 2500 ppm. Other EOs like clove exhibited MIC value of 2500 ppm against all bacterial strains and lemongrass with MIC value of 2500 ppm against *E. coli* O157:H7, *L. monocytogenes*, *S. aureus* and 5000 ppm against *B. cereus*, *S.* Typhimurium, *E. sakazakii*. Cinnamon exhibited MIC value of 1250 ppm against *E. coli* O157:H7 and *S. aureus* and that of 2500 ppm against the rest of the bacterial strains. On the other hand, organic acids and salts showed comparatively a lower antibacterial activity. The MIC value of lactic acid occurred at 2500 ppm against *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium, *S. aureus* and that of 5000 ppm against *B. cereus*, *E. sakazakii*, citric acid with MIC value of 5000 ppm against *E. coli* O157:H7, *S. aureus*, *L. monocytogenes* and >5000 ppm against the other bacterial strains demonstrating less effectively against pathogens. Among all the tested compounds, sodium carbonate had the lowest antibacterial activity with MIC value >5000 ppm against all bacterial strains. The lowest MIC recorded was 312.5 ppm for citrus extract against all bacterial strains, among EOs oregano showed a greater antibacterial activity at 625 ppm and among organic acids lactic acid displayed comparatively higher bactericidal effect with MIC value of 2500 ppm.

Table 3.2. Minimum inhibitory concentration of essential oils, Citrus extract, organic acids and salts

Compound name	Bacterial strains - MIC in ppm					
	***E. coli* O157:H7**	***S. aureus***	***L. monocytogenes***	***E. sakazakii***	***S.* Typhimurium**	***B. cereus***
Oregano	625	625	625	625	625	1250
Mediterranean formulation	1250	1250	1250	1250	1250	2500
Cinnamon	1250	1250	2500	2500	2500	2500
Lemongrass	2500	2500	2500	5000	5000	5000
Clove	2500	2500	2500	2500	2500	2500
Citrus extract	312.5	312.5	312.5	312.5	312.5	312.5
Lactic acid	2500	2500	2500	5000	2500	5000
Citric acid	5000	5000	5000	>5000	5000	>5000
Sodium carbonate	>5000	>5000	>5000	>5000	>5000	>5000

3.2. FIC index of selected EOs and organic acids

The checkerboard assay results showcasing the FIC index of the interaction binary interactions of EOs and between organic acids are presented in **Table 3.3a**. In EOs, Mediterranean formulation and oregano combination showed synergistic effect against *E. coli* O157:H7 and *S. aureus*, and additive effect against other bacterial strains. Other EO combinations cinnamon and lemongrass, clove and lemongrass showed an additive effect against all the tested bacterial strains. On the other side, lactic acid and citric acid combination showed non-interactive effect against all the bacterial strains.

Table 3.3a. Fractional inhibitory concentrations (FIC) of the combination of different EOs and the combination of organic acids

EOs and Organic acids	FICI					
	***E. coli* O157:H7**	***S. aureus***	***L. monocytogenes***	***E. sakazakii***	***S.* Typhimurium**	***B. cereus***
Oregano Mediterranean formulation	**0.375 (S)**	**0.375 (S)**	1 (AD)	0.75 (AD)	0.625 (AD)	0.75 (AD)
Cinnamon Lemongrass	1 (AD)	1 (AD)	1 (AD)	1 (AD)	1 (AD)	1 (AD)
Clove Lemongrass	0.625 (AD)	0.625 (AD)	0.625 (AD)	0.625 (AD)	0.625 (AD)	0.625 (AD)
Lactic acid Citric acid	1.5 (NI)	1.5 (NI)	1.5 (NI)	1.5 (NI)	1.5 (NI)	1.5 (NI)

3.3. FIC index of triple combination of EOs with other EOs, citrus extract and organic acids

The FIC index results corresponding to triple combinations of EOs with other EOs, citrus extract, organic acids and salts are showed in **Table 3.3b**. Combination of EOs of oregano and mediterranean formulation, cinnamon and lemongrass along with citrus extract exhibited a synergistic effect against all the bacterial strains except *E. sakazakii*, showing an additive effect. The other combination of oregano and mediterranean formulation along with cinnamon displayed an additive effect against all the tested pathogens. Meanwhile, the triple combinations between cinnamon/ lemongrass/ citric acid and oregano/ mediterranean formulation/ lactic acid showed a non-interactive effect against all the tested strains.

Table 3.3b. Fractional inhibitory concentrations (FIC) of the combination of EOs with other EOs, Citrus extract (F440D) and organic acids

Antibacterial Combinations tested	FICI					
	***E. coli* O157:H7**	***S. aureus***	***L. monocytogenes***	***E. sakazakii***	***S.* Typhimurium**	***B. cereus***
Oregano + Mediterranean formulation	**0.281 (S)**	**0.281 (S)**	**0.281 (S)**	0.563 (AD)	**0.281 (S)**	**0.281 (S)**
Citrus extract						
Cinnamon + Lemongrass	**0.25 (S)**	**0.375 (S)**	**0.375 (S)**	0.563 (AD)	**0.375 (S)**	0.5 (AD)
Citrus extract						
Oregano + Mediterranean formulation	0.75 (AD)	0.75 (AD)	0.75 (AD)	0.75 (AD)	0.75 (AD)	0.75 (AD)
Cinnamon						
Oregano + Mediterranean formulation	2.5 (NI)	2.5 (NI)	2.5 (NI)	2.5 (NI)	2.5 (NI)	2.5 (NI)
Lactic acid						
Cinnamon + Lemongrass	2.5 (NI)	2.5 (NI)	2.5 (NI)	2.5 (NI)	2.5 (NI)	2.5 (NI)
Citric acid						

3.4. FIC index of the quadruple antibacterial formulations

The FIC index results of developed quaternary formulations with oregano, thyme, citrus extract and lemongrass, cinnamon, citrus extract along with citric acid and lactic acid are shown in **Table 3.3c**. All the four formulations: oregano/ mediterranean formulation/ citrus extract/ lactic acid; oregano/ mediterranean formulation/ citrus extract/ citric acid; cinnamon/ lemongrass/ citrus extract/ lactic acid and cinnamon/ lemongrass/ citrus extract/ citric acid exhibited a synergistic effect against all the tested bacterial strains causing the contamination in mother's milk.

Table 3.3c. Fractional inhibitory concentrations (FIC) of the synthesized antimicrobial formulation using Oregano, Mediterranean formulation, citrus extract (F440D) with organic acids and Cinnamon, Lemongrass, citrus extract (F440D) with organic acids.

Antibacterial formulations	FICI					
	***E. coli* O157:H7**	***S. aureus***	***L. monocytogenes***	***E. sakazakii***	***S.* Typhimurium**	***B. cereus***
Oregano + Mediterranean formulation + Citrus extract	**0.156 (S)**	**0.375 (S)**	**0.186 (S)**	**0.313 (S)**	**0.281 (S)**	**0.375 (S)**
Lactic acid						
Oregano + Mediterranean formulation + citrus extract	**0.375 (S)**	**0.375 (S)**	**0.186 (S)**	**0.186 (S)**	**0.375 (S)**	**0.375 (S)**
Citric acid						
Cinnamon + Lemongrass + citrus extract	**0.375 (S)**	**0.186 (S)**	**0.313 (S)**	**0.313 (S)**	**0.375 (S)**	**0.281 (S)**

Lactic acid						
Cinnamon + Lemongrass + citrus extract	**0.375 (S)**	**0.375 (S)**	**0.186 (S)**	**0.375 (S)**	**0.281 (S)**	**0.375 (S)**
Citric acid						

3.5. Inactivation of bacteria in frozen milk samples using γ-irradiation combined with synthesized antibacterial formulations

The D10 (kGy) values of the combined treatment of γ-irradiation along with antibacterial formulation and control (irradiation alone) and the effect of the four developed antibacterial formulations in combination with γ-irradiation on the bacterial strains radiosensitivity are displayed in **Table 3.4a**. The irradiation dosage (kGy) required to reduce 6 Log bacterial population in mother's milk by irradiation treatment alone (control) and in combination with antibacterial formulations are presented in **Table 3.4b**. For *E. coli* O157:H7 a D10 value between 0.130- 0.132 kGy with the radiosensitivity ranging from 2.57 to 2.61 were observed in combined treatments, while the D10 value for the control was 0.340 kGy. The lowest D10 recorded against *E. coli* O157:H7 was 0.130 kGy for the combined treatment respective to formulation 3 showing a radiosensitivity of 2.61 **(Table 3.4a)**. The dose required to reduce the limit of detection of *E. coli* O157:H7 (6-Log) for control was 2.04 kGy and it was reduced to 0.78 kGy with the combined treatment respective to formulation 3 which was the lowest when compared to the other formulations 1, 2 and 4 **(Table 3.4b)**.

For *S. aureus* both formulation 1 and 2 exhibited a similar D10 value and radiosensitivity of 0.175 kGy and 2.48. These values changed to 0.170 kGy and 2.55 for formulation 3 and 4 respectively **(Table 3.4a)**. The D10 values of the combined treatment with all the four formulations against *S. aureus* is significantly different ($p < 0.05$) from what was recorded for the control 0.433 kGy **(Fig. 3.1d)**. The lowest dose required for the 6-log reduction of *S. aureus* was 1.02 kGy for the combined treatment respective to formulation 3 and 4. For control, the dose required for the 6-log reduction was 2.59 kGy **(Table 3.4b)**.

For *B. cereus* higher radiosensitivity was observed with the values of 2.03 and 2.06 for formulation 3 and 4, compared to the respective values for formulation 1 and 2 (1.38 and 1.4). Changes of radiosensitivity were in parallel with those of D10 as for formulation 3 and 4 required less dose (0.176 and 0.174 kGy) compared to formulation 1 and 2 (0.261 and 0.256 kGy) **(Table 3.4a)**. The D10 value for control was 0.358 kGy against *B. cereus* which is higher and significantly different ($p < 0.05$) compared to the combined treatment. The lowest dose

required for the 6-log reduction of *B. cereus* was 1.04 kGy for the combined treatment respective to formulation 4. For control, the dose required for the 6-log reduction was 2.14 kGy **(Table 3.4b)**.

For *E. sakazakii* (**Fig. 3.1b**), a D10 value of 0.372 kGy was recorded for control. For combined treatment pertaining to formulation 1 and 2, D10 values of 0.277 and 0.270 kGy were recorded respectively, which are significantly higher when compared to the respective D10 values of formulation 3 and 4 (0.176 kGy) **(Table 3.4a)**. A radiosensitivity of 1.34 and 1.38 was recorded for formulation 1 and 2 respectively, which are lower when compared to formulation 3 and 4 (2.11). The lowest dose required for the 6-log reduction of *E. sakazakii* was 1.06 kGy for the combined treatment with respect to formulation 3 and 4. For control, the dose required for the 6-log reduction was 2.23 kGy **(Table 3.4b)**.

For *L. monocytogenes*, the D10 value of control was 0.538 kGy and for the combined treatment along with formulation 3 and 4 the values were 0.298 and 0.314 kGy respectively, which are significantly lower ($p < 0.05$) compared to the respective D10 values of 0.380 and 0.381 kGy for formulation 1 and 2 **(Table 3.4a)**. The highest radiosensitivity of 1.81 against *L. monocytogenes* was achieved for formulation 3, followed by 1.71 for formulation 4. Formulation 1 and 2 adopted the lowest radiosensitivity of 1.41 against *L. monocytogenes* (**Fig. 3.1a**). The dose required to reduce the limit of detection of *L. monocytogenes* (6-Log) for control was 3.23 kGy, while it decreased to 1.78 kGy with for the combined treatment respective to formulation 3 which was the lowest when compared to the other formulations 1, 2 and 4 **(Table 3.4b)**.

For *S.* Typhimurium (**Fig. 3.1c**), the highest radiosensitivity of 3.99 among all the tested bacterial strains was found with formulation 3. The D10 value of control was 0.528 kGy diminishing to 0.270, 0.268, 0.132 and 0.133 kGy for combined treatments regarding to formulation 1, 2, 3 and 4 respectively. The D10 values of the combined treatments were significantly different ($p < 0.05$) compared to the control **(Table 3.4a)**. Like other bacterial strains, formulation 3 and 4 (3.99 and 3.97) were more effective in increasing the radiosensitivity of *S.* Typhimurium when compared to formulation 1 and 2 (2.0 and 2.35). The lowest dose required for the 6-log (CFU/mL) reduction of *S.* Typhimurium was 0.792 kGy for the combined treatment respective to formulation 3 showing notable loss in comparison with that of control (3.17 kGy) **(Table 3.4b)**.

Regarding sporulated form of *B.* cereus, a D10 value of 1.513 kGy was observed for the control. The introduction of formulations of 1, 2, 3 and 4 decreased D10 values to 0.986, 0.984, 0.796 and 0.801 kGy respectively **(Table 3.4a)**. The radiosensitivity for formulation 1 and 2 (1.53 and 1.54) was lower when compared to formulation 3 and 4 (1.90 and 1.89). Sporulated form of *B. cereus* is the most resistant bacteria and the required dose for its 6-log reduction under spore condition (9.08 kGy) reduced to 4.78 kGy by applying formulation 3 **(Table 3.4b)**.

Table 4.4a. D10 (kGy) values for control (irradiation alone) and combined treatment of irradiation and antibacterial formulation. And Radiosensitivity (R_s) of Bacterial strains for the combined effect of irradiation and antibacterial formulation

Bacteria (6 log)	Control – D10 (kGy)	Formulation 1 – D10 (kGy)	Radiosensitivity (R_s) – Formulation 1	Formulation 2 – D10 (kGy)	Radiosensitivity (R_s) – Formulation 2	Formulation 3 – D10 (kGy)	Radiosensitivity (R_s) – Formulation 3	Formulation 4 – D10 (kGy)	Radiosensitivity (R_s) – Formulation 4
E. coli O157:H7	0.340 ± 0.02 aB	0.132 ± 0.01 aA	2.59	0.131 ± 0.02 aA	2.59	0.130 ± 0.01 aA	2.61	0.132 ± 0.01 aA	2.57
S. aureus	0.433 ± 0.01 bB	0.175 ± 0.01 aA	2.48	0.175 ± 0.03 abA	2.48	0.170 ± 0.00 aA	2.55	0.170 ± 0.00 aA	2.55
B. cereus (vegetative cells)	0.358 ± 0.02 abC	0.261 ± 0.03 bB	1.38	0.256 ± 0.04 bB	1.4	0.176 ± 0.02 aA	2.03	0.174 ± 0.02 aA	2.06
S. Typhimurium	0.528 ± 0.08 cB	0.270 ± 0.07 bB	2.0	0.268 ± 0.08 bB	2.35	0.132 ± 0.01 aA	3.99	0.133 ± 0.01 aA	3.97
E. sakazakii	0.372 ± 0.04 abC	0.277 ± 0.03 bB	1.34	0.270 ± 0.02 bB	1.38	0.176 ± 0.00 aA	2.11	0.176 ± 0.01 aA	2.11
L. monocytogenes	0.538 ± 0.02 cB	0.380 ± 0.05 cA	1.41	0.381 ± 0.05 cA	1.41	0.298 ± 0.04 bA	1.81	0.314 ± 0.02 bA	1.71
B. cereus (spores)	1.513 ± 0.03 dC	0.986 ± 0.04 dB	1.53	0.984 ± 0.03 dB	1.54	0.796 ± 0.02 cA	1.9	0.801 ± 0.03 cA	1.89

For each bacterial strain, D10 values in columns followed by a same lowercase letter are significantly indifferent and the different uppercase letter behind the D10 values are significantly different within the same row ($p \geq 0.05$)

Table 4.4b. Irradiation dosage (kGy) required to reduce 6 Log of bacterial population with combined treatment of irradiation along with 4 different antibacterial formulations and irradiation treatment alone (control)

Bacteria (6 Log)	Irradiation dosage required to reduce 6 Log (kGy)				
	Control	Formulation 1	Formulation 2	Formulation 3	Formulation 4
E. coli	2.04	0.792	0.792	0.78	0.792
S. aureus	2.59	1.05	1.05	1.02	1.02
B. cereus (vegetative cells)	2.14	1.57	1.57	1.05	1.04
S. Typhimurium	3.17	1.62	1.62	0.792	0.798
E. sakazakii	2.23	1.662	1.62	1.06	1.06
L. monocytogenes	3.23	2.28	2.28	1.78	1.88
B. cereus (spores)	9.08	5.92	5.89	4.78	4.81

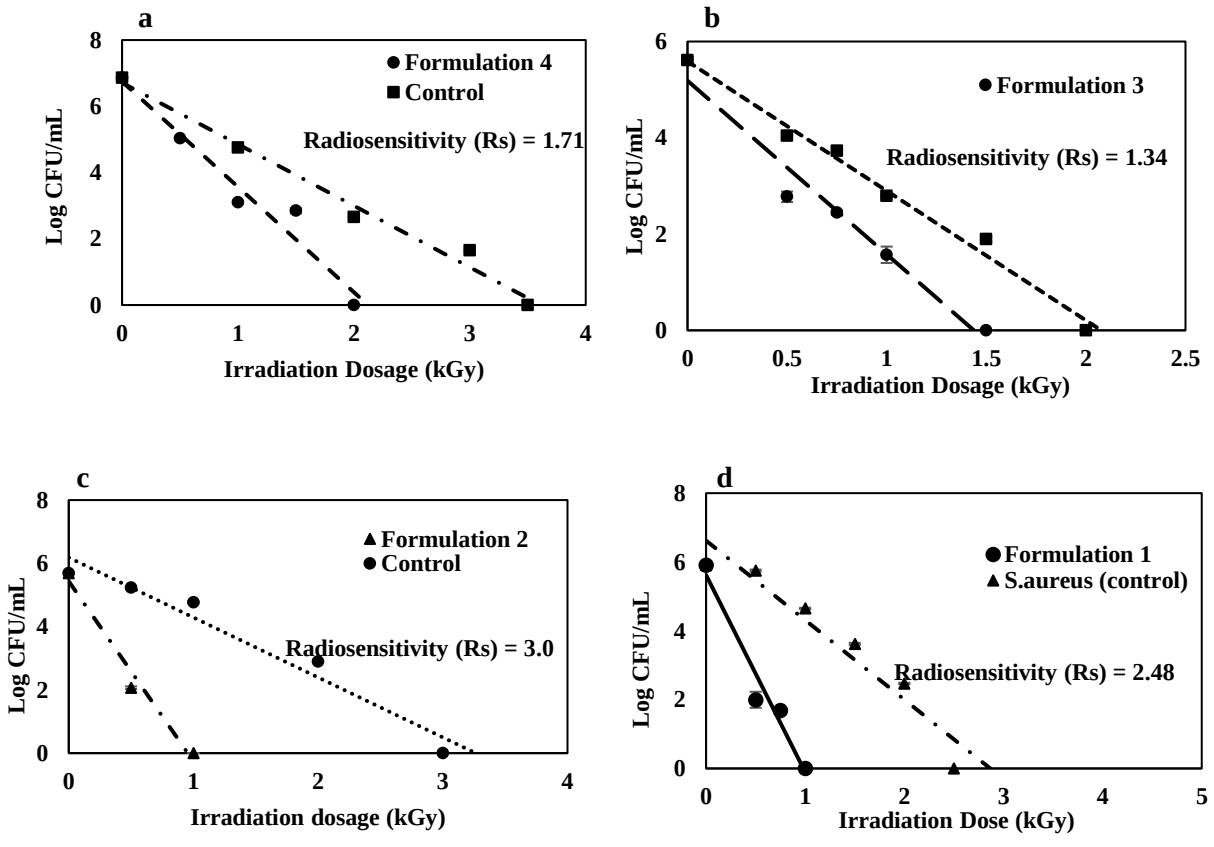

Figure 1. D10 curves of *L. monocytogenes* in frozen breast milk in combination with formulation 4 ($y = -1.86x+6.71$; $R^2 = 0.98$) and respective control ($y = -3.18x+6.75$; $R^2=0.981$) **(a);** D10 curves of *E. sakazakii in* frozen breast milk in combination with formulation 3 ($y = -2.69x+5.5879$; $R^2=0.99$) and respective control ($y = -3.607x+5.1853$; $R^2=0.96$) ***(b);*** D10 curves of *S.* Typhimurium in frozen breast milk in combination with formulation 2 ($y = -1.89x+6.18$; $R^2 = 0.95$) and respective control ($y=-3.60x+5.18$; $R^2=0.96$) ***(c);*** D10 curves of *S. aureus* in frozen breast milk in combination with formulation 1 ($y=-2.31x+6.62$; $R^2 = 0.93$) and respective control ($y=-5.72x+5.60$; $R^2 = 0.95$) **(d)**

4. Discussion

Natural antimicrobials like essential oils, phytoalexins, organic acids, plant extracts and their phytochemicals (tannins, terpenoids, alkaloids, phenols, flavonoids, etc) are used as food preservatives for many years **(Oussalah *et al*., 2006; Burt, 2004; Hyldgaard *et al*., 2012)**. These natural antimicrobials from plant origin helps in the defense mechanism against invading bacterial species, fungus and other pathogens **(Aisha Sithika *et al*., 2018; Akash Emmanuel *et al*., 2012)**. The lack of toxicity to the consumers, low cost, wide spectrum and effectiveness in its natural form at very low concentration are all general aspects considered for natural antimicrobials **(Karthik *et al*., 2019; Davidson *et al*., 2015)**. Organic acids (lactic acid, citric acid, malic acid, tartaric acid, etc) are weak acids and are obtained from plant and microbial sources. They are used as antimicrobials, antioxidants, acid regulators and emulsifiers **(Robichaud *et al*., 2021; Codex alimentarius, 1981)**. The antibacterial activities of citric acid and malic acid against various bacterial strains have been already proved. Citric acid has MIC values of 500, 500, 1000 and >1000 µg/mL against *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus* respectively. Malic acid shows MIC values of 500, 500, 1000 and 1000 µg/mL against *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus* respectively **(Adamczak *et al*., 2019)**.

Oussalah *et al*. 2007, demonstrated the antibacterial effect of cinnamon, coriander, Spanish oregano, lemongrass, clove, thyme, savory against four foodborne pathogens: *E. coli* O157:H7, *S.* Typhimurium, *S. aureus*, *L. monocytogenes* among which cinnamon, thyme and oregano were recorded with highest antibacterial activity. The lowest MIC value of 0.013% with Spanish oregano was recorded against *S. aureus*. For cinnamon they reported the lowest MIC value of 0.025 % against *S.* Typhimurium and *S. aureus* respectively. Likewise, they reported a MIC value of 0.025 % against *S. aureus* which was the lowest for thyme **(Oussalah *et al*., 2007)**. Another research showed the effect of cinnamon EO against *B. subtilis*, *K. pneumoniae*, *P. vulgaris*, *P. aeruginosa* with MIC values of 1600, 3200, 1600 and 800 ppm respectively **(Gavaric *et al*., 2015)**. These research studies also predicted that antibacterial activity was probably due to the presence of secondary metabolites like cinnamaldehyde, thymol, carvacrol, eugenol, geranial, etc **(Oussalah *et al*., 2007; Oussalah *et al*., 2007)**.

The FIC results presented in **Table 3.3c** shows that all four antibacterial combinations presented in **Table 3.1** affect all the tested pathogens in a synergistic way. According to Oussalah *et al*. 2006, Spanish oregano and cinnamon has proven to disrupt the membrane integrity of *E. coli* O157:H7 and *L. monocytogenes* and induced depletion of the intracellular

ATP concentration **(Lacroix, 2010)**. Supporting the previous research, Gavaric *et al*. 2015 also proved that thymol and carvacrol in combination with eugenol had a synergistic effect against *E. coli*, which can be attributed the cell membrane disintegration caused by carvacrol and thymol facilitating the penetration of eugenol into the cell cytoplasm and the antibacterial properties **(Oussalah *et al*., 2007)**. Ji *et al*. 2019 demonstrated that garlic and thyme combination showed synergistic effect against *Penicillium corylophilum* strains **(Ji *et al*., 2019)**. **Ayari *et al*., 2020** discovered that the combination of oregano/thyme, thyme/tea tree showed synergistic effect against *P. amylolyticus* and *B.* cereus. Additionally, cinnamon/thyme and thyme/peppermint EOs combination showed synergistic effect against *B. cereus*. Sadekuzzaman *et al*. **(2018)** also discovered that thyme/tea EOs showed significant antibacterial effect against *E. coli*, *L. monocytogenes* and *S.* Typhimurium. Parallelly, another study revealed oregano/nisin, thyme/nisin and *S. montagna* EO/pediocin showed additive effect against *E. coli* O157:H7, *L. monocytogenes* and *B. cereus* **(Turgis *et al*., 2012)**. Still, there are ongoing research studies trying to understand the root cause of the synergistic effect between the combinations of antibacterial compounds. Our study revealed the synergy between EOs, citrus extract and organic acids and their potential application as preservatives in food industry. Furthermore, an in-depth study in the future will help us to explore the mechanism behind the synergistic effect.

In summary, this study showed that formulation 3 and 4 have high antibacterial activity and increased the bacterial radiosensitivity of all the tested bacterial strains (**Table 3.4a**) as compared to formulation 1 and 2. Citrus extract's antibacterial activity has been demonstrated in the form of MIC (ppm) as displayed in **Table 3.2**. It exhibited the highest antibacterial activity among the tested compounds with the MIC value of 312.5 ppm against all bacterial strains and probably the most important reason for greater bacterial radiosensitivity of formulation 3 and 4 as the ratio of citrus extract is high when compared to formulation 1 and 2. Maherani *et al*. **(2019)**, have also proved that antimicrobial formulation containing citrus extract increased the radiosensitivity of fungal species *A. niger*, *P. chrysogenum* and *S. cerevisiae* by 1.54, 2.32 and 2.10 respectively in orange juice. Spore forming bacterial strains are highly resistant to irradiation and requires a higher amount of dosage to eliminate them. A study revealed a D10 value of 2.37 kGy to eliminate *B. cereus* spores in infant formula with γ-irradiation **(Robichaud *et al*., 2020)**. Current research showed the combined treatment of γ-irradiation along with natural antimicrobial formulation reduced the dose required to reduce 6-log *B. cereus* (sporulated) was 9.08 kGy for control and it got reduced to a dose of 4.78 kGy

with the combined treatment with formulation 3 **(Table 3.4b)** and increased its radiosensitivity by 1.90 **(Table 3.4a)**. It has been reported in previous research studies that the combined effect of γ-irradiation with natural antimicrobial formulation was a very effective technique for the growth inhibition of potential foodborne pathogens especially, *E. coli* O157:H7, *S. aureus, L. monocytogenes, B. cereus, S.* Typhimurium in fresh vegetables, meat, mother's milk and rice **(Robichaud *et al*., 2021; Maherani *et al*., 2019; Turgis *et al*., 2008; Begum *et al*., 2020)**. The combined effect of Spanish oregano and Chinese cinnamon EOs along with γ-irradiation increased the radiosensitivity of *E. coli* O157:H7 and *S.* Typhimurium by 3.57 and 3.26 in ground beef **(Sadekuzzaman *et al*., 2018)**. It has been reported that the radiosensitivity of sporulated *B. cereus* was increased up to 1.5 times by the addition of cinnamaldehyde (1.47% w/w) in minced meat **(Ayari *et al*., 2012)**. The addition of oregano/thyme EO in combination with the γ-irradiation increased the radiosensitization of *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* in rice by 1.20, 1.27 and 1.17 respectively **(Begum *et al*., 2020)**. Robichaud *et al*. 2021 **(Robichaud *et al*., 2021)**, investigated the combined effect of food additives like sodium carbonate and sodium citrate against *E. coli* O157:H7, *S. aureus, L. monocytogenes, B. cereus, S.* Typhimurium in powder and frozen infant formula. It showed an increase in radiosensitivity in both frozen and powdered infant formula in the range of 1.0 to 4.1 for sodium citrate and sodium carbonate. According to Turgis *et al*. 2008 **(Turgis *et al*., 2008)**, the presence of cinnamon and oregano essential oils minimized the radiation dose from 1.2 kGy to 0.35 kGy for *E. coli* and 1.4 kGy to 0.6 kGy for *Salmonella* Typhimurium elimination in beef. Chiasson *et al*. 2005 **(Chiasson *et al*., 2005)** revealed that the combined effect of γ-irradiation along with the addition of carvacrol and tetra-sodium pyrophosphate in beef reduces the radiation dose needed to eliminate foodborne pathogens. From 1.55 kGy the irradiation dose was reduced to 0.25 kGy in the presence of carvacrol and tetra-sodium pyrophosphate for *S.* Typhimurium and for *E. coli* it got reduced from 0.65 kGy to 0.55 kGy in the presence of carvacrol and tetra-sodium pyrophosphate.

Ionizing radiations like X-rays and γ-irradiation inhibit the bacterial growth by numerous ways such as causing damage to DNA of the bacterial cells through breaking the chemical bonds or altering the membrane permeability causing the destruction of cell membrane or cellular dysfunction **(Robichaud *et al*., 2021; Lacroix, 2010; Begum *et al*., 2020)**. The combined treatment of EOs and γ-irradiation leads to a synergistic effect adversely affecting the integrity of bacterial membrane and composition of murein which results in the release of cell constituents from bacterial cells thereby altering the internal pH and ATP

(Lacroix, 2010; Robichaud *et al.*, 2020; Turgis *et al.*, 2008). Furthermore, the reactive diffusible free radical formed due to the radiolysis of water, such as hydroxyl radical, hydrated electron, H^+ radical, hydrogen peroxide, and hydrogen radical can have indirect destruction ability. H_2O_2 and OH^- are strong oxidizing agents, while H^+ radical is a strong reducing agent. The H^+ and OH^- radicals are highly reactive, and cause oxidation and reduction on materials as well as the breakdown of carbon–carbon bonds, bonds of other molecules, and single and double strands in DNA at the sugar–phosphate bond. **(Lacroix, 2010; Robichaud *et al.*, 2020)**. Sporulated bacterial strains are highly resistant to γ-irradiation, lysozyme, heat and chemical disinfection as the endospore consist of spore coat made up of 30 spore-specific proteins covering the supercoiled DNA **(Robichaud *et al.*, 2021; Fiester *et al.*, 2012)**. This study has shown that γ-irradiation and natural antimicrobial treatments in combination are more effective for inhibition of the growth of foodborne pathogens and sporulated bacterial strains in mother's milk than the use of irradiation alone.

5. Conclusion

This study exhibited the strong antibacterial properties of EOs, citrus extract, organic acids and salts through MIC against six bacterial strains. The interaction between EOs, organic acids and citrus extract against all the tested bacterial strains was also demonstrated. Four developed antibacterial formulations showed synergistic effect against the tested pathogens. The combined treatment of γ-irradiation and the newly developed antibacterial formulations increased the radiosensitivity of bacterial strains and antibacterial activity and two formulations (3 and 4) containing the highest amount of citrus extract were especially more effective. Combined cold pasteurization treatment of γ-irradiation along with developed natural antibacterial formulations is a very effective technique to assure the safety of frozen mother's milk which is more efficient in eliminating the bacterial when compared to thermal pasteurization technique.

Conflicts of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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The connection between article 1 and article 2

The main objective of this research work was to develop a cold pasteurization technique based on γ-irradiation in combination with natural antimicrobial formulations for the preservation of frozen human milk. Article 1 clearly explained the selection of natural antibacterial compounds such as EOs, organic acids and citrus extract; determination of their antibacterial activity in form of MIC against selected pathogens. The synergistic interaction between the different antibacterial compounds were also determined. Based on the interaction between the compounds quaternary antibacterial formulation was developed. The combined effect of γ-irradiation and developed antibacterial formulations against selected pathogens at various dosages was determined using D_{10} values and the radiosensitivity was calculated. Article 2 explained the selection of single irradiation dosage required by the combined treatment of irradiation and antibacterial formulations to eliminate all the selected pathogens in mother's milk. Based on the D_{10} values, single irradiation dosage (which is 5 kGy) was selected for the combined treatment. The effect of the combined treatment at 5 kGy for the elimination of selected bacterial strains was determined. The effect of the combined treatment at 5 kGy on lactose & immunoglobulin content, lipid oxidation potential, *in vitro* protein digestibility, peptide profile and physicochemical properties of frozen mother's milk was also determined to check the efficiency of this combined treatment of γ-irradiation and antibacterial formulations in preservation of human milk.

Chapter 4

Effect of γ-irradiation in combination with natural antimicrobial formulation on microbial inactivation, protein digestibility and quality of mother's milk

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Contribution of the authors

Prof Monique Lacroix is the project lead. She coordinated the research activities, she completed the corrections of the paper and participated on the scientific discussion. I have performed the research, statistical analysis and written the article, Dr. Zahra Allahdad helped me with figure illustrations and article correction.

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Abstract:	In this study the combined effect of γ-irradiation (5 kGy) and four antimicrobial formulations developed for their antimicrobial properties: 1 (Oregano/Mediterranean formulation/Citrus extract/Lactic acid), 2 (Cinnamon/Lemongrass/Citrus extract/Citric acid), 3 (Oregano/Mediterranean formulation/Citrus extract/Citric acid), and 4 (Cinnamon/Lemongrass/Citrus extract/Lactic acid) was investigated in terms of microbial inactivation of selected foodborne pathogens, lactose and immunoglobulin composition, in vitro protein digestibility, peptide profile, physicochemical properties of mother's milk in frozen condition. γ-Irradiation treatment along with formulation 3 and 4 eliminated all tested pathogens including sporulated B. cereus that was resistant to γ-irradiation in the presence of formulation 1 and 2. Furthermore, combined treatment did not significantly alter the lactose, immunoglobulins and lipid oxidation of mother's milk. Protein digestibility increased in the irradiated milk and hydrolysis of larger proteins to smaller peptides was demonstrated. Irradiated samples had a minimal change in color and viscosity. Hence, combined treatment was effective in preservation of the quality of frozen mother's milk.
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Résumé

Dans cette étude, l'effet combiné de l'irradiation γ (5 kGy) et de quatre formulations antimicrobiennes développées pour leurs propriétés antimicrobiennes : 1 (formulation Origan/Méditerranée/extrait d'agrumes/acide lactique), 2 (Cannelle/Lemongrass/extrait d'agrumes/acide citrique), 3 (formulation Origan/Méditerranée/extrait d'agrumes/acide citrique), et 4 (Cannelle/Lemongrass/Extrait d'agrumes/Acide lactique) ont été étudiés en termes d'inactivation microbienne de pathogènes alimentaires sélectionnés, de composition du lactose et des immunoglobulines, de digestibilité des protéines in vitro, de profil peptidique, de propriétés physicochimiques du lait maternel à l'état congelé. Le traitement par irradiation γ avec les formulations 3 et 4 a éliminé tous les pathogènes testés, y compris *B. cereus* sporulé qui était résistant à l'irradiation γ en présence des formulations 1 et 2. En outre, le traitement combiné n'a pas modifié de manière significative le lactose, les immunoglobulines et l'oxydation des lipides du lait maternel. La digestibilité des protéines a augmenté dans le lait irradié et l'hydrolyse des plus grosses protéines en peptides plus petits a été démontrée. La couleur et la viscosité des échantillons irradiés ont peu changé. Le traitement combiné a donc été efficace pour préserver la qualité du lait maternel congelé.

Mots clés

lait maternel; γ-irradiation ; profil peptidique; digestibilité des protéines in vitro; formule antibactérienne.

Abstract

In this study the combined effect of γ-irradiation (5 kGy) and four antimicrobial formulations developed for their antimicrobial properties: 1 (Oregano/Mediterranean formulation/Citrus extract/Lactic acid), 2 (Cinnamon/Lemongrass/Citrus extract/Citric acid), 3 (Oregano/Mediterranean formulation/Citrus extract/Citric acid), and 4 (Cinnamon/Lemongrass/Citrus extract/Lactic acid) was investigated in terms of microbial inactivation of selected foodborne pathogens, lactose and immunoglobulin composition, *in vitro* protein digestibility, peptide profile, physicochemical properties of mother's milk in frozen condition. γ-Irradiation treatment along with formulation 3 and 4 eliminated all tested pathogens including sporulated *B. cereus* that was resistant to γ-irradiation in the presence of formulation 1 and 2. Furthermore, combined treatment did not significantly alter the lactose, immunoglobulins and lipid oxidation of mother's milk. Protein digestibility increased in the irradiated milk and hydrolysis of larger proteins to smaller peptides was demonstrated. Irradiated samples had a minimal change in color and viscosity. Hence, combined treatment was effective in preservation of the quality of frozen mother's milk.

Keywords

mother's milk; γ-irradiation; peptide profile; *in vitro* protein digestibility; antibacterial formulation.

1. Introduction

Mother's milk is a complex mixture of nutrients and bioactive compounds helping in the growth and development of immune system in new born and infants **(Ballard & Morrow, 2013; Martin, 2016)**. Therefore, the necessity to preserve and provide milk for the infants whenever needed is met securely by human milk banks, where the mother's milk samples are collected from various donors, pasteurized (62.5 ˚C for 30 min) and stored safely in frozen condition **(Haiden & Ziegler, 2016)**. However, mother's milk can be contaminated at any point of this process by foodborne pathogens like *E. sakazakii*, *E. coli* O157:H7, *S. aureus*, *L. monocytogenes*, *B. cereus* and *S. Typhimurium*. Heat pasteurization is a widely used promising technique for elimination of bacterial pathogens and to assure product safety. But thermal treatments involving high temperature leads to nutritional loss in the form of Maillard reaction, loss of vitamins, aggregation of proteins, isomerization of lactose to lactulose and protein digestibility **(Wazed, Ismail, & Farid, 2020)**. Moreover, thermal pasteurization doesn't completely help to eliminate the total bacterial population, spore forming bacteria like *B. cereus* and the endotoxins that are produced by *S. aureus* are highly resistant and can survive at high temperatures **(Forsythe, 2005; Keim et al., 2013)**. As a result, non-thermal pasteurization has attracted a great attention in recent years and is used as an alternative method to extend the shelf life and improve the food security while maintaining the nutritional value **(Lacroix & Ouattara, 2000; Arroyo et al., 2017)**.

Non thermal pasteurization technique using ionizing radiations such as γ-irradiation is very effective in inactivation of bacterial pathogens in food stuffs (Mahrour, Caillet, Nketsa-Tabiri, & Lacroix, 2003; Lacroix, 2010), while the macronutrient content of the food products such as carbohydrates, proteins and fats are not altered. However, the nutritional matrix is preserved depending on the environmental condition, food composition and irradiation dosage (kGy). Lipid oxidation and few micronutrients tend to be affected by the irradiation in a dose dependent manner as reported in previous research studies **(Dionísio, Gomes, & Oetterer, 2009; Robichaud et al., 2020)**. As per the regulations approved by Food and Drug Administration (FDA) up to 45 kGy of irradiation dosage is allowed to be used for sterilization of frozen food products **(Maherani et al., 2016)**. Irradiation can improve the protein digestibility by improving the hydrolysis of proteins into smaller peptides **(Shawrang et al., 2007)**. Therefore, various researches have been done in search of a way to reduce the required irradiation dose in commercial food processing and it has been reported that the combined effect of γ-irradiation along with natural antimicrobials like essential oils (EOs), plant extracts,

organic acids and bases (as a formulation or an individual compound) was able to reduce the irradiation dosage required to eliminate foodborne pathogens **(Tawema et al., 2016; Maherani et al., 2019; Robichaud et al., 2021)**. It has also been reported that EOs, plant extracts, bacteriocins, organic acids and bases are used as food preservatives for their high antimicrobial properties and also as food additives, acid regulators and stabilizers **(Join FAO/WHO Codex Alimentarius Commission, 1995; Tawema et al., 2016; Robichaud et al., 2021)**.

Therefore, the main objectives of this research project were to evaluate the radiosensitivity of the targeted foodborne pathogens in combination with natural antibacterial formulations as well as the effect of γ-irradiation on the lactose, protein digestion, peptide profile, lipid oxidation, color and texture of mother's milk.

2. Materials and Methods

2.1. Materials

The culture media for bacterial growth like Tryptic soy broth (TSB), Mueller Hinton Broth (MHB), Tryptic soy agar (TSA), PALCAM agar, B. cereus agar, Mannitol agar, MacConkey agar, DCLS agar, S. aureus agar and peptone water were purchased from Alpha Biosciences Inc. (Baltimore, MD, USA) and Thermo Fisher Scientific (Nepean, ON, Canada). The essential oils were purchased from Zayat Aroma (Bromont, QC, Canada), mediterranean formulation was bought from BSA (Montreal, QC, Canada), citrus extract was bought from Kerry int. (Woodstock, ON, Canada), organic acids and all other chemicals including pepsin (from lyophilized powder from porcine gastric mucosa ≥ 3200 units/mg protein, 65% purity) and trypsin (from bovine pancreas 10,000 BAEE units/mg protein, 93% purity) were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). The falcon tubes, petri plates and other utensils were purchased from SARSTEDT (St. Leonard, QC, Canada). Mother's milk was provided by the HEMA Quebec in frozen condition. it was then freeze-dried under sterile conditions.

2.2. Preparation of bacterial strains and inoculation

Enterobacter sakazakii (ATCC 29004), *Escherichia coli* O157:H7 (ATCC 43895), *Staphylococcus aureus* (ATCC 29213), *Listeria monocytogenes* (HPB 2812 serovar 1/2a), *Bacillus cereus* (ATCC 14579) and *Salmonella Typhimurium* (ATCC SL1344) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were maintained in -80 ˚C using TSB-glycerol mixture (10% v/v) for prolonged storage. Before each

experimentation, 10^8 CFU/mL of bacterial stock cultures were cultured by passing along through 2 consecutive cycles in TSB and incubated at 37 ˚C for 24 h (per cycle).

2.3. Preparation of natural antimicrobial formulation from EOs, citrus extract and organic acids

EO (Mediterranean formulation, Oregano, Lemongrass, Clove and Cinnamon) emulsions was prepared by mixing each EOs (2.5% v/v) with Tween 80 (Laboratoire Mat, QC, Canada) as a stock solution. These mixtures were homogenized for 5 min with an Ultra-Turrax homogenizer (model TP18/1059, Germany) at 15,000 g to obtain the final colloidal suspension and were stored in 4 ˚C before use **(Ayari, Shankar, Follet, Hossain, Lacroix, 2020)**. The Citrus extract, organic acids and bases (Citric acid, Lactic acid and Sodium carbonate) were diluted from its initial concentration to 2% stock concentration. Four antibacterial formulations were developed by combining different ratios of each compound. From the previous study, the interaction between the various antibacterial compound's composition against target pathogens were determined based on the Fractional Inhibitory Concentration (FIC) index. The degree of synergy between antibacterial agents is often expressed in terms of FIC. Four antibacterial formulations were developed based on the synergistic effect exhibited by the quaternary combination of compounds (not shown). The four antibacterial formulations are Formulation 1 (Oregano/Mediterranean formulation/Citrus extract/Lactic acid), Formulation 2 (Cinnamon/Lemongrass/Citrus extract/Citric acid), Formulation 3 (Oregano/Mediterranean formulation/Citrus extract/Citric acid), Formulation 4 (Cinnamon/Lemongrass/Citrus extract/Lactic acid). From the stock solution a working solution of 0.023% (v/v) of each formulation was prepared and mixed well to obtain colloidal suspension using Ultra-Turrax homogenizer and filtered by 0.2 µm sterile filter and then used for experiments **(Ayari, Shankar, Follet, Hossain, Lacroix, 2020)**. The composition of each antibacterial formulation is displayed in **Table 4.1**. The milk pH did not show any significant changes upon addition of antimicrobial formulations.

Table 4.1. Composition of newly developed antimicrobial formulations

Formulation	Composition
1	Oregano + Mediterranean formulation + Citrus extract + Lactic acid (1:1:2:1)
2	Cinnamon + Lemongrass + Citrus extract + Citric acid (2:1:5:2)
3	Cinnamon + Lemongrass + Citrus extract + Lactic acid (2:1:5:2)
4	Oregano + Mediterranean formulation + Citrus extract + Citric acid (1:1:2:1)

2.4. Preparation of mother's milk samples

The frozen mother's milk from HEMA Quebec was freeze dried and stored in 4 ˚C as a stock. When needed, the freeze-dried mother's milk was reconstituted into its original form (liquid) by adding sterile water under sterilized conditions (approximately 71.3 g water was added to 5 g of freeze-dried milk) and used for experiments. Then, the reconstituted milk was inoculated with selected bacterial pathogens (10^6 CFU/mL) and antibacterial formulation (0.25%). The milk, bacteria and antimicrobial formulation were added in the ratio of 9:1:1 ml. Thus, the final concentration of antibacterial formulation is 0.023% **(Robichaud et al., 2021)**.

2.5. γ-Irradiation treatment and bacterial count determination

γ-Irradiation of the samples was done by UC-15A irradiator device with a ^{60}Cobalt source (Nordion Inc., Laval, QC, Canada) and it was certified by national Institute of Standards and Technology (Gaithersburg, Md.) based on the protocol developed by **Maherani et al., 2019; Robichaud et al., 2021**. Two kinds of samples were irradiated, one was mother's milk inoculated with bacterial culture (10^6 CFU/mL) and antibacterial formulation in the ratio of 9:1:1; the other one was free of antimicrobial formulations which was considered as control. Treatment used for experiments were 0 kGy and 5 kGy respectively. The irradiation dose rate of the device used was 6.318 kGy/h and the samples were irradiated in frozen condition. Same procedure was repeated against all the tested bacterial strains with four different antibacterial formulations. Immediately after irradiation the frozen samples were kept inside the laminar airflow chamber until it brought back to its original liquid form and 100 µL from each sample was taken and inoculated in specific agar media in petri plates using spread plate technique and incubated at 37 ˚C for 24 h depending on the bacterial strain. The next day, colony counting was done and Log CFU/mL was calculated. Irradiation treatment was carried out in triplicates.

2.6. Determination of Lactose

The concentration of lactose in combined treatment of irradiation (5 kGy) and antibacterial formulation (3 and 4) in mother's milk and non-irradiated mother's milk was measured and compared using Agilent 1260 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) with a ZORBAX carbohydrate column (4.6*250 mm, pore size 70 Å, 5 µm particle size). The samples were prepared by adding equal amount of Trichloroacetic acid (TCA) 12% (w/v) into the both irradiated and non-irradiated mother's milk. Then samples were subjected to centrifugation (Sorvall® Instrument, Du Pond, USA) at 10,000 g for 20 min at 4 ˚C to remove the fat and casein content. The supernatant was filtered using 0.2 µm syringe filter and stored in the freezer at -20 ˚C prior to use. Lactose separation using HPLC was done under isocratic conditions. Mili-Q water was used as the mobile phase at a flow rate of 1.5 mL/min. The sample volume injected to the column was 20 µL. The chromatogram was analysed by Chemstation v.2.0 (Agilent). The standard curve was prepared using lactose concentration of 0 to 50 mg/mL. Refractive index detector was used as detector at the temperature 50 ˚C and the column was set at 80 ˚C **(Robichaud et al., 2020)**.

2.7. *In vitro* protein digestion and determination of nitrogen release

The *in vitro* protein digestibility (IVPD) of combined treatment of irradiation (5 kGy) and antibacterial formulation (3 and 4) in mother's milk and non-irradiated breast milk were determined by sequential digestion using pepsin and trypsin as per the method mentioned by **Manus et al. (2021)**. As per protocol, an aliquot containing at least 0.5 g of protein should be used for the experiment. Concisely, 40 g of mother's milk protein (100 g of mother's milk contains 1.25 g of protein) was suspended in 9.5 mL of HCl (0.1 M). Upon HCl addition, the pH changed to 2 and then 0.5 ml of pepsin (11 mg/ml in 0.1 M HCl) was added to the solution. The protein substrate to enzyme ratio was 1:50 w/w. The mixture was incubated at 37 ˚C for 120 min at 100 g. The digested solution was adjusted to pH 8 using NaOH (1.0 M) and 10 mg of trypsin was added (ratio between enzyme and initial protein was 1:50 w/w). Following incubation at the same condition, 1-mL samples were collected at various digestion times and subjected to heat for 5 min at 95 ˚C in a water bath to stop the enzyme reaction. After that, it was centrifuged at 10,000 g for 20 min and the supernatant was collected and filtered using 0.2 µm syringe filter to determine the molecular weight distribution of peptides using SEC-HPLC on a Biosep-SEC 2000 column.

The percentage of nitrogen release during the digestion process was calculated from the supernatant obtained after precipitation with trichloroacetic acid (TCA) as per the protocol explained by **Manus et al. (2021)**. Digestibility is proportional to the hydrolysis of High molecular weight peptides (HMW) into Medium molecular weight peptides (MMW) and Low molecular weight peptides (SMW). Greater the protein hydrolysis greater the digestibility. Briefly, 6 mL of the enzyme digested protein hydrolysate sample was mixed with equal volume of TCA (20 % w/w) followed by centrifugation at 10,000 g for 20 min. Then, the supernatant was subjected to Kjeldahl method (N * 6.25) as per the method mentioned by **AOAC International (2000)**. The formula used for the percentage nitrogen release concentration was:

% N release = (Nitrogen in the supernatant / Nitrogen in the sample) * 100

2.8. Peptide analysis

Peptide molecular weight distribution and immunoglobulin content of combined treatment of irradiation (5 kGy) and antibacterial formulation (3 and 4) in mother's milk and non-irradiated mother's milk were determined as the protocol described by Manus et al. (2021). Size exclusion HPLC was used for the analysis with a Biosep-SEC 2000 column (300 * 7.8 mm, pore size 145 Å, 5 mm particle size) from Phenomenex (Torrance, CA, USA). The HPLC system used for the experiment was Agilent 1260 HPLC infinity system (Agilent Technologies, Palo Alto, CA, USA). Briefly, the mother's milk samples before and during *in vitro* protein digestion were centrifuged at 10,000 g for 20 min and the supernatant was filtered using 0.2 µm syringe filter. Then, 10 µL of the filtered supernatant was injected into the column. The mobile phase used here was 100 mM sodium phosphate buffer solution (pH 6.8). The sample was injected at the flow rate of 1 ml/min for 20 min. Diode array detector at 280 nm was used for detection, and the standards used for the analysis were Bovine thyroglobulin (670 KDa), gamma globulin (300 KDa), IgG (150 KDa), ovalbumin (44 KDa), myoglobin (17 KDa) and uridine (244 Da).

2.9. Thiobarbituric acid-reactive substances assay (TBARS)

The lipid oxidation potential of breast milk under combined treatment of irradiation (5 kGy) and antibacterial formulation (3 and 4) was evaluated by the determination of Thiobarbituric acid reactive substances (TBARS) based on the protocol described by **Criado et al. (2020)**. A 10-mL portion of mother's milk sample was blended with 10 mL of 15% trichloroacetic acid. Then, the mixture was centrifuged at 5900 g for 5 min and the supernatant was filtered through 0.45 µm syringe filter. After that, 2 mL of 0.06 M Thiobarbituric acid

(TBA) dissolved in 10% acetic acid was added to 8 mL of the filtered supernatant, vortexed well and placed into a water bath at 80 ˚C for 90 min and immediately cooled in ice. Finally, the absorbance was read at 520 nm using Cary 1 spectrophotometer (Agilent Technologies inc., Mississauga, ON, Canada) and the concentration of malondialdehyde (MDA) was determined. Serial dilutions of a solution of 1,1,3,3 tetramethoxypropane (97% v/v) was used as standard.

2.10. Colour and viscosity analysis

The colour variation of the mother's milk samples (both irradiated and non-irradiated) were measured using Color reader CR10-Plus (Konica Minolta, Ramsey, NJ, USA). Color parameters such as L^* (lightness, blank = 0, white = 100), a^* (redness > 0, greenness = 0, blue < 0), b^* (blue-yellow from -300 to +299) and the global colour difference (ΔE^*) were determined **(Ben-Fadhel et al., 2021)**. The total colour difference ΔE^* was calculated using the following formula:

$$\Delta E^* = \sqrt{[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]}$$

The viscosity of the combined treatment of irradiation (5 kGy) and antibacterial formulation (3 and 4) in mother's milk and non-irradiated milk (control) was measured using DV-II+ viscometer (Brookfield engineering laboratories, Middleboro, MA, USA). YULA-15 spindle was used for the experiment. All the measurements were calculated at 100 g and 23.5 ˚C ± 0.1 ˚C **(Okyere & Odamtten, 2014)**.

2.11. Statistical analysis

All experiments were performed with same batch of milk. Each treatment was irradiated in triplicates and parameter was measured for each replicate. Results were reported as mean ± standard deviation. The results obtained from the experiments were subjected to one-way analysis of variance (ANOVA) using Ducan test ($p \leq 0.05$) in SPSS v23 software (SPSS, Chicago, USA).

3. Results

3.1. Inactivation of bacteria using combined treatment of γ-irradiation and novel antimicrobial formulation

The effect of the combined treatment of antimicrobial formulations along with γ-irradiation at 5 kGy against the selected foodborne pathogens are displayed in **(Table 4.2)**. Results showed that the combined treatment of formulation 3 and 4 along with γ-irradiation at 5 kGy eliminated all the tested bacterial strains (6 log) even sporulated *B. cereus* in mother's

milk **(Table 4.2)** which is the most resistant species to irradiation**.** While, the combined treatment of formulation 1 and 2 along with γ-irradiation at 5 kGy limited the 6-log detection of all the foodborne pathogens except the sporulated form of *B. cereus.* The initial content of *B. cereus* spores in the mother's milk was 6.25 ± 0.03 log CFU/mL. After the irradiation treatment at 5 kGy without antimicrobial formulation (control) a value of 3.64 ± 0.01 log CFU/mL of sporulated *B. cereus* has been recorded **(Table 4.2)**. The incorporation of formulation 1 and 2 in the mother milk further reduced *B. cereus* by 1. 25 and 1.19 log CFU/ mL, respectively. In the presence of formulation 3 and 4 the content of *B. cereus* spores was nil **(Table 4.2)**. From the results, it can be observed that combined treatment of γ-irradiation with formulation 3 and 4 was significantly more effective ($p > 0.05$) to eliminate all tested pathogens (both sensitive and resistant strains) when compared to formulation 1 and 2. For this reason, formulations 3 and 4 were selected as best-suited for the following experiments.

Table 4.2. Effect of combined treatment of antimicrobial formulation and γ-irradiation in 5 kGy

Bacteria	Log CFU/mL					
	0 kGy	Irradiation alone (5 kGy)	Formulation 1 + 5 kGy	Formulation 2 + 5 kGy	Formulation 3 + 5 kGy	Formulation 4 + 5 kGy
E. coli	5.80 ± 0.03	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
S. aureus	5.91 ± 0.02	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
B. cereus (vegetative cells)	5.89 ± 0.05	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
S. Typhimurium	5.70 ± 0.03	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
E. sakazakii	5.61 ± 0.06	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
L. monocytogenes	6.63 ± 0.02	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
B. cereus (spores)	6.25 ± 0.03	3.64 ± 0.01	1.29 ± 0.14	1.18 ± 0.15	0 ± 0	0 ± 0

3.2. Lactose content and lipid oxidation in irradiated and non-irradiated mother's milk

In the present study, the lactose content of frozen mother's milk was not altered after the combined treatment of irradiation and antibacterial formulations (3 and 4) when compared to the control (0 kGy) **(Fig. 4.1a).** The lactose content in non-irradiated sample (control) was 7.7 ± 0.16 g/100 mL and there was no tangible alteration in the irradiated samples with formulation 3 and 4 which respectively exhibited 7.9 ± 0.29 g/100 mL and 7.4 ± 0.16 g/100 mL.

Similar to lactose content, there was no difference in the malondialdehyde (MDA) content of frozen mother's milk before and after the combined treatment (Fig. 1b). The MDA

content of control sample (0 kGy) was 2.16 ± 0.11 µg/g changing to 2.26 ± 0.13 µg/g and 2.21 ± 0.08 µg/g for the sample contained with formulation 3 and 4 and exposed to 5 kGy.

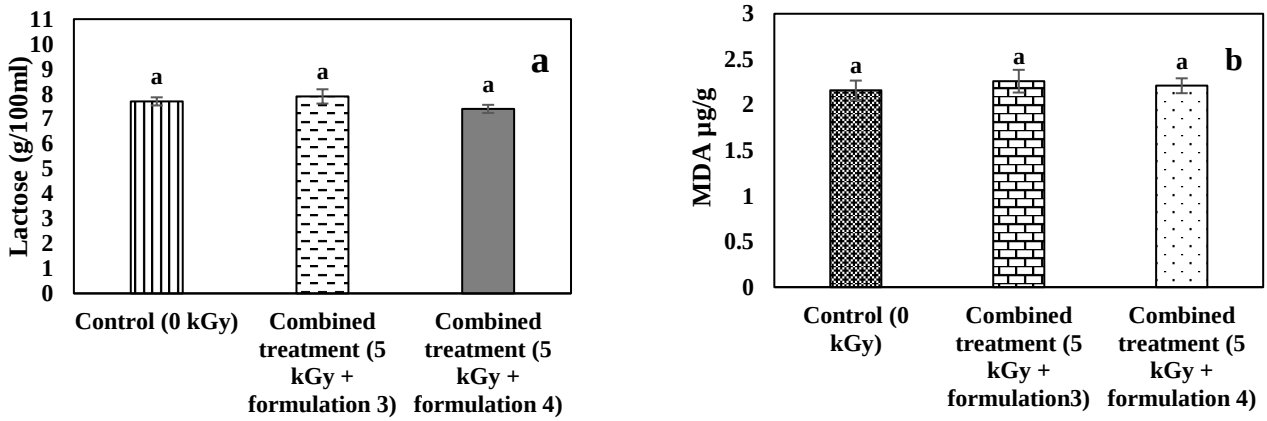

Figure 1. Effect of γ-irradiation and antibacterial formulation on the lactose concentration of mother's milk (a); Effect of γ-irradiation and antibacterial formulation on the lipid oxidation in mother's milk (b)

3.3. Peptide profile and immunoglobulin content

The molecular weight distribution of peptides of the control sample (non-irradiated mother's milk) and combined treatment of irradiation (5 kGy) and antibacterial formulations (3 and 4) in mother's milk are presented in **Table 4.3**. Enzymatic digestion with pepsin and trypsin breaks down proteins and larger peptides into smaller peptides and free amino acids. Peptides were divided into three different groups; Those with molecular weight >2700 Da (polypeptides) are considered as high molecular weight (HMW); between 2700 and 200 Da are known as medium molecular weight (MMW) (oligopeptides and tripeptides) and <200 Da are low molecular weight group (LMW) (dipeptides and free aminoacids). The total proportion of HMW and MMW peptides reduced during the digestion for both irradiated and non-irradiated samples while proportion of LMW increased. Comparing the difference in protein hydrolysis efficiency during enzymatic digestion of combined treatment and non-irradiated samples, it showed that combined treatment of γ-irradiation at 5 kGy and antimicrobial formulation 3 has led to the reduction of HMW peptides in mother's milk by 7% which is significantly greater ($p \leq 0.05$) when compared to that of the non-irradiated sample. Similarly, combined treatment of γ-irradiation and antimicrobial formulation 4 has led to a reduction of HMW by 10 %. The proportion of MMW peptides was reduced by 15% and 21% in irradiated samples contained with formulation 3 and 4 which is significantly higher ($p \leq 0.05$) than that of non-irradiated

samples leading to the increase in the total area under the curve for LMW. These results demonstrated that combined treatment of γ-irradiation and antibacterial formulations of mother's milk at 5 kGy increased the protein hydrolysis during enzymatic digestion resulting in the release of LMW peptides ($p \leq 0.05$) when compared to non-irradiated samples.

The immunoglobulin (IA, IgG, IgM) content of combined treatment of irradiation and antibacterial formulation (3 and 4) in mother's milk and non-irradiated mother's milk was compared using the HPLC analysis. The comparison of immunoglobulin concentration is displayed in **Table 4.4**. The immunoglobulin content in mother's milk of the combined treatments was not significantly modified ($p > 0.05$) when compared to the control sample.

Table 4.3. Impact of the combined effect of gamma irradiation and antibacterial formulation on molecular distribution of peptides

Molecular weight (Da)	Non irradiated	Area under the curve Irradiation (5 kGy) + Formulation 3	Irradiation (5 kGy) + Formulation 4
>2700	3533.15 ± 66.37a	3262.535 ± 41.96b	3179.44 ± 62.64b
2700-212	54685.54 ± 667.39a	46340.36 ± 784.57b	42785.85 ± 76.55b
<212	14630.63 ± 146.92a	26134.13 ± 292.43b	27602.62 ± 123.84b

Data are expressed in mean ± standard deviation done in triplicates. Means with same lower-case letters within same row are significantly indifferent ($p \leq 0.05$).

Table 4.4 Comparison of immunoglobulin content before and after combined treatment of γ-irradiation and antibacterial formulation

Immunoglobulins	Concentration (mg/mL)		
	Control	Combined treatment (5 kGy + Formulation 3)	Combined treatment (5 kGy + Formulation 4)
IgA	0.870 ± 0.03 a	0.862 ± 0.04 a	0.879 ± 0.03 a
IgG	0.659 ± 0.01 a	0.651 ± 0.01 a	0.653 ± 0.04 a
IgM	0.975 ± 0.03 a	0.977 ± 0.02 a	0.963 ± 0.01 a

Data are expressed in mean ± standard deviation done in triplicates. Means with same lower-case letters within same row are significantly indifferent ($p \leq 0.05$).

3.4. *In vitro* protein digestibility (IVPD)

The IVPD of combined treatment of irradiation (5 kGy) and antibacterial formulation (3 and 4) in mother's milk and non-irradiated mother's milk was determined by the nitrogen release during *in vitro* digestion using pepsin and trypsin was displayed in **Fig 4.2**. Results showed that both the control (0 kGy) and irradiated samples (5 kGy) have the same pattern of nitrogen release during *in vitro* digestion. The percent of nitrogen release after pepsin digestion

(0 to 120 min) was 73.31% that increased to 94.73% after trypsin (120 to 240 min) digestion for the control. Both the combined treatments at 5 kGy also showed a similar nitrogen release pattern in mother's milk after pepsin and trypsin digestion. So that the total nitrogen release after 240 min (pepsin + trypsin digestion) for the irradiated samples in combination with formulation 3 and 4 was 95.86 % and 95.23 %, respectively.

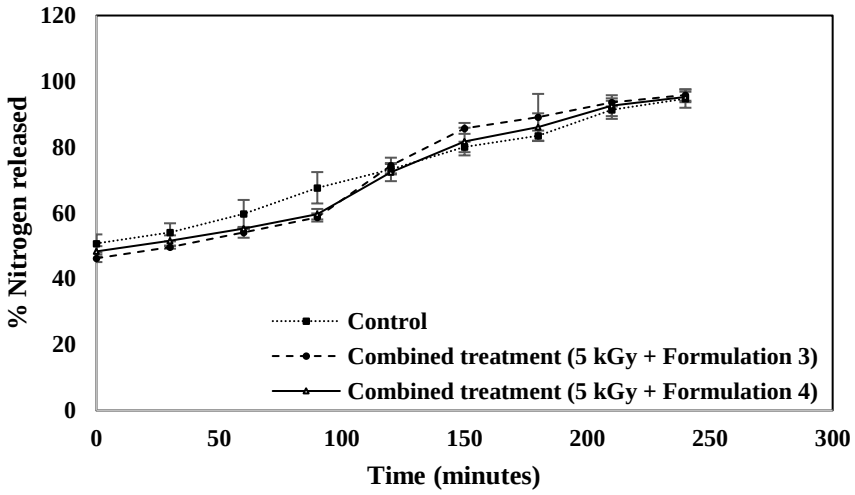

Figure 2. In vitro protein digestibility of mother's milk using pepsin and trypsin

3.5. Color and viscosity of irradiated and non-irradiated mother's milk

The color parameters (L^*, a^*, b^* and ΔE) of combined treatment of irradiation (5 kGy) and antibacterial formulation (3 and 4) in mother's milk and non-irradiated mother's milk under frozen condition were presented in **Table 4.5.** There wasn't any significant effect on L^* (brightness), a^* (redness) of mother's milk subjected to combined treatment compared to the control. But, b^* (yellowness) of both the samples subjected to combined treatment showed less values when compared to that of the control. The ΔE value was 1.39 ± 0.07 which shows a very small difference in color.

From **Table 4.5** it can be also observed that combined treatment of irradiation (5 kGy) and antibacterial formulation (3 and 4) showed slight increase in the viscosity of mother's milk when compared to the non-irradiated sample. The viscosity of non-irradiated sample was 3.2 ± 0.06 mPa.s and for irradiated samples in combination with formulation 3 and 4 was 3.02 ±0.08 and 3.05 ± 0.07, respectively.

Table 4.5. Effect of combined treatment of γ-irradiation and antibacterial formulation on the colour and texture of mother's milk

Analysis	Parameters	Samples		
		Control	Combined treatment (5 kGy + Formulation 3)	Combined treatment (5 kGy + Formulation 4)
Color	L^*	72.13 ± 0.39 a	71.40 ± 0.16 b	71.01 ± 0.49 b
	a^*	-15.6 ± 0.43 a	-14.8 ± 0.31 a	-14.9 ± 0.28 a
	b^*	2.2 ± 0.16 b	1.4 ± 0.13 a	1.4 ± 0.08 a
	ΔE	-	1.39 ± 0.07	1.38 ± 0.04
Texture	viscosity (mPa.s)	3.19 ± 0.06 b	3.02 ± 0.08 a	3.05 ± 0.07 a

ΔE is the total color change. Data are expressed in mean ± standard deviation done in triplicates. Means with different lower-case letters within same row are significantly different ($p \leq 0.05$).

4. Discussion

Previous studies have reported that the γ-irradiation treatment in combination with natural antimicrobial formulations is able to increase the radiosensitivity of potential foodborne pathogens. **Robichaud et al. (2020**) showed that 14.2 kGy is needed to reach a 6-Log reduction of *B. cereus* spores, while in our studies, irradiation at 5 kGy in combination with formulation 3 and 4 was effective on all tested pathogens including sporulated *B. cereus* suggesting that combined treatment of irradiation with antibacterial formulation is more efficient when compared to irradiation alone. **Turgis et al. (2008)** proved that the combined effect of Chinese cinnamon and Spanish oregano EOs along with γ-irradiation at 0.3, 0.4, 0.5, 0.58 and 0.6 kGy increased the radiosensitivity (a measure showing its effectiveness compared to the control) of *E. coli* O157:H7 and *S. Typhimurium* by 3.57 and 3.26 in ground beef. **Maherani et al. (2019)** showed that addition of natural antimicrobial formulation containing citrus extract in orange juice along with γ-irradiation at 1 kGy increased the radiosensitivity of fungal species *A. niger*, *S. cerevisiae* and *P. chrysogenum* by 1.54, 2.10 and 2.32 respectively. **Robichaud et al. (2021)** reported that the incorporation of acid citric in powdered and frozen infant formula had a radiosensitivity of 2.1 and 1, respectively, against *B. cereus*. These values increased to 4.1 and 2.1 for sodium carbonate in powdered and frozen infant formula, respectively. **Chiasson et al. (2005)** also investigated the combined effect of γ-irradiation along with carvacrol and tetra-sodium pyrophosphate in beef reducing the radiation dose needed to limit the detection of *S.* Typhimurium from 1.55 kGy to 0.25 kGy. This value was reduced from 0.65 kGy to 0.55 kGy in the presence of carvacrol and tetra-sodium pyrophosphate for *E. coli*. Sporulated bacterial strains requires a higher amount of irradiation dose to limit their detection in food products.

Ionizing radiations inactivate the microorganisms by disrupting the double standard DNA (breaking the sugar-phosphate bond) thereby preventing microorganism from replicating **(Lacroix, 2012)**. **Caillet & Lacroix (2006)** reported that simultaneous treatment of EOs and γ-irradiation at 1.2 kGy had synergistic effect adversely affecting the integrity of bacterial membrane and murein as well as damages to the double standard DNA resulting in cellular dysfunction and release of cell constituents from bacterial cells thereby altering the internal pH and ATP.

It is well known that under appropriate operational conditions (dose rate, temperature, presence of oxygen and food type) the nutritional values are unaffected by irradiation treatment up to 45 kGy **(Maherani et al., 2016)**. **Robichaud et al. (2021)** proved that γ-irradiation up to 10 kGy had no significant effect on the lactose, protein and MDA content of liquid infant formula which coincided with the data obtained in our studies. Similarly, **Ham et al. (2009)** also proved that lactose content of plain yogurt wasn't altered by γ-irradiation at various doses (1, 3, 5 and 10 kGy). Employing higher doses for instance at 25 kGy exhibited an increasing effect on MDA of powdered infant formula **(Tesfai et al., 2014)** supporting the results that we had obtained on MDA concentration using TBARS assay. Increase in the formation of lipid oxides is due to the change in the environmental conditions of irradiation treatment by producing oxygen radicals **(Stefanova, Vasilev, & Sapassov, 2010)**. No significant change was observed in immunoglobulin concentration after the combined treatment. The obtained results were in parallel with that of **Tran et al. (2004)**. They reported that structural and functional activity of IgG molecule was not damaged by γ-irradiation up to 50 kGy. Similarly, **Smeltzer et al. (2015)** also proved that level of IgG was not altered and integrity of its polypeptide chain was completely protected at irradiation dose of 50 kGy at -80 ˚C. It indicates the conformational integrity of immunoglobulins kept intact and was not affected by γ-irradiation.

Results showed that the irradiation decreased the HMW peptides compared to non-irradiated sample **(Table 4.3)**. A decrease of 7% and 10% of the HMW was observed in the samples subjected to the combined treatment with formulation 3 and 4 after the digestion with pepsin and trypsin as compared to the treated control. **Robichaud et al. (2020)** proved that powdered and liquid infant formula irradiated at 10 kGy decreased the proportion of >500 kDa peptides by 18% when compared to non-irradiated samples. Irradiation through generating free radicals released from water radiolysis disrupts the disulphide bonds of HMW peptides and consequently structural weakening. Partial unfolding makes it susceptible to proteolytic attack.

(Hassan et al., 2018). Irradiation can also induce deamination which can further lead to protein unfolding **(Dogbevi et al., 2000)**. Following protein unfolding, the hydrophobic residues buried in the interior will be exposed and readily affected by the pepsin and trypsin enzymes **(Murray et., 2009)**. According to **Fombang et al. (2005)**, peptide bonds are exposed to proteolytic enzymes due to the modification in the secondary and tertiary structures of protein caused by γ-irradiation.

Despite more content LMW peptides in irradiated samples compared to the control, our results showed no significant difference in the *in vitro* protein digestibility when compared to the control sample. On contrary, some studies reported an increase of the IVPD after protein hydrolysis **(Manus et al., 2021; Dridi et al., 2021)**. Induced unfolding of the protein exposes non-polar groups and increase protein hydrophobicity. Hence, there is the possibility of greater presence of hydrophobic peptides in the samples under the effect of the combined treatments. By considering the fact that the peptides size and hydrophobicity affect their solubility in TCA, the similar results for soluble nitrogen imply the same total solubility for peptides accumulated in the supernatant after TCA precipitation.

Color differences can be analytically classified as very distinct (ΔE>3), distinct (1.5< ΔE<3) and small difference (1.5>ΔE) **(Lin et al., 2020)**. **Park & Ha (2019)** showed that irradiation at 0.8 kGy did not significantly alter the L^*(lightness), a^* (redness), b^* value (yellowness) of sliced cheese. Results showed that viscosity of the mother's milk after combined treatment of irradiation (5 kGy) and antimicrobial formulation (3 and 4) was marginally reduced when compared to the control **(Table 4.5)**. **Okyere & Odamtten (2014)** showed that increasing the irradiation dose from 0 to 10 kGy correspondingly decreased the viscosity of tiger nut milk. The reduction in viscosity of irradiated sample was caused by the formation of free radicals by irradiation **(Sokhey & Hanna, 1993)**. Hydroxyl and superoxide anion radicals generated by γ-irradiation, could modify primary structure of proteins, resulting in distortions of secondary and tertiary structures. In addition, exposure of proteins to oxygen radicals results in both non-random and random fragmentation that can be the possible reasons for the reduced viscosity **(Lee et al., 2003)**.

5. Conclusion

In the present study, the inactivation of foodborne pathogens in frozen mother's milk using combined treatment of γ-irradiation and antimicrobial formulations was demonstrated. The lactose, immunoglobulin content and the lipid oxidation in frozen mother's milk weren't

affected by the combined treatment. γ-Irradiation at 5 kGy contributed to the hydrolysis of large protein molecules into smaller peptides, but no effect on digestibility was observed *in vitro*. The color difference (ΔE) and viscosity difference between irradiated and non-irradiated mother's milk were minimal. This research demonstrated that the combined treatment of γ-irradiation along with antimicrobial formulations is effective in reducing the irradiation dose for the elimination of bacteria and assures the innocuity of frozen mother's milk.

Conflicts of Interest

No conflict of interest between the authors.

Acknowledgment

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Chapter 5: General Discussion & Conclusion

5.1. Natural antimicrobial formulation

The present study confirmed the enhanced inhibitory effect of four quaternary antibacterial formulations against foodborne pathogens. All the four antibacterial formulations: oregano/ mediterranean formulation/ citrus extract/ lactic acid; oregano/ mediterranean formulation/ citrus extract/ citric acid; cinnamon/ lemongrass/ citrus extract/ lactic acid and cinnamon/ lemongrass/ citrus extract/ citric acid showed synergistic effect against all the tested bacterial strains (*E. sakazakii*, *E. coli* O157:H7, *B. cereus, L. monocytogenes, S. aureus* and *S.* Typhimurium). Sadekuzzaman *et al*. **(2018)** found a significant reduction of *E. coli,* Salmonella, and *Listeria monocytogenes* by the application of Thyme/Tea tree EOs. In the present study, Cinnamon EO alone exhibited the best antibacterial activity against *P. amylolyticus*, but this compound was non-interactive in most of the combinations. In contrast, Cinnamon EO alone was not efficient against *B. cereus* but a synergistic inhibition was achieved when it was combined with Thyme EO. Another study revealed oregano/nisin, thyme/nisin and *S. montagna* EO/pediocin showed additive effect against *E. coli* O157:H7, *L. monocytogenes* and *B. cereus* **(Turgis *et al*., 2012)**. Gavaric *et al*. **(2015)** also proved that thymol and carvacrol in combination with eugenol had a synergistic effect against *E. coli*, which can be attributed the cell membrane disintegration caused by carvacrol and thymol facilitating the penetration of eugenol into the cell cytoplasm exhibiting antibacterial properties.

5.2. Combined effect of irradiation and antibacterial formulation

An enhancement in radiosensitivity and reduction in D10 values (kGy) was observed against all bacterial strains under the combined treatment of γ-irradiation and antibacterial formulations in frozen mother's milk. All four formulations improve the bacterial radiosensitivity but comparatively formulation 3 and 4 were more effective against all pathogens including sporulated *B. cereus* which is the most resistant bacteria. Also, combined treatment of irradiation at 5 kGy in the presence of formulations 3 and 4 eliminated all the tested bacterial strains (6-Log). The Radiosensitivity of *B. cereus* spores in the presence of formulations 3 and 4 was increased by a value of 1.53 and 1.54 respectively. The highest radiosensitivity of 3.99 has been observed for *S.* Typhimurium in presence of formulation 3.

For *E. coli* O157:H7 a D10 value of 0.130 kGy has been observed in the presence of formulation 3 which was the lowest.

Both irradiation and natural antimicrobial agents showed greater inhibition against microbial pathogens individually, many research experiments were carried out simultaneously to prove the combined effect of radiation and natural antimicrobial. It has been demonstrated that the combined effect of irradiation in the presence of various natural antimicrobials can result in reduction of required dosage to inhibit bacterial growth. Takala *et al*. **(2011)** proved that the combined effect of organic acids in combination with irradiation against *S.* Typhimurium, *E. coli*, *L. monocytogenes* in fresh broccoli, due to the combined effect the radiosensitivity of the bacterial strains increased significantly by 0.05. Turgis *et al*. **(2008)** showed the presence of Cinnamon and Oregano essential oils minimized the radiation dosage from 1.2 kGy to 0.35 kGy for *E. coli* and for *Salmonella* Typhimurium it got reduced from 1.4 kGy to 0.6 kGy respectively in beef. The use of carvacrol and tetra-sodium pyrophosphate when used along with irradiation in beef reduces radiation dosage from 1.55 kGy to 0.25 kGy for *S.* Typhimurium while for *E. coli* it got reduced from 0.65 kGy to 0.55 kGy (**Chiasson *et al*., 2005**).

Recent studies demonstrate that the application of low radiation dosage on food samples, followed by the application of small amounts of natural antimicrobial formulations could help to control food borne pathogens and extend the shelf life of food samples. Gamma irradiation at 1 kGy combined with spraying of antimicrobial formulations containing oregano or lemongrass essential oil plus citrus extract and lactic acid was the most suitable treatment to inhibit the growth of pathogenic bacteria like *E. coli* and *L. monocytogenes* yeasts and molds in fresh cut cauliflower (**Tawema *et al*. 2016**). The mechanism of action of this synergistic effect is also analysed. The use of oregano essential oil and irradiation treatment increased the radiosensitivity of *E. coli* and *L. monocytogenes*. As a result of combined treatment, the internal ATP concentration and the murein wall of both bacteria were also affected. The muropeptides was found to be greatly modified, resulting in major effects on the physical integrity of the cell wall. These modifications are dependent of the radiation dose applied to the bacterial cell or the essential oil concentration used **(Lacroix *et al*. 2009)**.

5.3. Effect of combined treatment on mother's milk

5.3.1 Lactose, lipid oxidation and immunoglobulin content

In the present study, the concentration of lactose and immunoglobulins in frozen mother's milk was not significantly altered ($p \leq 0.05$) by the combined treatment of irradiation and antibacterial formulations (3 and 4). Similarly, the lipid oxidation of milk was not significantly modified ($p \leq 0.05$) by the combined treatment. The addition of any energy to food will breakdown its nutrients, therefore the effect of irradiation on the nutritional status of food has been studied extensively. When compared to the thermally sterilized foods, the nutrient and chemical content is relatively less altered in products sterilized by radiation. Microorganisms will be destroyed primarily as the hydroxyl radicals formed within their cells react with the base and sugar molecules of DNA, which results in breakage of sugar-phosphate bonds and loss of the replication function **(Lacroix *et al.*, 2010)**. Robichaud *et al*. **(2020)** showed for liquid formulation of infant formula treated with ɣ-irradiation, significant changes that were not detected in the MDA level up to 10 kGy. However, powdered samples submitted to ɣ-irradiation presented significant dose-dependent increase in lipid oxidation up to 10 kGy, which led to detect greater number of secondary products of lipid oxidation including MDA by TBARS assay

Macronutrients like carbohydrates, fats, lipids and proteins are not significantly altered in terms of nutritional value. Research studies prove that biological value of proteins and amino acids are not adversely affected by irradiation dosage up to 45 kGy **(Maherani *et al.*, 2016)**. The carbohydrates and fatty acids have no or only marginal alterations due to radiation **(Woodside 2015; Robichaud *et al.*, 2020)**.

5.3.2. *In vitro* protein digestibility and peptide profile

The *in vitro* protein digestibility of frozen mother's milk under combined treatment was not significantly affected after pepsin and trypsin digestion. Results showed that the irradiation decreased the HMW peptides compared to non-irradiated sample **(Table 4.3)**. A decrease of 7% and 10% of the HMW was observed in the samples subjected to the combined treatment with formulation 3 and 4 after the digestion with pepsin and trypsin as compared to the treated control. **Robichaud *et al*. (2020)** proved that a decrease of 18% of the fraction with molecular weight >500 kDa and a complete elimination of the fraction from 500 to 50 kDa was observed on both samples treated at 10 kGy as compared to the non-irradiated sample. A reduction of

more than 20% of the fraction <50 kDa was also observed on both samples treated at 10 kGy. Fragmentation and aggregation could occur during the irradiation of proteins (**Zarei *et al*., 2017**). However, in the presence of oxygen, mostly fragmentation was observed **(Davies, 1987)**. The γ-irradiation generates free radicals, which can disrupt hydrogen and disulphide bonds, resulting in a loss of conformational integrity. An increase in the exposition of hydrophobic amino acids like aromatic amino acids was observed, resulting in higher accessibility to the proteases and improving protein digestibility (**Hassan *et al*., 2018; Osman *et al*., 2014**).

Also, irradiation can produce deamination through protein unfolding (**Dogbevi *et al*., 2000**). According to Shih and Kalmar (1987), conversion from amide group to acid group, during deamination reaction, can be responsible for increasing protein solubility during irradiation. These results showed that the total protein content was not affected by irradiation treatment. However, the concentration of the fraction <50 kDa in liquid formulation samples treated at 5 kGy was increased compared to the non-irradiated control. **El-Niely (2007)** reported that the digestibility of cowpeas, lentils and kidney beans was increased by 27.7%, 27.9% and 15.9% at 10 kGy respectively. γ-Irradiation is accompanied by protein unfolding causing its denaturation; hence the hydrophobic residues that are buried within will be exposed and readily affected by the pepsin and trypsin enzymes **(Murray et., 2009)**.

5.4. Color and texture analysis of mother's milk

The measurement of color parameters (L*, a*, b*, ΔE) was performed to see the effect of the combined treatment on the color of mother's milk. The results obtained showed that there was a very mild difference between control and combined treatments of irradiation and antibacterial formulations (3 and 4) ($p > 0.05$) with the ΔE value less than 1.5 (very slight difference).

The results of the measurements of the lightness parameter L* (which is a parameter varying from 0 corresponding to black to 100 corresponding to white) showed that combined treatment of irradiated and antibacterial formulations (3 and 4) slightly affected ($p > 0.05$) the clarity parameter L* with a value of 71.40 ± 0.16 and 71 ± 0.49 respectively.

The results of measurements of the parameter a* (which indicate the variation of the color from green (-300) to red (+299) indicate that the application of the combined treatment did not significantly affect negatively affected this parameter.

The parameter b* represents the color change from blue (-300) to yellow (+299) showed that the combined treatment slightly affected the b* parameter, with a value between 1.4 ± 0.13 and 1.4 ± 0.08.

The viscosity of mother's milk under combined treatment was measured. Results showed a slight reduction of viscosity of mother's milk under combined treatment of irradiation and antibacterial formulation (3 and 4) with a value of 3.02 ± 0.08 and 3.05 ± 0.07 mPa.s respectively.

5.5. General conclusion

In this dissertation, we were interested in the development of treatments in combination with γ-irradiation and quaternary antibacterial formulations to ensure the safety of frozen mother's milk against foodborne bacterial strains without altering their nutritional, bioactive and nutritional properties. Totally, nine natural antimicrobials were screened including five EOs; three organic acids and bases; citrus extract capable of eliminating food pathogens. The effect of the most efficient and strong antibacterial properties of EOs, citrus extract, organic acids and salts was exhibited through MIC against all tested pathogens. The interaction between the most efficient antibacterial compounds was also demonstrated through FIC index. Based on the FIC index four quaternary antibacterial formulations were developed. The effectiveness of the combination of γ-irradiation and antibacterial formulations to eliminate the bacterial strains in frozen mother's milk was evaluated. The effect of this combined treatment on the nutritional, bioactive, *in vitro* protein digestibility, peptide profile, lipid oxidation and physicochemical properties was also evaluated.

The four developed quaternary formulations: Formulation 1 (Oregano/Mediterranean formulation/Citrus extract/Lactic acid), Formulation 2 (Cinnamon/Lemongrass/Citrus extract/Citric acid), Formulation 3 (Oregano/Mediterranean formulation/Citrus extract/Citric acid), Formulation 4 (Cinnamon/Lemongrass/Citrus extract/Lactic acid) showed synergistic effect and very effective in eliminating all the selected bacterial strains (*E. sakazakii*, *E. coli* O157:H7, *S. aureus*, *L. monocytogenes*, *S.* Typhimurium, *B. cereus*). The combined treatment of γ-irradiation and the newly developed antibacterial formulations increased the radiosensitivity of bacterial strains at a range of 1.34 to 3.99. Especially, two formulations (3 and 4) containing the highest amount of citrus extract were especially more effective and greatly induced antibacterial effect in combination with irradiation at 5 kGy against all pathogens including sporulated *B. cereus*. The combined treatment also reduced the irradiation

dosage (kGy) required to eliminate the pathogens when compared to the control (irradiation alone).

The combined treatment of irradiation at 5 kGy along with formulations 3 and 4 did not significantly affect the lactose, immunoglobulin content and the lipid oxidation potential in frozen mother's milk. The color difference (ΔE) and viscosity difference between irradiated and non-irradiated mother's milk were minimal. The combined treatment at 5 kGy in the presence of formulation 3 and 4 facilitated the hydrolysis of large protein molecules into smaller peptides.

The combination of treatments applied to donor breast milk may be subject to modifications and improvements which can be very interesting to do as future work. It will be possible to combine the UV or X ray treatment with the antimicrobial formulation to assess their potential to inhibit human milk pathogens. Also, further study on the effect of these treatments on morphology and microbial integrity can also be very interesting to exploit in order to understand and find effective solutions for the resurrection of bacteria in milk during storage. Moreover, nano emulsion of these antibacterial formulations can be done and combined along with ionizing radiations to determine their effectiveness as nano emulsion may improve the antibacterial property of the compounds used in antibacterial formulation. Other work may search in this direction and find a solution to this problem. All these results show us that indeed the combined treatments can be an interesting alternative to ensure the safety of human milk. Treatments used in combination can act synergistically thus reducing the dose or the time of processing to ensure safety and physicochemical stability without altering the properties nutritional. These results may be of great interest to milk banks and food industry for food preservation and especially for neonates and small children with weak immune system. This work presents a new alternative for this category of people by protecting them from food-borne illnesses and preserving the nutritional quality of human milk.

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