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Production d'un aliment fonctionnel à base de lait maternel enrichi en probiotiques sur les propriétés anticancer et la prévention du syndrome métabolique

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RÉSUMÉ

Depuis les 20 dernières années, la santé mondiale est en déclin et le taux de dénutrition et d'obésité est en augmentation. Ces problèmes liés à la nutrition ont de multiples conséquences sur la santé de la population tel que le syndrome métabolique et autres maladies chroniques dégénératives. La création d'un aliment fonctionnel à base de lait maternel et de probiotiques ayant des capacités nutritionnelles et médicales éprouvées pourrait être une alternative envisagée pour améliorer ou prévenir les problèmes de santé lié à l'obésité et à la vieillesse chez les personnes à risque.

En premier lieu, dans le but de produire un produit à base de lait maternel microbiologiquement sûr, il est impératif de mettre au point une technologie de décontamination efficace et rentable qui saura préserver les qualités nutritionnelles et immunologiques du produit. En effet, la présence de bactéries et surtout de spores bactériennes est une préoccupation pour les banques de lait maternel et les industries de production de préparation pour nourrissons. L'objectif de cette étude est de démontrer que l'irradiation gamma est une technologie de décontamination fiable pour les produits thermosensibles (lait maternel, lait maternisé) de différents formats (poudre ou liquide congelé), et qui pourra être applicable à différentes industries alimentaires visant des populations au système immunitaire plus fragile tel que les nouveau-nés ou les personnes âgées. Les résultats ont montré qu'à l'exception de *L. monocytogenes* et *S.* Typhimurium, tous les agents pathogènes testés étaient plus sensibles à l'irradiation sous forme reconstituée et congelée que dans le produit en poudre. En outre, le traitement par irradiation n'a entraîné aucune altération significative de la teneur en lactose et en protéines. Par contre, les propriétés antioxydantes et la quantité de vitamine C ont été altérées et une augmentation de la peroxydation des lipides a également été remarquée lorsqu'irradié sous la forme congelée. L'étude a démontré que les additifs alimentaires testés (carbonate de sodium, citrate de sodium et acide citrique) dans le lait en formulation ont induit une radiosensibilisation de 1,3 jusqu'à 4,1 pour les préparations pour nourrissons en poudre et de 1,2 à 2,4 pour les préparations liquides congelées. L'utilisation de tous les additifs augmente considérablement l'efficacité du rayonnement pour inactiver la bactérie sporulante *B. cereus*. De façon générale, le carbonate de sodium est l'additif le plus efficace parmi les additifs testés pour induire la radiosensibilisation des différentes bactéries pathogènes alimentaires, particulièrement *E. coli* et *B. cereus*.

Ensuite, les qualités nutritionnelles et immunologiques du lait maternel ont été évaluées après le processus d'atomisation et d'irradiation. Sur la base des analyses de protéines, lactose et lipides, le lait maternel séché par pulvérisation n'était pas significativement différent du lait maternel non transformé et congelé immédiatement après l'excrétion. Le lait atomisé avait une bonne rétention des immunoglobulines et a montré des propriétés antioxydantes et antimicrobiennes. Ensuite, il a été démontré qu'une dose

d'irradiation de 5 kGy a entraîné une élimination de 99,7% de la flore aérobie totale. Les micronutriments et les macronutriments ainsi que certains composés bioactifs clés (lysozyme, amylase) dans le lait maternel liquide congelé et en poudre séché par pulvérisation sont restés intacts même après un traitement à 10 kGy. Les propriétés antioxydantes n'ont pas été affectées ($p>0.05$), cependant une augmentation de la peroxydation lipidique a été notée ($p \leq 0.05$).

Finalement, des tests *in vitro* et *in vivo* du lait maternel enrichi en probiotiques ont été effectuées afin de démontrer que cet aliment pourrait réduire les risques de maladies chroniques. Les résultats *in vitro* du lait maternel enrichi avec la formulation Bio-K Plus^{MD} contenant 9.0 UFC/mL d'un mélange de *Lactobacillus acidophilus* CL1285^{MD}, *Lacticaseibacillus casei* LBC80R^{MD} et *Lacticaseibacillus rhamnosus* CLR2^{MD} ont montré que le lait maternel voit son effet antioxydant potentialisé par l'ajout des probiotiques de 25%. Cet aliment fonctionnel provoque l'apoptose des cellules tumorales intestinales et hépatiques. La quinone réductase, un enzyme reconnu pour son action anticancéreuse en raison de ses propriétés antioxydantes, détoxifiantes et antiprolifératives, est induite jusqu'à 1,8 fois dans la lignée cellulaire Hepa 1c1c7. Des résultats similaires ont été obtenus pour les cellules saines CHO-K1. L'étude *in vivo* chez des rats Wistar a montré que l'administration de lait maternel enrichi en probiotiques à des rats nourris avec un régime alimentaire riche en graisses réduisait le gain de poids de 10% et entraînait une réduction des dommages oxydatifs des lipides et des protéines rénales supérieure à 50%. Dans le sang, les taux d'insuline et de l'inhibiteur de l'activateur du plasminogène 1 (PAI-1) étaient considérablement réduits de 22% et le rapport glucose / insuline est augmenté. Les rats ont retrouvé des valeurs de stress oxydatif et de marqueur de l'inflammation proches de la normale.

En conclusion, cette étude suggère que le développement d'un aliment fonctionnel à base de poudre de lait maternel enrichi en probiotiques pourrait être utilisé pour moduler le taux d'inflammation et les dommages oxydatifs aux tissus induits par des maladies liées à l'obésité et que l'irradiation gamma est une technologie sûre, efficace et pratique pour s'assurer de la sécurité microbiologique d'un tel produit tout en préservant un maximum de ses qualités nutritionnelles et immunologiques.

Mots clés : Irradiation, lait humain, préparation pour nourrisson, décontamination, valeur nutritive, syndrome métabolique, probiotique

ABSTRACT

For the past 20 years, global health has been in decline and the rate of undernutrition and obesity has been increasing. These nutritional problems have multiple consequences on the health of the population such as metabolic syndrome and other chronic degenerative diseases. The creation of a functional food based on human milk and probiotics with proven nutritional and medical properties is an alternative considered to improve or prevent health problems linked to obesity and old age in people at risk.

First, in order to produce a microbiologically safe human milk product, it is imperative to develop an effective and profitable decontamination technology that will preserve the nutritional and immunological qualities of the product. In fact, the presence of bacteria and especially bacterial spores is a concern for human milk banks and infant formula production industries. The objective of this study is to demonstrate that gamma irradiation is a reliable decontamination technology for heat-sensitive products (human milk, infant formula) of different formats (powder or liquid frozen), and which could be applicable to different food industries targeting populations with weaker immune systems such as newborns and the elderly. The results showed that, with the exception of *L. monocytogenes* and *S.* Typhimurium, all the pathogens tested were more sensitive to irradiation in reconstituted and frozen form than in the powdered product. In addition, the irradiation treatment did not cause any significant alteration in the lactose and protein content. On the other hand, the antioxidant properties and the amount of vitamin C were altered and an increase in lipid peroxidation was also noted when irradiated in the frozen form. The study demonstrated that the food additives tested (sodium carbonate, sodium citrate and citric acid) induced radiosensitization from 1.3 to 4.1 for powdered infant formula and from 1.2 to 2.4 for liquid frozen infant formula. The use of all additives considerably increases the effectiveness of the radiation to inactivate the spore-producing bacteria *B. cereus*. In general, sodium carbonate is the most effective additive among the additives tested to induce radiosensitization of various food pathogenic bacteria, particularly *E. coli* and *B. cereus*.

Then, the nutritional and immunological qualities of human milk were assessed after the atomization and irradiation process. Based on protein, lactose and fat analyzes, spray-dried human milk was not significantly different from the unprocessed frozen human milk. Atomized milk had good immunoglobulin retention and showed antioxidant and antimicrobial properties. It was shown that a dose of 5 kGy irradiation resulted in the elimination of 99.7% of the total aerobic flora. The micronutrients and macronutrients as well as some key bioactive compounds (lysozyme, amylase) in frozen and spray dried powdered human milk were found to be intact even after treatment with 10 kGy. The antioxidant properties were not affected ($p > 0.05$), however an increase in lipid peroxidation was noted ($p \leq 0.05$).

Finally, *in vitro* and *in vivo* tests of human milk enriched with probiotics were carried out to demonstrate that this food could reduce the risk of chronic diseases. The *in vitro* results of human milk enriched with the Bio-K Plus formulation containing 9.0 CFU / mL of a mixture of *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R and *Lacticaseibacillus rhamnosus* CLR2 have shown that human milk has its antioxidant effect increased by 25% with the addition of probiotics. It induced apoptosis of intestinal and hepatic tumor cells. The study of quinone reductase, an enzyme recognized for its anticancer action due to its antioxidant, detoxifying and antiproliferative properties, is induced up to 1.8 times in the Hepa 1c1c7 cell line. Similar results were obtained for healthy CHO-K1 cells. The *in vivo* study in Wistar rats showed that administering probiotic-enriched human milk to rats fed a high-fat diet reduced weight gain by 10% and reduced oxidative lipid damage and kidney protein by greater than 50%. In the blood, insulin and plasminogen activator inhibitor 1 (PAI-1) levels are significantly reduced by 22% and the glucose / insulin ratio is increased. The rats fed with human milk had oxidative stress and inflammation marker levels closer to normal.

In conclusion, this study suggests that the development of a functional food based on human milk powder enriched with probiotics could be used to modulate the rate of inflammation and the oxidative damage to tissues induced by diseases linked to obesity and that gamma irradiation is a safe, effective and practical technology to ensure the microbiological safety of such a product while preserving a maximum of its nutritional and immunological qualities.

Key words: Irradiation, human milk, infant formula, decontamination, nutritional value, metabolic syndrome, probiotic

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CHAPITRE 1 – INTRODUCTION

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1. Irradiation

1.1. Généralités

Quand cela est possible, il est reconnu que l'allaitement d'un nourrisson par sa mère est la meilleure façon de nourrir un enfant afin de s'assurer de sa croissance et de sa santé. Le lait maternel contient un équilibre spécifique de protéines, gras et sucre en plus de contenir des facteurs bioactifs tels que des enzymes, anticorps, facteurs de croissance, nucléotides, bactéries bénéfiques et oligosaccharides. Il a été démontré que le lait humain chez les enfants prématurés est particulièrement important pour prévenir diverses pathologies telles que l'entérocolite nécrosante (Commonwealth of Australia, 2014). Également, l'administration de lait humain suite à un diagnostic ou à une chirurgie a été démontré comme étant grandement bénéfique comparativement au lait en formulation pour le rétablissement du nourrisson (Commonwealth of Australia, 2014). Pour pallier aux situations pour lesquelles un nouveau-né ne peut bénéficier du lait de sa mère et pour lesquelles ceci s'avère important (prématuré, post-chirurgie ou à risque d'entérocolite nécrosante), des banques de lait maternel ont été mises en place à travers le monde entier. Puisque le lait maternel est un produit sécrété par l'humain, des considérations concernant la sécurité alimentaire sont à prendre en compte. Pour contrôler la charge virale et microbiologique du lait maternel, la pasteurisation à la chaleur est la méthode établie dans la majorité des établissements. Puisque le lait contient des cellules vivantes et des substances biologiquement actives, le traitement à la chaleur ainsi que les cycles de congélation/décongélation peuvent diminuer le pouvoir du lait maternel (Commonwealth of Australia, 2014).

Tableau 1. Effet de la pasteurisation sur des composantes du lait humain

Peu ou pas d'effets	Réduction significative	Destruction ou inactivation
- Lactose - Oligosaccharides - Vitamine A, D et E - Facteurs de croissance insulinoïde - Protéines liant les facteurs de croissance insulinoïde - Gangliosides - Glucoroconjugué - Acide gras polyinsaturés à longue chaîne - Facteur de croissance épidermique	- Propriétés d'inhibition de la croissance bactérienne - Lysozyme (jusqu'à 86%) - Anticorps (IgG, IgA, IgM) - Lactoferrine (jusqu'à 65%) - Facteur C3 du complément - Glutathion peroxydase - Vitamines B et C - Acide folique - Amylase - Facteurs de croissance (IGF,EPO, HGF) - Hormones (insuline, adiponectine)	- Phosphatase alcaline - Lipases

(Tiré de l'encart informatif sur le lait maternel pasteurisé (Hema-Québec, 2020) et adapté de Peila *et al.* (2016))

Bien qu'il soit essentiel que le lait soit décontaminé, on peut se demander si la pasteurisation à chaleur est la meilleure méthode pour garantir la sécurité du lait sans détruire ses composants nutritionnels et immunologiques. Une des raisons supplémentaires est que la pasteurisation Holder, la plus souvent utilisée dans les banques de lait maternel, échoue souvent à éradiquer les spores bactériennes, ce qui entraîne le rejet et la perte des précieux dons et représente un risque pour les enfants receveurs (Cacho *et al.*, 2017).

1.2. Risques microbiologiques de la préparation pour nourrissons et le lait maternel

Bien que la pasteurisation Holder soit efficace, certaines bactéries peuvent survivre au processus de pasteurisation, particulièrement les bactéries sporulées telles que les *Bacillus*. Le genre *Bacillus* a plus de 250 espèces identifiées, dont certaines sont des pathogènes alimentaires et sont retrouvés sur la peau, tel que *Bacillus cereus*, et représentent donc un risque de se retrouver dans le lait humain (Froh, Vanderpool, & Spatz, 2018). En fait, selon les données d'Hema-Québec, la contamination des dons de lait fait en sorte que plus de 18% du lait donné a été perdu en 2019. Depuis la création de la banque de lait maternel, de nombreuses améliorations dans les processus sanitaires ont pu diminuer de beaucoup les pertes, puisque plus de 40% des dons étaient rejetés il y a quelques années. Le but visé par l'organisation serait entre 8 à

10% de pertes, mais il y a moins d'un an encore, plusieurs milliers de litres en précieux dons de lait humain ont été perdus (Le Soleil, 2020). Dans les aliments pour nourrissons, la teneur en spores ne devrait jamais dépasser 100 spores / g, mais les banques de lait maternel rejettent tout don positif pour les bactéries sporulantes (Holsinger, Rajkowski, & Stabel, 1997; Rigourd et al., 2018). Une étude de 261 échantillons de dons de lait maternel dans 17 pays a montré que 52% des échantillons contenaient entre 0,3 et 10 bactéries par gramme (Becker, Schaller, von Wiese, & Terplan, 1994). Plusieurs autres pathogènes alimentaires sont à risque de contaminer le lait maternisé et le lait maternel, soit par contamination féco-orale ou par contact direct. Ils sont susceptibles de causer des maladies pouvant aller jusqu'à la septicémie et la mort via des bactéries pouvant être retrouvées sur la peau, tels que *Staphylococcus aureus* et *Escherichia coli* (Carré, Dumoulin, Jounwaz, Mestdagh, & Pierrat, 2018; Widger, O'Connell, & Stack, 2010).

Le lait maternisé est traité thermiquement pendant la fabrication, mais pas suffisamment pour rendre le produit final stérile sur le plan commercial (NSF Food Authority, 2011). Ainsi, plusieurs contaminations ont été retrouvées dans ce produit au fil des années, et l'irradiation du produit a déjà été étudié en ce qui concerne l'inactivation de *Enterobacter sakazakii* (Osaili, Shaker, Abu Al-Hasan, Ayyash, & Martin, 2007). Également, la préservation de l'intégrité des protéines et acides aminés avait été étudiée (Matloubi, Aflaki, & Hadjiezadegan, 2004). Salmonella spp. est un agent pathogène d'origine alimentaire qui a entraîné plusieurs rappels de lait en poudre pour bébés en 2005, puis en 2017. Il aurait rendu au moins 38 bébés malades et a accusé plus de 300 millions d'euros en perte monétaire (BFM ÉCO, 2019). *Salmonella* est également un agent pathogène transmis de la mère au lait maternel (Widger *et al.*, 2010).

1.3. Irradiation gamma

L'irradiation est approuvée depuis 1981 par les Nations Unies comme traitement de conservation. Cette technologie est utilisée sur tous types de produits, frais, sec ou congelés. Une limite de 10kGy est couramment de mise pour les aliments consommables sans compromettre la qualité nutritionnelle et sensorielle ni causer de risques toxicologiques (Osaili *et al.*, 2007). Toutefois, un comité de l'Organisation Mondiale de la Santé a approuvé des doses de plus de 45 kGy pour la stérilisation des aliments sous forme congelé (Maherani *et al.*, 2016). Il a été admis que cette dose n'affectait pas les propriétés nutritives.

Le rayonnement gamma présente de nombreux avantages par rapport à la pasteurisation. Tout d'abord, il s'agit d'un procédé de pasteurisation à froid, ce qui signifie que les risques de dénaturation des protéines et d'analytes immunologiques sont réduits. Deuxièmement, ce processus peut être effectué sur les produits solides ou liquides, frais, cuits ou congelés et emballés ou non, ce qui rend la technologie versatile et

polyvalente. De plus, l'intérêt pour les consommateurs à consommer des aliments le plus frais possible tend à rendre obsolète les techniques de pasteurisation et stérilisation à la chaleur (Szczawinska, 2017).

Plusieurs études ont démontré que l'irradiation gamma permet de rendre les aliments plus sécuritaires contre les pathogènes alimentaires les plus communs, augmentant ainsi le temps de conservation et la qualité des aliments. Il peut inactiver les bactéries pathogènes et retarde la germination. Il est également possible de combiner divers traitements pour diminuer la dose d'irradiation demandée pour un produit. En effet, des travaux sur le bœuf haché ont permis de démontrer qu'il est possible d'augmenter de plus de 5 à 10 fois la radiosensibilité de certains pathogènes alimentaires tels que *Escherichia coli* et *Salmonella* Typhi (Chiasson, Borsa, Ouattara, & Lacroix, 2004; Huq, Vu, Riedl, Bouchard, & Lacroix, 2015).

La dose de rayonnement reçue est généralement mesurée en gray. Un gray est une unité dérivée de rayonnement ionisant et est définie comme l'absorption d'un joule d'énergie de rayonnement dans une masse d'un kilogramme (1 Gy = 1 J/kg)(Szczawinska, 2017). Le choix de la dose d'irradiation dépend de la réduction bactérienne recommandée par les normes. De faibles doses, inférieures à 2 kGy, sont utilisées pour retarder la germination des légumes et le vieillissement des fruits. Une dose comprise entre 1 et 10 kGy est utilisée pour réduire les niveaux d'organismes pathogènes. Enfin, une dose supérieure à 10 kGy est utilisée pour obtenir la stérilité du produit (Komolprasert & Morehouse, 2004). Les valeurs D_{10} sont utilisées en recherche et en industrie afin d'établir la dose d'irradiation demandée pour obtenir la stérilisation ou la décontamination d'un produit. Une valeur D_{10} est définie comme étant la dose requise d'irradiation pour éliminer 1 Log UFC bactérien pour une matrice alimentaire donnée, une température et un environnement gazeux donné (Szczawinska, 2017). Dans notre étude, nous avons déterminé la dose d'irradiation dans le lait maternel et les préparations pour nourrissons induisant une réduction d'au moins 5 Log des bactéries végétatives, sur la base des normes HACCP (Breidt, Hayes, Osborne, & McFeeters, 2005).

Le rayonnement gamma pénètre la matière sans la modifier et peut causer des lésions sur l'ADN, les protéines ou les lipides. Ainsi, les bactéries et autres microorganismes voient leur structure externe endommagés et leurs enzymes et matériel génétique modifiés, ce qui cause leur mort. De plus, l'irradiation interfère avec l'eau disponible, ce qui crée des radicaux libres qui à leur tour peuvent également créer des dommages létaux aux bactéries. La résistance des microorganismes à l'irradiation dépend de la matrice alimentaire et de la température d'irradiation. Ainsi, le pH, l'environnement gazeux, la température, la disponibilité en eau peuvent rendre les bactéries plus ou moins résistantes à l'irradiation. Également, la concentration en microorganismes, leur âge, le genre bactérien et le type de cellule (les spores sont plus résistantes que les bactéries végétatives) vont influencer leur résistance au traitement. Finalement, les

nourritures sèches ou congelées demandent de plus grandes doses d'irradiation, mais est également plus résistante aux dommages oxydatifs sur l'aliment tels que l'oxydation des lipides (Szczawinska, 2017).

Il a été démontré que l'irradiation n'altère pratiquement aucun des macronutriments (protéines, glucides et lipides), bien que cela dépende des doses d'irradiation et de la matrice alimentaire. Les vitamines sont connues pour être plus sensibles, mais cela dépend aussi de la nature de la nourriture et des vitamines (Woodside, 2015). Enfin, même si le processus d'irradiation génère des ROS (espèces réactives de l'oxygène), on peut se demander si des dommages oxydatifs se produisent ou non. En effet, la perte ou l'augmentation des propriétés antioxydantes varie avec les doses d'irradiation, la matrice alimentaire et le contenu nutritionnel (propriétés lipidiques, protéiques et antioxydantes) (Kavitha et al., 2015; Kim et al., 2009).

1.4. Utilisation d'additifs alimentaires pour induire la radiosensibilisation bactérienne

Dans l'optique d'améliorer l'efficacité des traitements antibactériens par irradiation gamma, l'utilisation d'additifs alimentaires est de plus en plus étudié par plusieurs industries. En effet, l'effet des traitements combinés permet d'augmenter la sensibilité bactérienne à l'irradiation, ce qui permet d'avoir un meilleur contrôle de la qualité microbiologique des produits, d'augmenter l'efficacité et de réduire les coûts sans compromettre la sécurité des aliments. Cela aide également pour la préservation de la qualité sensorielle et nutritionnelle (Huq *et al.*, 2015). Une sensibilité accrue aux radiations des micro-organismes cibles, en particulier des bactéries sporulantes, entraînerait des doses plus faibles requises pour la létalité, ce qui contribue à accroître la sécurité sanitaire des aliments tout en étant rentable (Lacroix, Caillet, & Shareck, 2009). Divers additifs naturels et synthétiques ont été prouvés efficace dans une grande variété de produits alimentaires. Par exemple, les carottes enrobées avec de la nisine et de carvacrol ont induit la radiosensibilisation de *Listeria monocytogenes* de 2,7 fois (Ndoti-Nembe, Vu, Doucet, & Lacroix, 2013).

En somme, l'irradiation est un bon moyen de décontamination pour les produits emballés ou non, sous forme solide, poudre, congelée, fraiche ou séchée. Il est possible de décontaminer ou stériliser un produit en ayant aucun effet significatif sur la valeur nutritionnelle et il est possible de réduire radicalement la dose d'irradiation visée par les traitements combinés, que ce soit par des additifs alimentaires, des huiles essentielles, par un pré-traitement d'ozonation, un traitement à la chaleur, ou en changeant les paramètres de l'atmosphère.

1.5. Aliment fonctionnel

Dans un monde industriel qui regorge de produits artificiels et transformés, les consommateurs demandent de plus en plus des ingrédients naturels et des produits non-transformés. De plus, ils demandent la disponibilité d'aliments possédant des bienfaits marqués pour la santé afin d'éviter le développement de maladies telles que l'obésité, le diabète, les maladies cardiovasculaires et le cancer, qui sont les véritables fléaux du 21e siècle. Ainsi, des aliments optimisés pour leurs bienfaits nutritionnels, antioxydants ou autres font l'apparition sur le marché en tant qu'aliments fonctionnels. Selon Santé Canada, un aliment fonctionnel est un aliment normal ou similaire en apparence, qui peut être consommé dans le cadre d'une diète normale et « qui procure, au-delà des fonctions nutritionnelles de base, des bienfaits physiologiques précisés par la documentation scientifique et qui réduit le risque de maladies chroniques ». Plusieurs de ces aliments sont disponibles sur le marché, tel qu'un yogourt enrichi en probiotiques, un œuf enrichi en oméga-3 ou un jus additionné de calcium. Ainsi, le lait maternel enrichi en probiotiques est un bon candidat pour être un aliment fonctionnel, puisqu'on lui attribue des propriétés anti-inflammatoires, antioxydantes, nutritionnelles (Gila-Diaz *et al.*, 2019).

1.6. Lait maternel

Le lait maternel est un fluide nutritif complet et dynamique. Il change tout au long de l'allaitement, selon le temps de la journée, l'indice de masse corporelle, l'environnement, la diète de la mère et peut même s'adapter à l'état de santé du nourrisson (Gila-Diaz *et al.*, 2019). Mis à part ses protéines, glucides, lipides et vitamines, il est également porteur d'éléments bioactifs aidant au développement de l'enfant ou sa protection immunitaire. L'ensemble des hormones, cytokines, facteurs de croissances, probiotiques, prébiotiques et antioxydants pourrait contribuer à promouvoir un développement sain de l'enfant, allant jusqu'à prévenir le développement de maladies chroniques telles que les maladies cardiovasculaires. En effet, les enfants prématurés nourris avec du lait maternel auraient un taux réduit de syndrome métabolique, d'hypertension ou de diabète comparativement aux enfants nourris avec de la préparation pour nourrissons (Gila-Diaz *et al.*, 2019).

1.7. Probiotiques

1.7.1 Probiotiques et syndrome métabolique

Il est accepté depuis plusieurs années déjà que le microbiote intestinal a un rôle majeur dans le développement du syndrome métabolique. Celui-ci est fortement influencé par la diète et par la présence de prébiotiques. Les bactéries présentent dans le tractus intestinal interviennent dans plusieurs aspects du métabolisme, notamment en influençant l'accumulation des gras, le métabolisme des glucides, le péristaltisme et l'appétit par la sécrétion et la dégradation de diverses molécules (Festi *et al.*, 2014).

Un des mécanismes les plus étudiés de la participation des probiotiques dans le métabolisme des gras est la production d'acides gras à chaîne courtes (AGCC) par l'hydrolyse et la fermentation des polysaccharides (Festi *et al.*, 2014). Par des mécanismes variés entre la stimulation de transporteurs et l'activation de récepteurs à la surface des intestins, les AGCC produits tels que propionate, butyrate et acétate influent sur la dépense énergétique, l'absorption d'énergie, le transit intestinal et l'appétit, tout en stimulant la lipogenèse et en diminuant le stress oxydatif (Festi *et al.*, 2014).

Les probiotiques ont également un rôle dans la modulation du stress oxydatif induit par le syndrome métabolique par la protection de la barrière épithéliale et la prévention de l'endotoxémie en resserrant les jonctions entre les cellules épithéliales et en stimulant les cellules dendritiques et des récepteurs immunitaires (Festi *et al.*, 2014). En effet, dans une étude récente, des souris obèses nourries avec 9 Log UFC (unité formant colonie)/jours de *Lacticaseibacillus casei* CRL 431 a permis de réduire l'inflammation dans le petit intestin, le foie ainsi que les tissus adipeux (Novotny Núñez, Maldonado Galdeano, de Moreno de LeBlanc, & Perdigón, 2015). Cela a été conclu par l'observation de la diminution de IFN-α, TNF-β, IL-6 ainsi qu'IL-17 et la diminution de l'infiltration de cellules immunitaire dans le foie.

1.7.2 Probiotiques et cancer

Le cancer est une des causes les plus importantes de mortalité au 21^{e} siècle. Depuis plusieurs années, les études démontrent qu'un microbiote intestinal sain est essentiel à la santé générale et à la balance homéostatique chez l'homme. En ce sens, il est démontré que l'ingestion de probiotiques, notamment des genres *Lactobacillus* et *Bifidobacterium*, promeut la santé intestinale et aide à la régulation de l'inflammation et du stress oxydatif au niveau intestinal et somatique. Plusieurs études *in vivo* et *in vitro* impliquant une variété de souches probiotiques ont démontré des effets antiprolifératifs et anti-tumeur et plusieurs mécanismes ont été étudiés pour en comprendre la cause, ce qui pourrait mener à une utilisation des probiotiques comme outil dans la lutte contre les cancers. En premier lieu, certaines souches

probiotiques ont démontré avoir un rôle significatif dans la modulation du système immunitaire lors de l'établissement des tumeurs (Górska, Przystupski, Niemczura, & Kulbacka, 2019).

Dans le même sens, certaines souches ont une influence sur la production de cytokines pro-inflammatoires, à la fois par l'augmentation ou la réduction de celles-ci. Cela a de nombreuses incidences, notamment, l'activation des phagocytes afin d'éliminer les cellules cancéreuses aux premiers stades de la pathologie (Górska *et al.*, 2019).

Ensuite, certaines souches bactériennes seraient responsables de la détection et la dégradation de molécules présumées carcinogènes et en faisant compétition à des couches bactériennes productrices de molécules et d'enzymes carcinogènes telles que b-glucuronidase, azoréductase et nitroréductase. Également, la production des AGCC aurait un rôle dans l'induction de l'apoptose et le contrôle de la prolifération, puisqu'ils sont connus pour participer à la signalisation de ces mécanismes de contrôle cellulaire et à l'intégrité de la barrière épithéliale (Górska *et al.*, 2019).

Une autre conséquence collatérale de la présence de bactéries lactiques dans le tractus intestinal est la production d'acide lactique, qui s'est révélée avoir un rôle dans la réduction de la croissance et l'agressivité des cancers dû à son pouvoir immunomodulateur, particulièrement sur les cellules épithéliales intestinales. Également, l'effet de l'abaissement du pH est aussi une conséquence physicochimique de la prolifération de ces bactéries et aurait un rôle direct dans l'inhibition de la carcinogénèse du cancer du côlon (Górska *et al.*, 2019).

1.7.3 Probiotiques, prébiotiques et lait humain

Le lait humain contient des prébiotiques, tels que les oligosaccharides, qui promeuvent la croissance spécifique ou facilitent la colonisation de certaines bactéries probiotiques telles que les bifidobactéries et les lactobacilles. Ces prébiotiques sont en fait la source majeure d'hydrates de carbone dans le lait humain et sont non-digestibles pour l'humain (Hoang et al., 2019; Sanders, Merenstein, Reid, Gibson, & Rastall, 2019). Pour être un prébiotique, cette substance doit également avoir certains bénéfices pour la santé. En effet, il a été démontré que les prébiotiques permettent la colonisation de bactéries bénéfiques dans les intestins des nouveau-nés (Hoang *et al.*, 2019) et pourraient également influencer leur fonction (Sanders *et al.*, 2019). Ainsi, certains prébiotiques ont été reconnus pour augmenter la colonisation de souches probiotiques productrices de butyrate dans les intestins, ayant ainsi un impact sur le métabolisme des lipides. Malgré cela, notre compréhension de l'impact des prébiotiques sur la santé globale est très incomplète. En général, il est établi qu'ils augmentent l'impact déjà observés chez les probiotiques, c'est-à-dire meilleure défense contre les pathogènes, meilleure immunité, meilleure absorption des minéraux, meilleur

péristaltisme et effet au niveau de différents métabolismes et de la satiété par les bactéries productrices de AGCC (Sanders *et al.*, 2019). Très peu d'études cliniques ont été effectuées chez les adultes. Toutefois, l'utilisation des prébiotiques dans l'optique de concevoir un aliment fonctionnel pour promouvoir la santé intestinale et la satiété est permise par les autorités européennes.

En ce sens, certaines études ont démontré que les enfants nourris avec du lait en formulation additionnée de prébiotiques et probiotiques voyaient une diminution de diarrhées, de maladies inflammatoires et d'allergies par rapport au groupe sans prébiotiques. Toutefois, de plus en plus d'études démontrent que les probiotiques, lorsqu'administré avec le lait humain plutôt que le lait en formulation pourrait procurer des bienfaits supérieurs pour la prévention de maladies inflammatoires et métaboliques chez les nouveau-nés, telles que l'entérocolite nécrosante (Repa *et al.*, 2015). Dans une étude avec des chiots, le lait humain enrichi avec *Lactobacillus reuteri* DSM 17938 a permis une stimulation maximale de l'activation immunitaire de l'intestin par rapport aux rats nourris avec le lait humain sans les probiotiques (Hoang *et al.*, 2019).

L'amplification des bienfaits pour la santé des probiotiques en présence de prébiotiques est appelée synbiotique. L'utilisation d'aliments synbiotiques en santé humaine est de plus en plus étudiée, notamment dans la commercialisation d'aliments fonctionnels (Maftei, 2019). Le lait humain enrichi en probiotiques peut être un aliment synbiotique puisqu'il peut potentialiser les bienfaits des probiotiques par la présence des prébiotiques et des facteurs immunologiques qu'il contient.

1.8. Syndrome métabolique (SM)

Cela fait des décennies que le lien entre l'obésité et les maladies cardiaques a été établi (Oda, 2018). Depuis plus de cent ans déjà, les médecins avaient identifié un groupe à risque de souffrir de maladies cardiovasculaires. En effet, les hommes obèses âgés de 45 à 55 ans présentant une surabondance de graisse étaient connus pour être à risque de problèmes de santé (Oda, 2018). Assez rapidement, l'hypertension, plus que l'accumulation de graisse, s'est avérée être le facteur de risque prédominant d'infarctus (Oda, 2018). Depuis lors, il a été prouvé que l'accumulation de graisse viscérale et l'hypertension artérielle sont des facteurs de risque parmi d'autres pouvant augmenter les risques de maladie coronarienne athéroscléreuse, de diabète, de tolérance au glucose, et beaucoup plus (Oda, 2018). Nos connaissances actuelles nous permettent de classer cinq facteurs de risque prédominants qui, pris indépendamment, augmentent le risque de souffrir de différents problèmes de santé métabolique. Ceux-ci comprennent une accumulation de graisse abdominale élevée, des taux de cholestérol et de triglycérides élevés (dyslipidémie), un taux élevé de glucose / résistance à l'insuline et une pression artérielle élevée (O'Neill & O'Driscoll, 2015). Pris ensemble, ces facteurs augmentent considérablement le risque de souffrir de diabète, de maladies cardiovasculaires et de certains cancers courants (Esposito, Chiodini, Colao, Lenzi, & Giugliano, 2012). En fait, les patients qui cumulent au moins trois de ces facteurs de risque souffrent du syndrome métabolique (MetS) et risquent de décéder prématurément (Katzmarzyk, Church, Janssen, Ross, & Blair, 2005).

1.8.2 Inflammation chronique de bas grade

L'inflammation est une réaction normale du système immunitaire face à une menace (bactérie, virus) et à des lésions organiques. Les facteurs inflammatoires (cytokines, chimiokines, facteurs de croissance, etc.) sont rapidement sécrétés par les cellules immunitaires, mais aussi par les cellules locales. Ils permettent la communication entre les systèmes afin que le corps puisse combattre les infections et se protéger. Une inflammation de bas grade est une caractéristique de la plupart des maladies chroniques non infectieuses de tous types. Elle est mesurée par la quantification de différentes cytokines et par le recrutement des leucocytes dans le sang. Ils maintiennent le corps en état d'alerte constante, ce qui peut être stressant pour le corps. À long terme, il peut contribuer à causer des dommages, un stress oxydatif et affaiblir le système immunitaire. Il peut également mener à la dysfonction des organelles, notamment le réticulum endoplasmique et les mitochondries (Monteiro & Azevedo, 2010). L'inflammation peut être mesurée à l'aide de différents facteurs sanguins ou directement dans les tissus. Chez les sujets obèses, on observe souvent une inflammation de faible intensité associé à la résistance à l'insuline, ce qui est corrélé avec une élévation des cytokines et des adipokines inflammatoires dans les tissus et le sang tels que PAI-1, IFN-g, TNF-a, IL-1B et IL- 6 (Francisqueti et al., 2017; Tack, Stienstra, Joosten, & Netea, 2012; Wu, Zhang, Dai, Han, & Chen, 2016).

L'inflammation et la dyslipidémie surviennent après des semaines de conditions d'ingestion excessive d'énergie. Les adipocytes présentent alors une accumulation excessive de graisse due à l'hypertrophie, qui conduit à une infiltration de macrophages, une inflammation et un dysfonctionnement du tissu adipeux endocrinien (Tsatsoulis, Mantzaris, Bellou, & Andrikoula, 2013).

1.8.3 Stress oxydatif

Le bien-être du corps humain repose sur les interactions complexes entre l'apport énergétique via la nutrition, la transformation de cette énergie via le système digestif et l'utilisation de cette énergie pour permettre aux différents systèmes, organes et cellules d'être actifs et en bonne santé. Chacune de ces opérations nécessite une communication complexe et précise vers et entre les cellules et les organes, qui est réalisée via des hormones et des protéines. Mais la communication intrinsèque au sein des différents organites est essentiellement réalisée par des métabolites tels que les espèces réactives de l'oxygène (ROS). Les ROS sont des métabolites secondaires principalement produits par les mitochondries lors de la production d'ATP via la chaîne respiratoire ou résultant d'activités enzymatiques telles que la NADPH oxydase (Carrier, 2017). Ils sont générés lorsqu'un électron libre interagit avec des molécules réactives telles que l'O_2, qui engendre un intermédiaire réactif en oxygène (Sabharwal & Schumacker, 2014). Le résultat est une molécule réactive capable de signalisation intracellulaire via la modification de la protéine cible sensible au rédox, mais pouvant également avoir un effet cytotoxique par sa capacité à provoquer la peroxydation des lipides, des dommages sur les protéines et une modification des acides nucléiques (Srivastava, Sinha, Saha, Marthala, & D'Silva, 2014).

Il est donc essentiel que la cellule maintienne un équilibre entre la quantité de ROS présente dans la cellule et maintienne la voie de signalisation essentielle sans subir de dommages collatéraux, en particulier dans les cellules très actives, ce qui produit une plus grande quantité de ROS. Ceci est accompli grâce à un métabolite antioxydant et à une enzyme produite par la cellule elle-même, capable de maintenir le niveau optimal de ROS (Srivastava *et al.*, 2014). Parmi les enzymes les plus courantes, il y a la superoxyde dismutase (SOD), qui élimine les anions superoxyde en produisant du peroxyde d'hydrogène, qui est ensuite rapidement éliminé par des enzymes telles que les catalases, la glutathion peroxydase (GSH) et la glutathion réductase (GRX) (Gopčević *et al.*, 2013).

Il a été démontré que l'obésité induite par un régime gras chez le rat modifie de nombreux composés biochimiques présents dans le sérum et les organes tels que le foie, les reins et le cœur. Le niveau élevé de stress oxydatif élevé et le manque d'antioxydants disponibles entraînent une réaction des ROS avec les lipides ou les protéines, conduisant à des dommages irréversibles. Ainsi, la peroxydation lipidique est un

dommage irréversible et est toxique pour la cellule. C'est un excellent marqueur au niveau des tissus et du sang de la présence de stress oxydatif et est mesuré par la présence de malondialdéhyde (MDA). Les ROS peuvent également réagir avec des protéines et produire des dérivés de protéines, soit des aldéhydes et des cétones (PCO). La modification des protéines est également irréversible et peut être inoffensive et non-enzymatique ou au contraire, peut directement inactiver ou affecter des systèmes cellulaires et causer des dommages préjudiciables (Pirinccioglu, Gökalp, Pirinccioglu, Kizil, & Kizil, 2010). Ainsi, MDA et PCO sont des marqueurs couramment utilisés pour estimer le stress oxydatif dans le sang et les tissus et sont significativement augmentés dans le syndrome métabolique (MetS), chez les sujets obèses et dans certains cancers (Monteiro & Azevedo, 2010).

1.8.4 Cancer et syndrome métabolique

Les résultats de plusieurs études épidémiologiques effectuées dans le monde ont montré que le syndrome métabolique est associé de façon constante à un risque accru de plusieurs cancers chez les adultes, particulièrement le cancer du pancréas, du foie, du rein et colorectal, autant chez les hommes que chez les femmes (Esposito *et al.*, 2012). En ce sens, le risque de mourir de cancer est augmenté de 56% lorsque le patient souffre de syndrome métabolique (MetS), et de nombreuses études ont montré que les facteurs reliés au syndrome métabolique jouent un rôle important dans l'aggravation de la progression ainsi que le pronostic de certains cancers (Braun, Bitton-Worms, & LeRoith, 2011).

L'obésité semble favoriser les tumeurs locales, et semble être associé à plus de chances de rechutes ainsi qu'à de la mortalité. Les femmes obèses ont également de plus grandes chances de développer des cancers ayant un récepteur pour l'œstrogène, et jusqu'à 50% des mortalités dues au cancer du sein sont reliées à l'obésité. La dyslipidémie, quant à elle, augmenterait les risques de cancers du sein et de la prostate dû au mauvais fonctionnement endocrine des cellules lipidiques. Le haut taux de glycémie et le diabète sont également associés à l'augmentation du taux de mortalité suite à un cancer. Cela est attribué à l'inflammation, à la résistance à l'insuline et au stress oxydatif qui est associé au syndrome métabolique (Braun *et al.*, 2011).

L'inflammation est depuis longtemps connue comme un facteur causal de plusieurs cancers. Les individus souffrant du MetS ont un niveau élevé de cytokines circulantes, en particulier IL-6 et TNF-α produits par les macrophages infiltrés dans les tissus lipidiques et les adypocytes inflammés (Braun *et al.*, 2011). Ces cytokines sont connues pour être liées au développement du cancer. L'adiponectine, sécrétée par les cellules adipeuses, est considérée comme anti-inflammatoire et anti-tumorale. Cette protéine est fortement diminuée dans un contexte de syndrome métabolique, ce qui est une cause de l'augmentation de la fréquence des

cancers (Braun *et al.*, 2011). Le rôle du stress oxydatif et des ROS dans la prolifération ou la destruction du cancer est toujours à l'étude. En effet, il est admis que les ROS peuvent induire une prolifération dans les cellules cancéreuses, l'angionénèse et de l'inflammation. En fait, tous les facteurs favorisant l'invasion et la prolifération des cellules cancéreuses peuvent augmenter (Genova & Lenaz, 2015; Liou & Storz, 2010). Il agit selon différentes voies et varie selon les types de cellules. Un exemple est les peroxydes produits par le métabolisme de l'oestrogène qui augmentent la prolifération cellulaire. Il est également connu que la signalisation induite par le stress oxydatif peut induire une prolifération via la signalisation MAPK (protéine kinase activée par le mitogène) et la NF-KB (Liou & Storz, 2010).

1.9. Tests *in vitro*

1.9.1 Mesure des capacités antioxydantes

Les radicaux libres étant indéniablement et continuellement produits par notre organisme, les industries alimentaires utilisent de plus en plus la présence d'antioxydants dans la nourriture et en vantent les bienfaits sur la santé de leurs produits. En effet, il est accepté que les radicaux libres peuvent mener au vieillissement de l'organisme, à des mutations menant à des cancers et aux troubles cardiovasculaires. Il est également connu que les capacités antioxydantes naturelles de nos cellules diminuent avec le temps. Ainsi, il est très intéressant de pouvoir palier à ce déficit par l'ingestion d'aliments fortement antioxydants (Liguori *et al.*, 2018). Une méthode répandue et universelle pour une première évaluation des propriétés antioxydantes d'un aliment est le test de piégeage des radicaux libres du radical libre stable α, α-diphenyl-β-picrylhydrazyl (DPPH). En effet, cette méthode colorimétrique simple et peu coûteux permet d'estimer le pouvoir antioxydant d'une substance par le taux de réduction de la molécule stable violette qui absorbe à 517nm (Kedare & Singh, 2011). En testant plusieurs concentrations d'une substance, il est possible de déterminer la concentration capable de réduire 50% de la solution de DPPH, ce qui permet ainsi de comparer le pouvoir antioxydant des substances entre elles. Ce test peut aussi être utilisé pour observer la perte ou le gain de pouvoir antioxydant d'un aliment suite à un traitement.

1.9.2 Les tests antiprolifératifs sur lignées cellulaires

Les tests antiprolifératifs sont utilisés depuis des années afin de pouvoir évaluer le potentiel anticancer et chimiothérapeutique de différents agents en les testant sur des lignées cellulaires cancéreuse ou non-cancéreuses. Un test courant est l'utilisation du sel de tetrazolium bromide (MTT) afin de quantifier la vitalité des cellules après traitement avec différentes concentrations de l'agent anticancer testé (Florento *et al.*, 2012). Celui-ci réagit avec les cellules ayant un métabolisme actif, le sel est réduit par la succinate déshydrogénase des mitochondries pour former un crystal de formazan ayant une coloration violette qui se dissout dans le DMSO (Wang, Henning, & Heber, 2010). Ainsi, l'absorbance détectée à 570nm est directement proportionnelle à la vitalité cellulaire après un traitement de 24h ou 48h, ce qui permet de déterminer la concentration qui inhibe 50% de la croissance, le IC_{50}. Cette concentration est utile pour comparer le pouvoir antiprolifératif entre différents agents et pour étudier la spécificité du pouvoir antiprolifératif entre une lignée cancéreuse et une autre non cancéreuse. Ainsi, en observant le IC_{50} des cellules transformées sur celui des cellules saines, on peut évaluer le potentiel de spécificité du composé évalué. Ainsi, plus la concentration est faible, plus la molécule est efficace et plus la différence est grande, plus la spécificité est grande (Fortin *et al.*, 2017).

1.9.3 Apoptose

L'apoptose est définie comme étant un processus de mort cellulaire hautement régulé et organisé. Il peut se produire en condition pathologique ou physiologique normale et est une façon sûre de réguler la croissance et la multiplication des cellules sans provoquer d'inflammation ou alarmer inutilement le système immunitaire. Dans un contexte de développement de cancer, le processus d'apoptose est grandement inhibé, ce qui entraine une surcroissance cellulaire désorganisée et la formation de tumeurs. La cellule normale possède plusieurs mécanismes pour détecter des anomalies au niveau du cycle cellulaire et ainsi induire le phénomène d'apoptose afin d'empêcher la prolifération de ces cellules. Normalement, des dommages au niveau de l'ADN ou des protéines, le stress oxydatif ou une anomalie au niveau de la régulation du cycle cellulaire devrait enclencher le phénomène d'apoptose (Sznarkowska, Kostecka, Meller, & Bielawski, 2017; Wong, 2011). Ainsi, les aliments capables d'induire la mort cellulaire programmée chez les cellules cancéreuses présentent des propriétés anticancer fortement recherchées dans un contexte d'aliment fonctionnel.

1.9.4 NAD(P)H quinone réductase

La nicotinamide adénine dinucléotide phosphate hydrogène: quinone réductase (NQO1) est une enzyme fortement utilisée pour évaluer les propriétés chimiothérapeutiques potentielles de différentes substances. Cette enzyme est une enzyme de détoxication chimio-préventive du cancer de phase II connue pour protéger contre les métabolites toxiques directement impliqués dans la première phase de la cancérogenèse. Plusieurs études ont montré que l'induction de cette enzyme dans les cellules permettait de réduire les risques de carcinogénèse et que la mutation ou l'absence de cette enzyme étaient associés à un risque très élevé de plusieurs types de cancer (Oh & Park, 2015).

NQO1 est une enzyme exprimée dans plusieurs tissus et est régulée par le système ARE (antioxidant response element) en réponse au stress oxydatif, radiations, substances étrangères, RES (reactives électrophilic species), hypoxie et métaux lourds. Elle est souvent activée en même temps que d'autres enzymes de détoxification telles que la glutathione S-transferase (GST) et HO-1 (heme oxygenase). Cette enzyme prévient le développement des cancers par quatre actions. Elle empêche la toxicité et de la production de ROS par les quinones. Elle participe au mécanisme antioxydant endogène cellulaire, élimine directement les superoxydes et stabilise diverses protéines de régulation du cycle cellulaire et suppresseurs de tumeurs, telle que p53, en protégeant de la dégradation par les protéases (Oh & Park, 2015).

Il est toujours difficile de prévoir et d'observer de tels effets *in vivo*. Bien souvent, les problèmes de biodisponibilité, l'élimination naturelle, les limitations techniques ou les limitations pour obtenir une

concentration physiologique suffisante sont un frein pour faire les liens entre les expériences effectuées en culture cellulaire et l'effet physiologique observable et quantifiable. En ce qui conserve la NQO1, il est démontré qu'une ingestion d'environ 333 333U (où 1U est la dose requise pour induire une augmentation de deux fois la concentration de NQO1 dans des cellules d'hépatome murin Hepa 1c1c7 cultivées dans des puits de 150 µl par rapport aux cellules non-traitées) par jour permettait d'observer l'effet anticancer recherché (Kirlin, Cai, DeLong, Patten, & Jones, 1999). Ainsi, cette enzyme est un excellent indice des propriétés chimiopréventives d'un aliment.

1.10. But, hypothèses, objectifs

1.10.1 Problématique

Pour produire un aliment fonctionnel à base de lait humain, il faut s'assurer de fournir un lait maternel sécuritaire au niveau microbiologique tout en s'assurant de garder ses propriétés nutritionnelles et immunologiques optimales. Actuellement, les banques de lait maternel ont un système en place pour la collecte et la décontamination des dons afin de redistribuer dans les hôpitaux pour les bébés prématurés. Les donneurs doivent collecter leur lait de façon hygiénique à l'aide d'une pompe et le lait est immédiatement congelé. Il est ensuite envoyé à la banque de lait maternel, où le lait est pasteurisé et soumis à une analyse microbiologique pour s'assurer qu'il ne reste aucune bactérie pathogène ou autre contamination indésirable (Kim & Unger, 2010). Bien qu'il soit essentiel que le lait soit décontaminé, il est à se demander si la pasteurisation à la chaleur est la meilleure méthode pour garantir la sécurité du lait sans détruire ses composantes nutritionnelles et immunologiques. En effet, il est connu que la pasteurisation peut diminuer ou complètement détruire des composantes telles que plusieurs enzymes, vitamines, anticorps et hormones (Peila *et al.*, 2016). De plus, la pasteurisation Holder ne parvient pas à éradiquer les spores bactériennes, ce qui entraine le rejet de produits et la perte des précieux dons (Cacho *et al.*, 2017). Les spores sont difficiles à éliminer et requièrent des traitements qui risquent d'endommager les composantes du lait et très peu d'alternatives ont à ce jour été sérieusement envisagées en ce qui concerne le lait maternel ou les préparations pour les nourrissons (Holsinger *et al.*, 1997; Rigourd *et al.*, 2018; Becker *et al.*, 1994).

La seconde partie de l'étude vise à répondre à une problématique qui touche plus de 20% de la population en Amérique du nord. En effet, le syndrome métabolique est une combinaison de facteurs de risque comprenant le diabète, une augmentation du test de glucose sanguin, l'obésité abdominale, un taux de cholestérol élevé et l'hypertension artérielle, contribuant à l'accélération du vieillissement, au déclin fonctionnel et au risque accru de maladies cardiovasculaires et chroniques (Lalan *et al.*, 2018; Ogunsile *et al.*, 2019). Puisque l'obésité, l'inflammation chronique de bas grade et le stress oxydatif sont connus pour

être des facteurs de co-morbidité pour le développement du cancer (Marseglia *et al.*, 2014), la consommation d'un aliment fortement antioxydant et chimiopréventif et hautement nutritionnel est une alternative envisagée afin d'offrir aux adultes un aliment fonctionnel pour aider à la prévention des maladies liées au vieillissement et à l'obésité.

Ainsi, la première partie de ce projet de recherche a pour but de déterminer si l'utilisation de l'irradiation gamma en tant que méthode de stérilisation à froid serait une technologie de choix pour assurer la sécurité du lait maternel sans affecter ses importantes propriétés nutritionnelles et antioxydantes. Différentes formes physiques ainsi que des additifs alimentaires seront testés afin d'induire la radiosensibilisation dans le but de réduire la dose de traitement à administrer pour décontaminer différents pathogènes alimentaires communs (*B. cereus*, *L. monocytogenes*, *S. aureus*, *E. coli* O157: H7, et *S.* Typhimurium). Par la suite, le lait maternel sera étudié en combinaison avec des probiotiques (*L. acidophilus* CL1285, *L. casei* LBC80, *L. rhamnosus* CLR2) afin d'évaluer ses propriétés anticancer *in vitro* et son impact sur les facteurs de risque du syndrome métabolique et leur prévention *in vivo*.

1.10.3 Hypothèses

1) L'irradiation du lait en formulation et du lait maternel en poudre et liquide congelé permet une décontamination efficace de différentes souches de pathogènes alimentaires.
2) L'irradiation du lait en formulation avec des additifs alimentaires permettet d'augmenter la radiosensibilité bactérienne.
3) Les propriétés nutritionnelles et antioxydantes du lait maternel et du lait en formulation ne seront pas altérées par l'irradiation.
4) Le lait maternel, les probiotiques et une combinaison des deux ont des propriétés antioxydantes, antiprolifératives et chimioprotectrices.
5) La combinaison de lait maternel et de probiotique procure un effet de synergie.

1.10.4 Objectifs

1) Évaluer l'effet de radiosensibilisation selon la forme physique (poudre ou liquide congelé) du lait maternel et de la préparation pour nourrissons sur différents pathogènes alimentaires (Chapitres 2, 3 et 4),

2) Évaluer la dose d'irradiation nécessaire pour décontaminer le lait maternel (Chapitre 4),

3) Évaluer la qualité nutritive (protéine, lipide, vitamine C, lactose) et les propriétés antioxydantes (MDA, DPPH) du lait maternel et du lait en formulation ayant subi l'irradiation (Chapitres 2 et 4)

4) S'assurer de la qualité microbiologique, nutritionnelle et immunologique du lait maternel atomisé (Chapitre 4),

5) Produire un aliment fonctionnel à base de lait humain enrichi en probiotiques Bio-K Plus^{MD} (Chapitre 5),

6) Déterminer *in vitro* les propriétés anti-radicalaires, chimiopréventives et antiprolifératives du lait maternel et des probiotiques (Chapitre 5),

7) Déterminer la capacité du lait maternel enrichi en probiotiques à prévenir les maladies liées à l'obésité et au syndrome métabolique en modèle *in vivo* (Chapitre 5).

1.10.5 Méthodologie

1) Le lait maternisé en poudre commercial a été inoculé (entre 10^6 et 10^8 UFC/mL selon la souche bactérienne) avec cinq pathogènes alimentaires (*B. cereus*, *L. monocytogenes*, *S. aureus*, *E. coli* O157: H7, et *S.* Typhimurium). Différentes doses d'irradiation et additifs alimentaires ont été appliqués pour déterminer les valeurs de D_{10} et déterminer la dose requise pour obtenir une réduction bactérienne de 6 Log. Les valeurs de D_{10} ont été calculées par la méthode décrite par Hossain *et al.* (2014) (Chapitres 2 et 3),

2) Les quantifications du lactose et de la vitamine C ont été effectuées par HPLC inspirée par les méthodes décrites par Richmond, Harte, Gray, and Stine (1987) et Romeunadal, Morerapons, Castellote, and Lopezsabater (2006). Les propriétés antioxydantes (MDA, DPPH, FRAP) du lait maternel et du lait en formulation ayant subi l'irradiation ont été évaluées par les méthodes spectrophotométriques décrites par Blois (1958) et Joubran, Mackie, and Lesmes (2013) (Chapitres 2 et 4),

3) La qualité microbiologique du lait a été évaluée par ensemencement sur différents milieux sélectifs. La conservation des propriétés immunologiques du lait maternel a été évaluée par la quantification d'enzymes (amylase, lysozyme) par des méthodes spectrophotométriques. La purification de la lactoferrine par la méthode de Conesa *et al.* (2008), suivi de l'évaluation de ses propriétés antioxydantes (DPPH, FRAP) par les méthodes citées plus haut, et antibactériennes par diffusion sur agar ont été entreprises sur le lait maternel atomisé dans le but de s'assurer de l'intégrité et de la conservation des propriétés de cette protéine importante (Chapitres 4 et 5),

4) La détermination *in vitro* des propriétés anti-radicalaires, chimiopréventives et antiprolifératives du lait maternel a été évaluée par la quantification de l'induction de la Quinone réductase, un test de prolifération ainsi que l'évaluation de la capacité à induire l'apoptose par les méthodes de Prochaska (1988), de Vistica *et al.* (1991) et par cytométrie en flux (Chapitre 5),

5) La démonstration de la capacité du lait maternel enrichi en probiotiques à prévenir les maladies liées à l'obésité et au syndrome métabolique a été faite en modèle *in vivo* sur des jeunes rats nourris avec une diète haute en gras pendant 8 semaines. La prise de poids des rats et leur taux glycémique sont évalués tout au long de l'étude. À la fin de l'étude, le stress oxydatif sur les protéines et lipides rénaux a été mesuré par les méthodes de Levine *et al.* (1990), et Moghadam *et al.* (2015). Finalement, des facteurs inflammatoires sanguins (PAI-1, TNF-α, IL1-β, PON1) ont été mesurés à la fin de l'étude par immunodosage (Chapitre 5).

1.11 Références

Becker, H., Schaller, G., von Wiese, W. and Terplan, G. (1994). Bacillus cereus in infant foods and dried milk products. *International Journal of Food Microbiology*, *23*(1), 1-15. doi:10.1016/0168-1605(94)90218-6

BFM ÉCO, B. F. (2019, 7 February). Lactalis: l'affaire du lait infantile contaminé lui a coûté très cher. Repéré June 21, 2020, à https://www.bfmtv.com/economie/entreprises/industries/lactalis-l-affaire-du-lait-infantile-contamine-lui-a-coute-tres-cher_AN-201902070091.htmL

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, *181*(4617), 1199-1200. doi:10.1038/1811199a0

Braun, S., Bitton-Worms, K. and LeRoith, D. (2011). The link between the metabolic syndrome and cancer. *International Journal of Biological Sciences*, *7*(7), 1003-1015.

Breidt, F., Hayes, J. S., Osborne, J. A. and McFeeters, R. F. (2005). Determination of 5-log pathogen reduction times for heat-processed, acidified vegetable brines. *Journal of Food Protection*, *68*(2), 305-310. doi:10.4315/0362-028x-68.2.305

Cacho, N. T., Harrison, N. A., Parker, L. A., Padgett, K. A., Lemas, D. J., Marcial, G. E., Lorca, G. L. (2017). Personalization of the Microbiota of Donor Human Milk with Mother's Own Milk. *Frontiers in microbiology*, *8*, 1470. doi:10.3389/fmicb.2017.01470

Carré, M., Dumoulin, D., Jounwaz, R., Mestdagh, B. and Pierrat, V. (2018). Maternal adherence to guidance on breast milk collection process. *Archives de Pediatrie*, *25*(4), 274-279. doi:10.1016/j.arcped.2018.02.003

Carrier, A. (2017). Metabolic syndrome and oxidative stress: A complex relationship. *Antioxidants & Redox Signaling*, *26*(9), 429-431. doi:10.1089/ars.2016.6929

Chiasson, F., Borsa, J., Ouattara, B. and Lacroix, M. (2004). Radiosensitization of Escherichia coli and Salmonella Typhi in ground beef. *Journal of Food Protection*, *67*(6), 1157-1162. doi:10.4315/0362-028x-67.6.1157

Commonwealth of Australia. (2014). Donor Human Milk Banking in Australia- Issues and Background Paper. *Department of Health*. Repéré May 8, 2020, à https://www1.health.gov.au/internet/main/Publishing.nsf/Content/D94D40B034E00B29CA257BF0001CAB31/$File/Donor%20Human%20Milk%20Banking%20in%20Australia%20paper%20(D14-1113484).pdf

Conesa, C., Sánchez, L., Rota, C., Pérez, M.-D., Calvo, M., Farnaud, S. and Evans, R. W. (2008). Isolation of lactoferrin from milk of different species: calorimetric and antimicrobial studies. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, *150*(1), 131-139. doi:10.1016/j.cbpb.2008.02.005

Esposito, K., Chiodini, P., Colao, A., Lenzi, A. and Giugliano, D. (2012). Metabolic syndrome and risk of cancer: a systematic review and meta-analysis. *Diabetes Care*, *35*(11), 2402-2411. doi:10.2337/dc12-0336

Festi, D., Schiumerini, R., Eusebi, L. H., Marasco, G., Taddia, M. and Colecchia, A. (2014). Gut microbiota and metabolic syndrome. *World Journal of Gastroenterology*, *20*(43), 16079-16094. doi:10.3748/wjg.v20.i43.16079

Florento, L., Matias, R., Tuaño, E., Santiago, K., Dela Cruz, F. and Tuazon, A. (2012). Comparison of Cytotoxic Activity of Anticancer Drugs against Various Human Tumor Cell Lines Using In Vitro Cell-Based Approach. *International journal of biomedical science : IJBS*, *8*(1), 76-80.

Fortin, O., Aguilar-Uscanga, B., Vu, K. D., Salmieri, S., Zhao, J.-C. and Lacroix, M. (2017). Chemo-preventive Properties of Essential Oils Obtained from Boswellia carterii and B. sacra in Combination with Insoluble Î²-Glucan. *Journal of Nutraceuticals and Food Science*.

Francisqueti, F. V., Nascimento, A. F., Minatel, I. O., Dias, M. C., Luvizotto, R. de A. M., Berchieri-Ronchi, C., Corrêa, C. R. (2017). Metabolic syndrome and inflammation in adipose tissue occur at different times in animals submitted to a high-sugar/fat diet. *Journal of nutritional science*, *6*, e41. doi:10.1017/jns.2017.42

Froh, E. B., Vanderpool, J. and Spatz, D. L. (2018). Best practices to limit contamination of donor milk in a milk bank. *Journal of Obstetric, Gynecologic, and Neonatal Nursing*, *47*(4), 547-555. doi:10.1016/j.jogn.2017.12.002

Genova, M. L. and Lenaz, G. (2015). The interplay between respiratory supercomplexes and ROS in aging. *Antioxidants & Redox Signaling*, *23*(3), 208-238. doi:10.1089/ars.2014.6214

Gila-Diaz, A., Arribas, S. M., Algara, A., Martín-Cabrejas, M. A., López de Pablo, Á. L., Sáenz de Pipaón, M. and Ramiro-Cortijo, D. (2019). A review of bioactive factors in human breastmilk: A focus on prematurity. *Nutrients*, *11*(6). doi:10.3390/nu11061307

Gopčević, K. R., Rovčanin, B. R., Tatić, S. B., Krivokapić, Z. V., Gajić, M. M. and Dragutinović, V. V. (2013). Activity of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase in different stages of colorectal carcinoma. *Digestive Diseases and Sciences*, *58*(9), 2646-2652. doi:10.1007/s10620-013-2681-2

Górska, A., Przystupski, D., Niemczura, M. J. and Kulbacka, J. (2019). Probiotic bacteria: A promising tool in cancer prevention and therapy. *Current Microbiology*, *76*(8), 939-949. doi:10.1007/s00284-019-01679-8

Hema-Québec. (2020, 2 March). ENCART' ' INFORMATIF - LAIT MATERNEL PASTEURISÉ . Repéré May 6, 2020, à https://www.hema-quebec.qc.ca/userfiles/file/media/francais/lait%20maternel/PUB-00031.pdf

Hoang, T. K., Freeborn, J., Wang, T., Mai, T., He, B., Park, S., Liu, Y. (2019). Human Breast Milk Promotes the Immunomodulatory Function of Probiotic Lactobacillus reuteri DSM 17938 in the Neonatal Rat Intestine. *Journal of Probiotics & Health*, *7*(1). doi:10.35248/2329-8901.19.7.210

Holsinger, V. H., Rajkowski, K. T. and Stabel, J. R. (1997). Milk pasteurisation and safety: a brief history and update. *Revue scientifique et technique (International Office of Epizootics)*, *16*(2), 441-451. doi:10.20506/rst.16.2.1037

Hossain, F., Follett, P., Vu, K. D., Salmieri, S., Senoussi, C. and Lacroix, M. (2014). Radiosensitization of Aspergillus niger and Penicillium chrysogenum using basil essential oil and ionizing radiation for food decontamination. *Food control*, *45*, 156-162. doi:10.1016/j.foodcont.2014.04.022

Huq, T., Vu, K. D., Riedl, B., Bouchard, J. and Lacroix, M. (2015). Synergistic effect of gamma (γ)-irradiation and microencapsulated antimicrobials against Listeria monocytogenes on ready-to-eat (RTE) meat. *Food microbiology*, *46*, 507-514. doi:10.1016/j.fm.2014.09.013

Joubran, Y., Mackie, A. and Lesmes, U. (2013). Impact of the Maillard reaction on the antioxidant capacity of bovine lactoferrin. *Food chemistry*, *141*(4), 3796-3802. doi:10.1016/j.foodchem.2013.06.096

Katzmarzyk, P. T., Church, T. S., Janssen, I., Ross, R. and Blair, S. N. (2005). Metabolic syndrome, obesity, and mortality: impact of cardiorespiratory fitness. *Diabetes Care*, *28*(2), 391-397. doi:10.2337/diacare.28.2.391

Kavitha, C., Kuna, A., Supraja, T., Sagar, S. B., Padmavathi, T. V. N. and Prabhakar, N. (2015). Effect of gamma irradiation on antioxidant properties of ber (Zizyphus mauritiana) fruit. *Journal of food science and technology*, *52*(5), 3123-3128. doi:10.1007/s13197-014-1359-x

Kedare, S. B. and Singh, R. P. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of food science and technology*, *48*(4), 412-422. doi:10.1007/s13197-011-0251-1

Kim, J.-H., Sung, N.-Y., Kwon, S.-K., Srinivasan, P., Song, B.-S., Choi, J.-I., Lee, J.-W. (2009). Gamma-irradiation improves the color and antioxidant properties of Chaga mushroom (Inonotus obliquus) extract. *Journal of medicinal food*, *12*(6), 1343-1347. doi:10.1089/jmf.2008.1281

Kim and Unger. (2010). Human milk banking. *Paediatrics & child health*, *15*(9), 595-602. doi:10.1093/pch/15.9.595

Kirlin, W. G., Cai, J., DeLong, M. J., Patten, E. J. and Jones, D. P. (1999). Dietary compounds that induce cancer preventive phase 2 enzymes activate apoptosis at comparable doses in HT29 colon carcinoma cells. *The Journal of Nutrition*, *129*(10), 1827-1835. doi:10.1093/jn/129.10.1827

Komolprasert, V. and Morehouse, K. M. (dir.). (2004). *Irradiation of food and packaging: recent developments* (vol. 875). Washington, DC: American Chemical Society. doi:10.1021/bk-2004-0875

Lacroix, M., Caillet, S. and Shareck, F. (2009). Bacterial radiosensitization by using radiation processing in combination with essential oil: Mechanism of action. *Radiation Physics and Chemistry*, *78*(7-8), 567-570. doi:10.1016/j.radphyschem.2009.03.012

Lalan, S., Jiang, S., Ng, D. K., Kupferman, F., Warady, B. A., Furth, S. and Mitsnefes, M. M. (2018). Cardiometabolic risk factors, metabolic syndrome, and chronic kidney disease progression in children. *The Journal of Pediatrics*, *202*, 163-170. doi:10.1016/j.jpeds.2018.06.007

Le Soleil, B. R.-C. (2020, 21 June). Donneuses de lait maternel recherchées | Santé | Actualités | Le Soleil - Québec. *Le Soleil*. Repéré June 21, 2020, à https://www.lesoleil.com/actualite/sante/donneuses-de-lait-maternel-recherchees-7a2486b0ca38a811593a53b62cfd68ac

Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A.-G., Stadtman, E. R. (1990). [49] Determination of carbonyl content in oxidatively modified proteins. In *Oxygen radicals in biological systems part B: oxygen radicals and antioxidants* (vol. 186, pp. 464-478). Elsevier. doi:10.1016/0076-6879(90)86141-H

Liguori, I., Russo, G., Curcio, F., Bulli, G., Aran, L., Della-Morte, D., Abete, P. (2018). Oxidative stress, aging, and diseases. *Clinical Interventions in Aging*, *13*, 757-772. doi:10.2147/CIA.S158513

Liou, G.-Y. and Storz, P. (2010). Reactive oxygen species in cancer. *Free Radical Research*, *44*(5), 479-496. doi:10.3109/10715761003667554

Maftei, N.-M. (2019). Probiotic, prebiotic and synbiotic products in human health. In R. Lidia Solís-Oviedo

and Á. de la Cruz Pech-Canul (dir.), *Frontiers and new trends in the science of fermented food and beverages*. IntechOpen. doi:10.5772/intechopen.81553

Maherani, B., Hossain, F., Criado, P., Ben-Fadhel, Y., Salmieri, S. and Lacroix, M. (2016). World market development and consumer acceptance of irradiation technology. *Foods*, *5*(4). doi:10.3390/foods5040079

Marseglia, L., Manti, S., D'Angelo, G., Nicotera, A., Parisi, E., Di Rosa, G., Arrigo, T. (2014). Oxidative stress in obesity: a critical component in human diseases. *International Journal of Molecular Sciences*, *16*(1), 378-400. doi:10.3390/ijms16010378

Matloubi, H., Aflaki, F. and Hadjiezadegan, M. (2004). Effect of γ-irradiation on amino acids content of baby food proteins. *Journal of Food Composition and Analysis*, *17*(2), 133-139. doi:10.1016/j.jfca.2003.09.005

Moghadam, A. R., Tutunchi, S., Namvaran-Abbas-Abad, A., Yazdi, M., Bonyadi, F., Mohajeri, D., Ghavami, S. (2015). Pre-administration of turmeric prevents methotrexate-induced liver toxicity and oxidative stress. *BMC complementary and alternative medicine*, *15*, 246. doi:10.1186/s12906-015-0773-6

Monteiro, R. and Azevedo, I. (2010). Chronic inflammation in obesity and the metabolic syndrome. *Mediators of Inflammation*, *2010*. doi:10.1155/2010/289645

Ndoti-Nembe, A., Vu, K. D., Doucet, N. and Lacroix, M. (2013). Effect of combination of essential oils and bacteriocins on the efficacy of gamma radiation against Salmonella Typhimurium and Listeria monocytogenes. *International journal of radiation biology*, *89*(10), 794-800. doi:10.3109/09553002.2013.797621

Novotny Núñez, I., Maldonado Galdeano, C., de Moreno de LeBlanc, A. and Perdigón, G. (2015). Lactobacillus casei CRL 431 administration decreases inflammatory cytokines in a diet-induced obese mouse model. *Nutrition*, *31*(7-8), 1000-1007. doi:10.1016/j.nut.2015.02.006

NSF Food Authority. (2011). Microbiological quality of powdered infant formula. Repéré à http://www.foodauthority.nsw.gov.au/_Documents/science/Microbiological_quality_powdered_formula.

O'Neill, S. and O'Driscoll, L. (2015). Metabolic syndrome: a closer look at the growing epidemic and its associated pathologies. *Obesity Reviews*, *16*(1), 1-12. doi:10.1111/obr.12229

Oda, E. (2018). Historical perspectives of the metabolic syndrome. *Clinics in Dermatology*, *36*(1), 3-8. doi:10.1016/j.clindermatol.2017.09.002

Ogunsile, F. J., Bediako, S. M., Nelson, J., Cichowitz, C., Yu, T., Patrick Carroll, C., Lanzkron, S. (2019). Metabolic syndrome among adults living with sickle cell disease. *Blood Cells, Molecules & Diseases*, *74*, 25-29. doi:10.1016/j.bcmd.2018.10.005

Oh, E.-T. and Park, H. J. (2015). Implications of NQO1 in cancer therapy. *BMB Reports*, *48*(11), 609-617. doi:10.5483/bmbrep.2015.48.11.190

Osaili, T. M., Shaker, R. R., Abu Al-Hasan, A. S., Ayyash, M. M. and Martin, E. M. (2007). Inactivation of Enterobacter sakazakii in infant milk formula by gamma irradiation: determination of D10-value. *Journal of Food Science*, *72*(3), M85-8. doi:10.1111/j.1750-3841.2007.00303.x

Peila, C., Moro, G. E., Bertino, E., Cavallarin, L., Giribaldi, M., Giuliani, F., Coscia, A. (2016). The Effect of Holder Pasteurization on Nutrients and Biologically-Active Components in Donor Human Milk: A

Review. *Nutrients*, *8*(8). doi:10.3390/nu8080477

Pirinccioglu, A. G., Gökalp, D., Pirinccioglu, M., Kizil, G. and Kizil, M. (2010). Malondialdehyde (MDA) and protein carbonyl (PCO) levels as biomarkers of oxidative stress in subjects with familial hypercholesterolemia. *Clinical Biochemistry*, *43*(15), 1220-1224. doi:10.1016/j.clinbiochem.2010.07.022

Prochaska, H. J. (1988). Purification and crystallization of rat liver NAD(P)H:(quinone-acceptor) oxidoreductase by cibacron blue affinity chromatography: identification of a new and potent inhibitor. *Archives of Biochemistry and Biophysics*, *267*(2), 529-538. doi:10.1016/0003-9861(88)90060-4

Repa, A., Thanhaeuser, M., Endress, D., Weber, M., Kreissl, A., Binder, C., Haiden, N. (2015). Probiotics (Lactobacillus acidophilus and Bifidobacterium infantis) prevent NEC in VLBW infants fed breast milk but not formula [corrected]. *Pediatric Research*, *77*(2), 381-388. doi:10.1038/pr.2014.192

Richmond, M. L., Harte, B. R., Gray, J. I. and Stine, C. M. (1987). Determination of sugars in yogurt and microbiological media by high performance liquid chromatography during processing and subsequent storage. *Journal of Dairy Science*, *70*(6), 1140-1147. doi:10.3168/jds.S0022-0302(87)80124-8

Rigourd, V., Barnier, J. P., Ferroni, A., Nicloux, M., Hachem, T., Magny, J. F., Bille, E. (2018). Recent actuality about Bacillus cereus and human milk bank: a new sensitive method for microbiological analysis of pasteurized milk. *European Journal of Clinical Microbiology & Infectious Diseases*, *37*(7), 1297-1303. doi:10.1007/s10096-018-3249-z

Romeunadal, M., Morerapons, S., Castellote, A. and Lopezsabater, M. (2006). Rapid high-performance liquid chromatographic method for Vitamin C determination in human milk versus an enzymatic method. *Journal of Chromatography B*, *830*(1), 41-46. doi:10.1016/j.jchromb.2005.10.018

Sabharwal, S. S. and Schumacker, P. T. (2014). Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? *Nature Reviews. Cancer*, *14*(11), 709-721. doi:10.1038/nrc3803

Sanders, M. E., Merenstein, D. J., Reid, G., Gibson, G. R. and Rastall, R. A. (2019). Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. *Nature Reviews. Gastroenterology & Hepatology*, *16*(10), 605-616. doi:10.1038/s41575-019-0173-3

Srivastava, S., Sinha, D., Saha, P. P., Marthala, H. and D'Silva, P. (2014). Magmas functions as a ROS regulator and provides cytoprotection against oxidative stress-mediated damages. *Cell death & disease*, *5*, e1394. doi:10.1038/cddis.2014.355

Szczawinska, M. E. (2017). Application of ionizing radiation for control of salmonella in food. In M. Mares (dir.), *Current topics in salmonella and salmonellosis*. InTech. doi:10.5772/67408

Sznarkowska, A., Kostecka, A., Meller, K. and Bielawski, K. P. (2017). Inhibition of cancer antioxidant defense by natural compounds. *Oncotarget*, *8*(9), 15996-16016. doi:10.18632/oncotarget.13723

Tack, C. J., Stienstra, R., Joosten, L. A. B. and Netea, M. G. (2012). Inflammation links excess fat to insulin resistance: the role of the interleukin-1 family. *Immunological Reviews*, *249*(1), 239-252. doi:10.1111/j.1600-065X.2012.01145.x

Tsatsoulis, A., Mantzaris, M. D., Bellou, S. and Andrikoula, M. (2013). Insulin resistance: an adaptive mechanism becomes maladaptive in the current environment - an evolutionary perspective. *Metabolism: Clinical and Experimental*, *62*(5), 622-633. doi:10.1016/j.metabol.2012.11.004

Vistica, D. T., Skehan, P., Scudiero, D., Monks, A., Pittman, A. and Boyd, M. R. (1991). Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production. *Cancer Research*, *51*(10), 2515-2520.

Wang, P., Henning, S. M. and Heber, D. (2010). Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. *Plos One*, *5*(4), e10202. doi:10.1371/journal.pone.0010202

Widger, J., O'Connell, N. H. and Stack, T. (2010). Breast milk causing neonatal sepsis and death. *Clinical Microbiology and Infection*, *16*(12), 1796-1798. doi:10.1111/j.1469-0691.2009.03071.x

Wong, R. S. Y. (2011). Apoptosis in cancer: from pathogenesis to treatment. *Journal of Experimental & Clinical Cancer Research*, *30*, 87. doi:10.1186/1756-9966-30-87

Woodside, J. V. (2015). Nutritional aspects of irradiated food. *Stewart Postharvest Review*, *11*(3), 1-6. doi:10.2212/spr.2015.3.2

Wu, P., Zhang, F., Dai, Y., Han, L. and Chen, S. (2016). Serum TNF-α, GTH and MDA of high-fat diet-induced obesity and obesity resistant rats. *Saudi pharmaceutical journal : SPJ : the official publication of the Saudi Pharmaceutical Society*, *24*(3), 333-336. doi:10.1016/j.jsps.2016.04.011

CHAPITRE 2:

EFFECT OF ϒ-IRRADIATION ON THE MICROBIAL INACTIVATION, NUTRITIONAL VALUE, AND ANTIOXIDANT ACTIVITIES OF INFANT FORMULA

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Contribution des auteurs

Valérie Robichaud a mis au point les protocoles, réalisé les manipulations avec l'aide d'un stagiaire et participé à la rédaction de l'article.

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Résumé

Cette étude visait à évaluer l'effet de l'irradiation (0 kGy, 5 kGy et 10 kGy) sur l'inactivation microbienne de certains agents pathogènes (*B. cereus, L. monocytogenes, S. aureus, E. coli O157: H7*, et *S.* Typhimurium), la composition nutritive (lactose, protéines, vitamine C et qualité des lipides), le profil peptidique et les activités antioxydantes d'une préparation pour nourrissons liquide congelée et en poudre. Selon les agents pathogènes, les valeurs de D_{10} (kGy) étaient comprises entre 0,28-2,37 et 0,57-6,13 pour les échantillons liquides congelés et en poudre, respectivement. Les résultats ont également montré qu'à l'exception de *L. monocytogenes* et *S.* Typhimurium, tous les agents pathogènes inoculés étaient plus sensibles à l'irradiation sous forme congelée. En outre, le traitement par irradiation n'a entraîné aucune altération significative de la teneur en lactose et en protéine des deux types d'échantillons, tandis que les propriétés antioxydantes et la vitamine C étaient affectées lorsqu'irradiés sous forme liquide congelé. En outre, les échantillons en poudre irradiés ont montré une augmentation significative dose-dépendante de l'oxydation des lipides jusqu'à 10 kGy. Une dose de 5 kGy favorise la libération d'une petite fraction protéique moléculaire qui peut avoir un effet positif sur la valeur nutritive de la préparation pour nourrissons irradiée.

Mots-clés: Préparation pour nourrissons, Décontamination, Valeur nutritive, ɣ-irradiation

Abstract

This study was aimed to assess the effect of ɣ-irradiation (0 kGy, 5 kGy, and 10 kGy) on the microbial inactivation of selected pathogens (*Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, Escherichia coli O157: H7, and Salmonella typhimurium*), nutrient composition (lactose, protein, vitamin C, and fat quality), peptide profile, and antioxidant activities of a liquid formulation and powdered infant formula. Depending on the pathogens, the D10 values (kGy) were range from 0.28-2.37 and 0.57-6.13 for liquid formulation and powdered samples, respectively. Results also showed that except for *L. monocytogenes* and *S. typhimurium*, all inoculated pathogens were more sensitive toward irradiation in the liquid formulation. Furthermore, irradiation treatment did not bring any significant alteration in lactose and protein content of both types of samples while antioxidant properties and vitamin C levels were affected when irradiated in liquid formulation. However, irradiated powdered samples showed a significant dose-dependent increment in lipid oxidation up to 10 kGy, which led to detect the greater level of malondialdehyde by thiobarbituric acid-reactive substances assay. A dose of 5 kGy favours the release of small molecular protein fraction which can have a positive effect on the nutritional value of the irradiated infant formula.

Key words: Infant formula, Decontamination, Nutritional value, ɣ-irradiation

1. Introduction

Infant milk formula is identified as the best alternative to provide the nutritional needs of newborns when breastfeeding is not possible. Therefore, the safety and security of the infant formula, as a sole source of nutrition for neonates and babies, is the major concern of the manufacturers. However, infant formula can be contaminated at any point in the food chain and some pathogens of concern including *Listeria monocytogenes*, *Bacillus cereus*, and *Salmonella* spp (Alanber, Alharbi, & Khaled, 2019; Cho et al., 2018). Heat treatment is a promising approach that used to provide the stability and safety of infant formula during storage and marketing. However, high-temperature treatment leads to Maillard reaction, which exhibits browning, cooked flavour, and nutritional loss. Furthermore, heat treatment induces vitamin losses, whey protein aggregation and lactose isomerization to lactulose, which leads to a decrement of milk protein solubility (Wazed, Ismail, & Farid, 2020). Due to their potential to enhance food security and to preserve the food quality and nutritional values, non-thermal hurdles have been considered in the food industry as a promising combined treatment for thermal processing (Arroyo et al., 2017; Lacroix & Ouattara, 2000). Ionizing radiation such as ɣ-irradiation is an effective non-thermal technique which inactivates foodborne pathogens in food products and extends shelf-life (Lacroix et al., 2013; Mahrour, Caillet, Nketsa-Tabiri, & Lacroix, 2003). According to the World Health Organization (WHO), irradiation of food up to an overall dose of 10 kGy is a safe technology for commercial food processing (Lacroix & Ouattara, 2000). In general, macronutrients including carbohydrates, proteins, and fats are not affected by irradiation treatment, but depend on food products (e.g. food composition, irradiation dose and factors such as temperature and presence or absence of oxygen in the irradiating environment); vitamin content and lipid oxidation have been reported to have dose-dependent effects (Dionísio, Gomes, & Oetterer, 2009; Lacroix, Bernard, Jobin, Milot, & Gagnon, 1990).

Therefore, the objectives of this study were to evaluate the inactivation effect of ɣ-irradiation to reduce the dose needed to eliminate pathogens which can contaminate infant formula (*B. cereus*, *L. monocytogenes*, *S. aureus*, *E. coli* O157: H7, and *S. typhimurium*) to determine: (i) the radiation sensitivity of the targeted microorganisms in powdered and liquid formulation infant formula (ii) the effect of ɣirradiation on the major nutrients including protein, lactose, vitamin C, peptide profile, and lipid oxidation as well as antioxidants properties of the infant formula.

2. Material and methods

2.1 Materials

Tryptic soy broth (TSB), Tryptic soy agar (TSA), and peptone water were purchased from Alpha Biosciences Inc. (Baltimore, MD, USA). All chemicals were purchased from Sigma-Aldrich Canada Ltd.

(Oakville, ON, Canada). Infant formula (stage 2, Nestlé Good Start®) was provided from a local store (IGA, Laval, QC, Canada). Pierce™ BCA protein assay kit was obtained from Thermo scientific (Montreal, QC, Canada).

2.2. Preparation of bacterial culture, samples and inoculation procedures

B. cereus (ATCC® 14579™), *L. monocytogenes* (HPB 2812 serovar 1/ 2a), *S. aureus* (ATCC® 29,213™), *E. coli* O157:H7 (EDL 993), and *S. typhimurium* (SL1344, INRS-IAF) were maintained at −80 °C in TSB containing glycerol (10% v/v).

Before each experiment, stock cultures were propagated through two consecutives 24 h growth cycles in TSB at 37 °C to obtain working cultures of approximately 10^8 (CFU/mL) for all target bacteria except for *B. cereus* for 10^6 (CFU/mL). The working cultures were obtained through centrifuging of 24 h cultures, followed by washing the collected pellets twice with 0.85% (w/v) of sterile saline solution. The powdered infant formula was then inoculated with the 5 target pathogens and thoroughly mixed.

In the case of liquid formulation sample, the powered infant formula was dissolved (14.5% w/v) in Milli-Q water while being stirred. Then inoculation was done as mentioned above and each tube that contained reconstituted infant formula was kept at −20 °C for 24 h.

2.3. ɣ- irradiation treatment

All tubes containing inoculated samples were double-sealed under normal atmosphere and stored the day before the irradiation at 4 °C and −20 °C for powdered and liquid formulation samples, respectively. The irradiation (9.172 kGy/h) was done at the Canadian Irradiation Center in a UC-15 A irradiator (Nordion Inc., Kanata, ON, Canada) equipped with a ^{60}Cobalt source. Dry ice was inserted in the radiator to keep the samples frozen.

The different doses of irradiation (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10 kGy) were applied to determine D10 values and to identify the irradiation dose required to obtain a 6-Log reduction. D10 values were calculated for all 5 pathogens considering the kinetics of target bacterial destruction and determined using linear regression considering the reciprocal of the slope of the line which was obtained by plotting bacterial populations (log CFU/g) against the dosages of irradiation (Hossain et al., 2014).

The dosages applied for in situ study were 0.0 (control), 5.0 and 10.0 kGy to investigate ɣ- irradiation effects on the chemical alteration in nutrients and antioxidant activity of either frozen liquid or powdered infant formula.

2.4. Microbiological analysis

The liquid formulation samples, which were frozen, were thawed and immediately diluted in sterile peptone water (0.1%). For the powdered infant formula, 1 g dissolved in 9 mL of sterile peptone water (0.1%) and then stirred. From each homogenate sample, serial dilutions were then prepared in peptone water (0.1% w/v) and 100 µl of appropriate dilution was then pour plated on TSA and incubated at 37 °C for 24 h.

2.5. Determination of lactose and vitamin C

Lactose content was measured using HPLC according to (Richmond, Harte, Gray, & Stine, 1987). Briefly, the samples were prepared by adding an equal part of trichloroacetic acid (TCA) 12% (w/v) into liquid infant formula. The samples were then centrifuged (Sorvall® Instrument, Du Pond, USA) at 10, 000 g for 20 min at 4 °C to remove casein and fat. The supernatant was then filtered through a 0.2 µm PVDF syringe filter (Sarstedt AG & Co. KG) and stored at −20 °C prior to use. A ZORBAX carbohydrate column (4.6 × 250 mm, 5 µm particle size, pore size 70 Å) connected to an Agilent 1260 series HPLC system (Agilent Technologies, Palo Alto, California, USA) was used for separation under isocratic conditions. The mobile phase was Milli-Q water at a flow rate of 1.5 mL/min and the volume of injection was 20 µL. The temperature of the reflective index detector and the column was set at 50 °C, and 80 °C, respectively. Data acquisition and processing were performed by Chemstation v. 2.0 (Agilent). Lactose solution (0, 1, 10, 20, 30, 40, 50 mg/mL) was used to prepare a standard curve.

To determine vitamin C, the samples were prepared by adding an equal part of 0.56% (v/v) phosphoric acid into the liquid infant formula (Romeu-Nadal, Morera-Pons, Castellote, & López-Sabater, 2006). The samples were then centrifuged, and the obtained supernatant was filtered using a 0.2 µm PVDF syringe filter as mentioned above and immediately injected into the HPLC.

HPLC analysis was done using an Agilent 1260 system, equipped with a DAD detector. A Poroshell 120 EC-C18 (4.6 × 50 mm, 2.7 µm particle size, pore size 120 Å) (Agilent, California, USA) was used for quantification under isocratic conditions at room temperature. The mobile phase consisted of 95% solution A (40 mM monobasic phosphate buffer, pH = 2.5) and 5% solution B, which was contained 60% methanol and 40% acetonitrile. The flow rate was 1 mL/min, and detection was performed at 243 nm. Ascorbic acid was used to obtain the standard curve (0, 5, 10, 15, 20, 25, and 30 µM).

2.6. Protein content

Protein concentration of the samples was determined using Bradford assay protein assay kit (Thermo scientific, Montreal, Canada) according to the manufacturer's specifications (Cheng, Wei, Sun, Tian, & Zheng, 2016). Bovine serum albumin (BSA, 20–2000 μg/mL) was used as a control and absorbance was read at 562 nm using a UV-VIS spectrophotometer (S-3100 Scinco, Triad Scientific, North Carolina, USA).

2.7. Antiradical activity

Briefly, 250 μL of liquid infant formula sample (1:10 dilution) was added to the 1 mL of DPPH methanolic solution (60 μM) (Blois, 1958). The mixture stirred and left to incubate for 60 min in the dark at room temperature. The absorbance of the samples was determined at 517 nm by a UV-VIS spectrophotometer (S-3100 Scinco) equipped with a microplate reader (EL800, Fisher BioTek, Toronto, Canada). The ascorbic acid (250 μL), and dimethyl sulfoxide (DMSO) (10%) were used as a positive control and negative control, respectively. Radical scavenging activity was calculated using the following equation: radical scavenging activity (%) = (DO control – DO sample)/DO control × 100. Ascorbic acid was used to obtain the standard curve (0, 5, 10, 15, 20, 25, and 30 μM).

2.8. Thiobarbituric acid-reactive substances (TBARS)

Malondialdehyde (MDA) content in infant formula was measured according to the method described by Botsoglou et al. (1994) with some modifications. Briefly, 1 mL of the reconstituted sample was mixed with 8 mL of 5% TCA and 5 mL of butylated hydroxyl anisole (0.8% in hexane). The mixture was mixed using a high sheer homogenizer (Ultra-Turrax T25, Montreal, Canada) at 18,000 rpm for 30 s followed by centrifugation at 3000 g for 3 min. Then, the top hexane layer was removed and the cloudy layer was made to 10 mL volume by adding 5% TCA. Subsequently, 2.5 mL of aliquot was mixed with 1.5 mL of 0.8% thiobarbituric acid and incubated at 70 °C for 30 min. The solution was then cooled down under tap water and it was analyzed by spectrophotometry against a blank reaction mixture. The derivative data were plotted against an MDA calibration curve (0–2 μg/mL).

2.9. Peptide analysis

Briefly, the samples were prepared by adding an equal part of TCA 12% (w/v) to the liquid infant formula. The mixture was then centrifuged 10,000 g at 4 °C for 10 min to remove casein and fat and the supernatant was filtered using a 0.2 μm PVDF syringe filter (González-Olivares, Añorve-Morga, Castañeda-Ovando, Contreras-López, & Jaimez-Ordaz, 2014). The samples were stored at −20 °C until being analyzed. A Biosep-SEC 2000 column (300 × 7.8 mm, 5 mm particle size, pore size 145 Å) from Phenomenex

(Torrance, Canada) connected to an Agilent 1260 series HPLC system was used. The mobile phase was a phosphate buffer solution (pH 6.8) and detection was done by a diode array detector at 280 nm. The analysis was performed at room temperature at a flow rate of 1 mL/min for 16 min. Mathematical modelling of the size exclusion chromatography was used to estimate the molecular weight of the peptides (Zelic & Nesek, 2006). Bovine thyroglobulin (670 KDa), gamma globulin (IgA, 300 KDa), IgG (150 KDa), ovalbumin (44 KDa), myoglobin (17 KDa) and uridine (244 Da) were used as standards (Phenomenex).

2.10. Statistical analysis

The experiment was done in triplicate and for each replicate, 3 samples were analyzed. The data were reported as mean ± standard deviation. The data obtained from the experiments were subjected to one-way analysis of variance (ANOVA) by IBM SPSS software version 23. Duncan test was used to determine any significant differences between the mean values at p level of 0.05.

The kinetic of bacteria destruction during irradiation treatment in presence of active compounds was evaluated by linear regression and the D10 values were determined by linear regression considering the reciprocal of the slope of the line produced by plotting bacterial counts (log CFU/mL) against the irradiation doses (Chiasson, Borsa, Ouattara, & Lacroix, 2004).

3. Results and Discussion

3.1. In activation of bacteria using ɣ-irradiation and D10 values

Target microorganisms associated with infant formula (either powdered or liquid formulation) were exposed to ɣ-irradiation at different dosages (0–10 kGy) to evaluate the effectiveness of the irradiation treatment to inactivate the tested pathogens. The decimal reduction dose or D10 values, which is defined as the irradiation dose (kGy) required to kill 90% of the microbial population, were obtained from the inverse of the slope of the regression lines (Figs. 1 and 2, shown as examples) for all microorganisms inoculated in the powered and liquid formulation infant formula (Table 1).

It can be observed that ɣ-irradiation treatment was effective in both types of samples to inactivate the five pathogens and that their viability reduced exponentially with increasing the dosage (Table 1). The results also showed that *E. coli* and *B. cereus* (under vegetative or spore forms) are more sensitive under frozen conditions than powder (Table 1). The radiosensitivity is respectively of 2 and 1.8 times for *E. coli* and *B. cereus* under vegetative form and *B. cereus* under spore form is 2.6 times more sensitive in liquid formulation form as compared to powder form (Table 1). Under powder and liquid formulation, *S. Typhimurium*, *L.*

monocytogenes and *B. cereus* (vegetative and spore form) are the most resistant to irradiation treatment. *B. cereus* under the spore condition is the most resistant bacteria. A dose of 14.2 and 36.8 kGy is needed to reduce by 6 Log (CFU/mL) *B. cereus* under the forms of liquid formulation and powder, respectively.

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 mechanism of action of ɣ-irradiation against microorganisms include direct (damage of the nucleic acids of microorganisms by a ɣ-ray) and indirect damage (by the products of water radiolysis) (Lacroix, 2012). The present study was designed to compare the effect of ɣ-irradiation to inactivate pathogens of concern in infant formula in liquid formulation and powered samples. The results demonstrated that ɣ-irradiation effectively suppressed the vegetative bacteria and spores. Indeed, indirect damage improved in the presence of available water (Dickson, 2001) and increased the effectiveness of direct action of ɣ-irradiation to inactivate tested pathogens in liquid formulation infant formula compared to powered ones. The obtained results are in good agreement with Song et al. (2014), who reported a D10 value of 0.4 and 0.6 kGy for *E. coli* and *S. typhimurium* in pepper, respectively. Based on the finding of this research, D10 value of 0.57 kGy was attained for *E. coli* in the powdered formula, which is higher than that obtained in pepper. In the case of *S. typhimurium*, D10 value of 0.70 kGy was obtained, which is almost the same as pepper. The D10 values of the same species varied depending on the chemical composition of foods and water activity (Maherani, Khlifi, Salmieri, & Lacroix, 2019).

Saroj et al. (2007) observed that in mung beans and chickpeas 2 kGy of ɣ-irradiation treatment resulted in 4.6 and 4.8-log reductions, respectively, of Salmonella whereas here 6-log reduction of *Salmonella* was obtained using 4.2 kGy. *Salmonella* is considered the most resistant pathogen to irradiation among gram-negative bacteria (Monk, Beuchat, & Doyle, 1995) it means that any irradiation dose sufficient to kill *Salmonella* would eradicate other gram-negative bacteria. These results also confirmed that D10 values and required dosage for having a 6-log reduction of *Salmonella* were higher than *E. coli* in both liquid formulation and powdered samples. It is also known that sporulated bacteria contribute to the development of bacterial resistance to irradiation. The endospore itself consists of an innermost core surrounded by an inner forespore membrane, cortex, outer forespore membrane, and spore coat covering supercoiled DNA. The spore coat is composed of 30 spore-specific proteins, associating with resistance against heat, lysozyme, chemical disinfection, and low-dose gamma irradiation (Fiester, Helfinstine, Redfearn, Uribe, & Woolverton, 2012).

3.2. Nutrients content and antioxidant proprieties

3.2.1. Lactose and protein content

In the present study, lactose and protein content of either powered or liquid formulation infant formula samples were not altered ($P > 0.05$) by ɣ-irradiation treatment up to 10 kGy compared to the control samples (0 kGy) (Fig. 3). This observation is consistent with Ham et al. (2009), who reported that γ-irradiation at 1, 3, 5 and 10 kGy had no affect on the lactose and protein content in plain yogurt. Previous studies on the shelf-life of dairy-like products irradiated at 1, 3, 5, and 10 kGy found no difference in the protein content and total solids which aligns with these obtained results (Odueke, Chadd, Baines, Farag, & Jansson, 2018). It is known that if the irradiation treatment is done under appropriate conditions (e.g. freezing, exclusion of oxygen, etc.), the nutritional value of the food is generally unaffected by γ-irradiation, even at 45 kGy (Roberts, 2016).

3.2.2. Vitamin C and antioxidant properties

The total vitamin C content of control and irradiated infant formula samples are presented in Fig. 4A. In liquid formulation samples treated with irradiation, vitamin C content showed a dose-dependent and significantly decreasing trend ($P \leq 0.05$) from 52.8 ppm for non-irradiated samples to 19.8 ppm for samples when irradiated at 10 kGy. However, there was no significant difference found in the vitamin C content of the irradiated powdered samples; whatever, the doses of irradiation used ($P > 0.05$). Wang and Chao (2003) studied the irradiation effects from 1.5 to 6 kGy on the vitamin C content of apple (Fuji) and they found that vitamin C was significantly reduced by increasing irradiation dosages. Vitamin C is one of the most sensitive vitamins to irradiation, but the sensitivity is also related to different factors like pH, presence of oxygen, and etc. (Dionísio et al., 2009). The use of combined treatment can reduce the dose needed to eliminate pathogens without affecting the nutritional value of the food (Hossain et al., 2014; Lacroix & Ouattara, 2000).

The effect of ɣ-irradiation on the antioxidant activity of infant formula either in liquid formulation or in powdered form was evaluated by the DPPH radical scavenging method (Fig. 4B). The results showed that ɣ-irradiation treatment did not significantly affect the antioxidant activity of the powdered samples, which was coherent with the level of vitamin C that also did not show any alteration with increasing irradiation dose. However, irradiated liquid formulation samples were shown to possess less antioxidant capacity ($P \leq 0.05$), but no difference was found between 5 and 10 kGy treated samples ($P > 0.05$). The decrement of antioxidant activity might be related to the depletion of free radical scavengers such as vitamin C and oxidative damages (Stefanova, Vasilev, & Spassov, 2010).

3.2.3. Oxidative stability or thiobarbituric acid-reactive substances (TBARS)

The indicator of lipid oxidation in ɣ-irradiated samples was measured by a TBARS assay of malondialdehyde (MDA) formation (Fig. 4C). In liquid formulation infant formula treated with ɣ-irradiation, significant changes were not detected in the MDA level up to 10 kGy. However, powdered samples submitted to ɣ-irradiation presented significant dose-dependent increment in lipid oxidation up to 10 kGy, which led to detect greater ($P \leq 0.05$) amount of secondary products of lipid oxidation including MDA by TBARS assay. The MDA content increased from 0.412 ± 0.083 (μg/g) for non-irradiated sample to 1.089 ± 0.143 (μg/g) for powdered infant formula, which treated by ɣ-irradiation at 10 kGy. These findings were in line with Tesfai, Beamer, Matak, and Jaczynski (2014), who reported that ionizing radiation (25 kGy) resulted in a significant increase of MDA in dehydrated infant formula. Moreover, Sohn et al. (2009) showed that the irradiation of ground beef samples (5 and 10 kGy) increased the amount of MDA. Ionizing radiation may initiate auto-oxidation of fat-containing food by producing oxygen radicals during ionizing radiation, which results in the formation lipid oxides (Stefanova et al., 2010).

In general, here MDA content of the liquid formulation was higher than the powdered sample, which could be due to moisture content, whereas the rate of fat oxidation in irradiated powdered samples was much more accelerated than irradiated liquid formulation. It is related to the immobilization of free water molecules by freezing process, which prevents any increment in lipid oxidation. On the other hand, when the food products with higher free water and water activity are irradiated, high concentrations of hydroxyl radicals are produced by water radiolysis, which may initiate lipid oxidation of unsaturated fatty acids (Mahrour et al., 2003).

3.2.4. Peptide profile

Results showed a significant reduction of all fractions with increasing the irradiation dose and the effect of irradiation was similar under powder and liquid formulation form (Table 2). A decrement 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, Bahreinipour, Eskandari, MousaviZarandi, & Ardestani, 2017). However, in the presence of oxygen, mostly fragmentation was observed (Davies, 1987). The γ-irradiation generates free radicals, which can disrupt hydrogen and disulfide 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, Mahmoud, Elmamoun, Adiamo, & Ahmed, 2018; Osman et al., 2014). Also, irradiation can produce deamination through protein unfolding (Dogbevi, Vachon, & Lacroix, 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. Then, a decrease of this fraction was observed when the sample was treated at 10 kGy. A rearrangement of the small molecular weight protein to a higher molecular weight could be observed during irradiation treatment (Afify & Shousha, 1988). A dose of 5 kGy may produce a release of small subunit fractions while a dose of 10 kGy favours a rearrangement to form a more complex protein fraction to form higher molecular weight. An increase by 69% of the fraction with > 500 kDa was observed in the liquid formulation sample treated at 10 kGy compared to the same sample treated at 5 kGy, showing an increase of the higher molecular weight.

4. Conclusion

The potential of ɣ-irradiation to inactivate foodborne pathogens in infant formula was demonstrated. The chemical composition consisting of lactose and total protein content were not affected by γ-irradiation. Irradiated liquid formulation samples had less vitamin C content and antioxidant capacity, but powdered samples submitted to ɣ-irradiation showed a significant dose-dependent increment in lipid oxidation. A dose of 5 kGy favours the release of small molecular protein fraction, which can have a positive effect on the nutritional value of the irradiated infant formula.

Declaration of competing interest

There is no conflict of interest. Acknowledgments This research was supported by the National Science Engineering research council (NSERC) discovery program (project number RGPIN2017-05947). This project is also a part of a research consultative program of the International Atomic Energy Agency on radiation inactivation of biohazards (code F23033).

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Table 1. D_{10} (kGy) and required irradiation does to reach 6-log reduction of *B. cereus*, *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7, and *S.* Typhimurium in the frozen and powdered infant formula. Data are expressed as mean ± standard deviation for triplicate tests. Means with different lower-case letters within a row are significantly different ($P \leq 0.05$); means with different capital letters within a column are significant different ($P \leq 0.05$).

Bacteria	D_{10} (kGy)		Irradiation dose to reach 6-Log reduction(kGy)		Sensitivity (powdered /frozen)
	powdered	frozen	powdered	frozen	
Escherichia coli	0.57 ± 0.08^{Aa}	0.28 ± 0.05^{Ab}	3.4	1.7	2.0
Staphylococcus aureus	0.48 ± 0.07^{Aa}	0.40 ± 0.04^{Ab}	2.9	2.4	1.3
Salmonella Typhimurium	0.71 ± 0.11^{Aa}	0.70 ± 0.08^{Ba}	4.2	4.2	1.0
Listeria monocytogenes	0.66 ± 0.07^{Aa}	0.64 ± 0.07^{Ba}	4.0	3.8	1.0
Bacillus cereus **(vegetative)**	0.77 ± 0.19^{Aa}	0.44 ± 0.03^{Bb}	4.6	2.6	1.8
Bacillus cereus **(spore)**	6.13 ± 0.47^{Ba}	2.37 ± 0.17^{Cb}	36.8	14.2	2.6

Table 2. Impact of different gamma-irradiation doses (0, 5, and 10 kGy) on the preservation of peptides of powdered and liquid frozen infant formula.

Group no.	Approx. MW(KDa)	Powder Area (% relative abondance)			Liquid frozen Area (% relative abondance)		
		0kGy	5kGy	10kGy	0kGy	5kGy	10kGy
1	**> 500**	25226 ± 333	23870 ± 856	20509 ± 1785	20672 ± 680	9951 ± 4137	16910 ± 2544
2	**500 to 50**	1770 ± 10	1680 ± 58	0 ± 0	1594 ± 36	0 ± 0	0 ± 0
3	**< 50**	4553 ± 32	4106 ± 35	3411 ± 193	3737 ± 138	15661 ± 4447	3013 ± 272

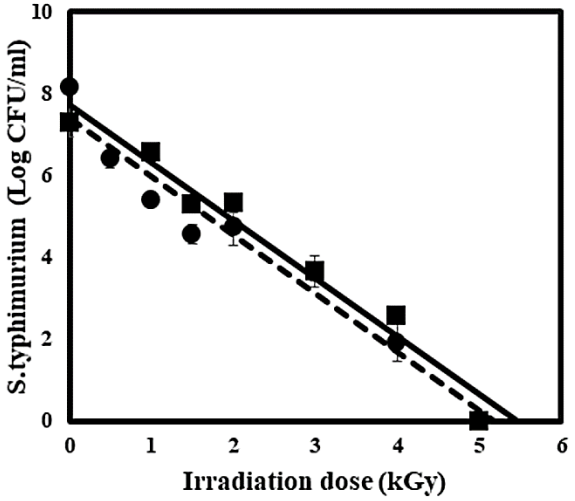

Figure 1. D_{10} (kGy) of *S.* Typhimurium in infant formula: liquid frozen sample (solid circle), powdered samples (solid square). Regression equation for plot are as follows: y = -1.43x + 7.39 (R^2 = 0.96) in liquid frozen and y = -1.41x + 7.72 (R^2 = 0.97) in powdered samples.

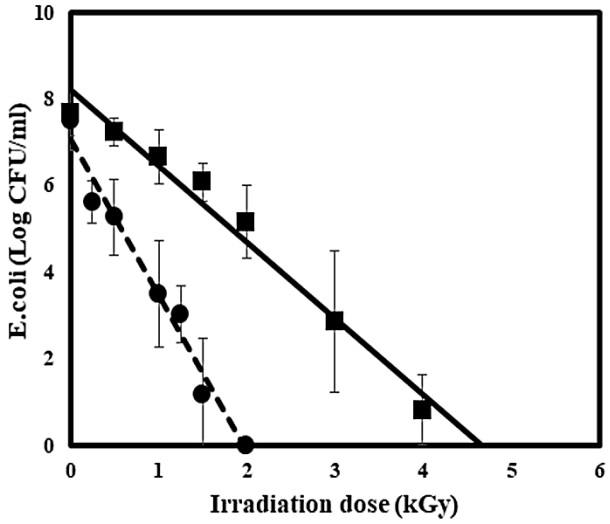

Figure 2. D_{10} (kGy) of *E. coli* in infant formula: liquid frozen sample (solid circle), powdered samples (solid square). Regression equation for plot are as follows: y = -3.60x + 7.08 (R^2 = 0.98) in liquid frozen and y = -1.76x + 8.24 (R^2 = 0.97) in powdered samples.

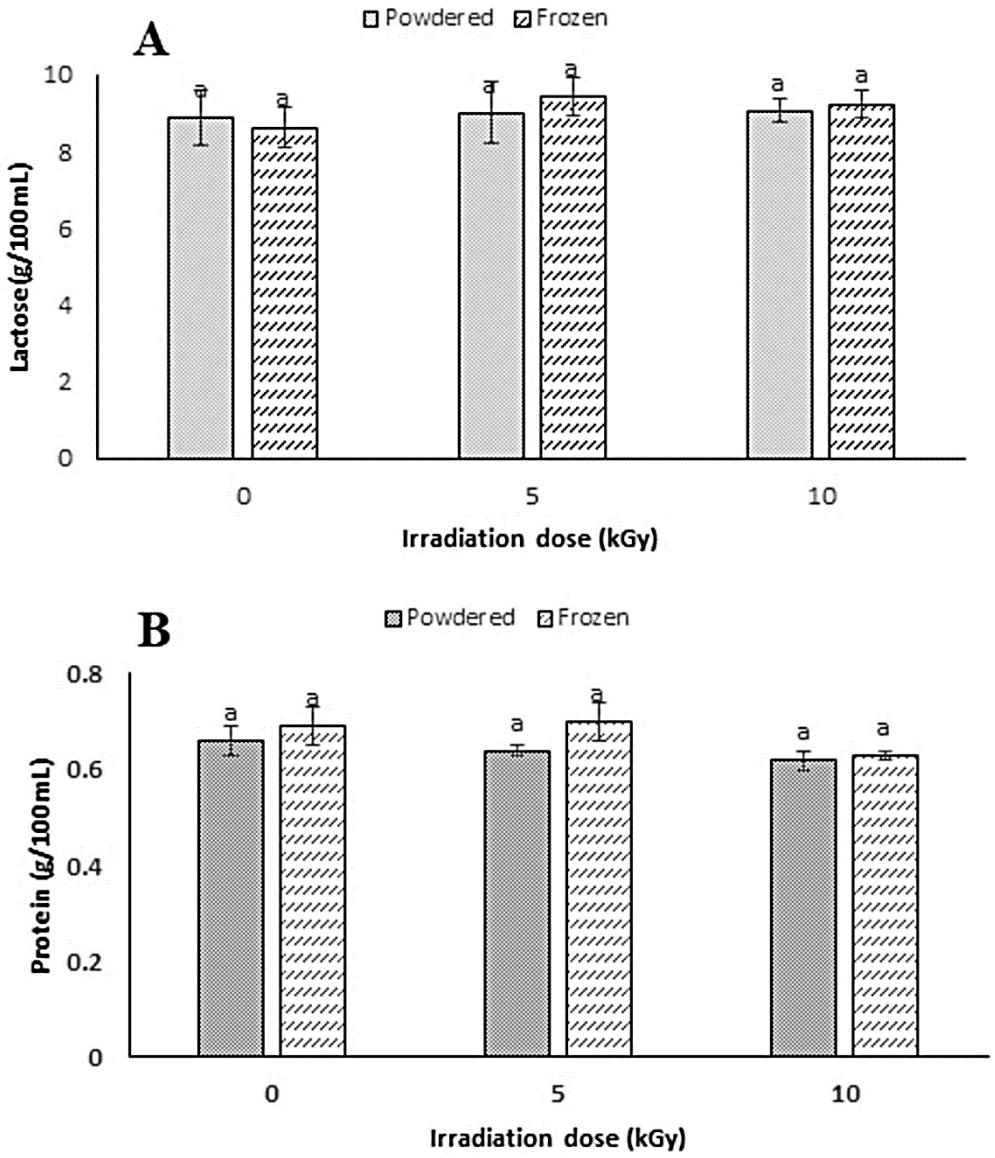

Figure 3. The effect of ɣ-irradiation dose on the lactose content (A) and protein content (B) of liquid frozen and powdered infant formula. Different superscripts indicate significant differences at $P < 0.05$.

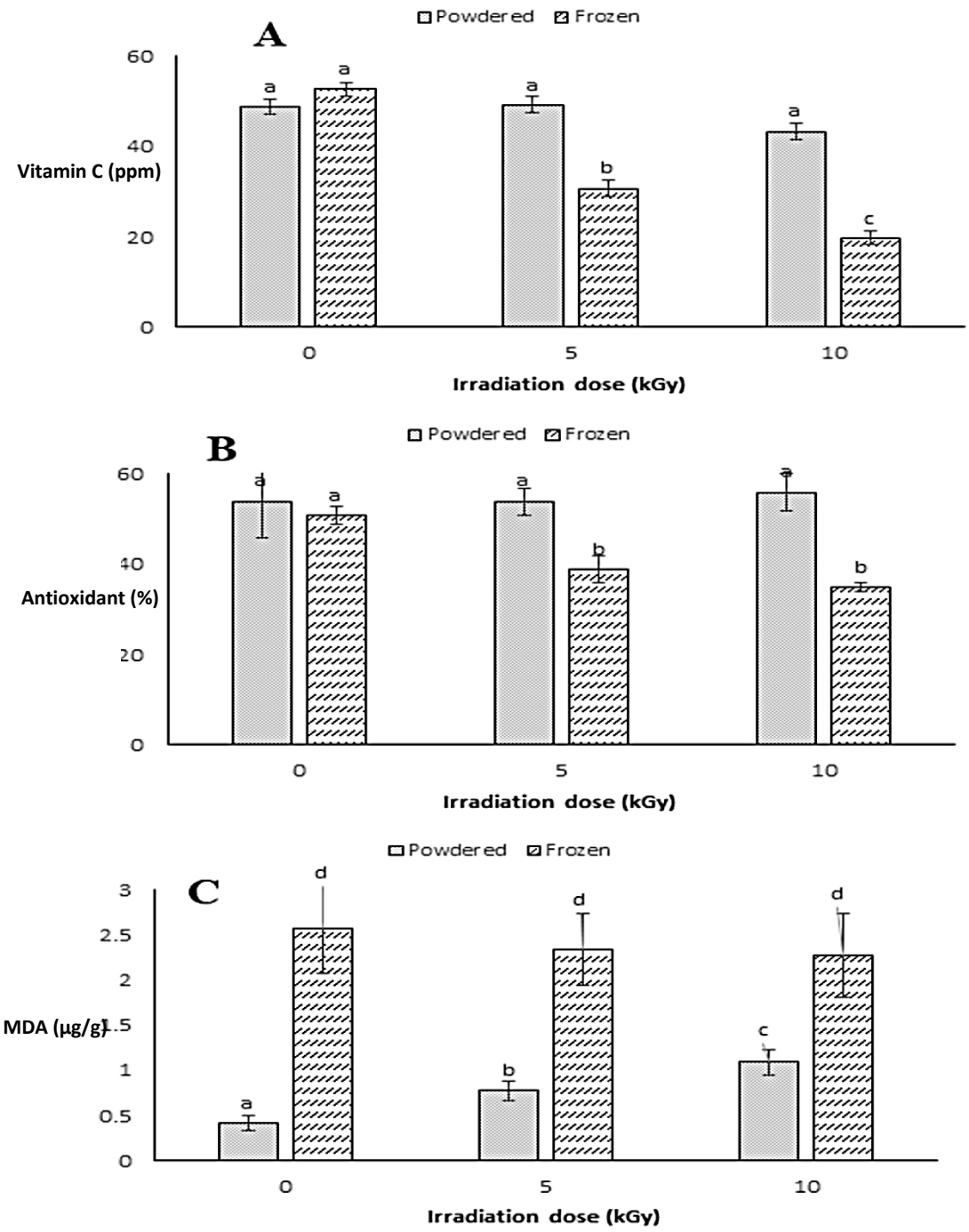

Figure 4. The effect of ɣ-irradiation dose on the vitamin C (A), antioxidant activity (B), and autoxidation of lipids content (C) of liquid frozen and powdered infant formula. Different superscripts indicate significant differences at $P < 0.05$.

References

Afify, A. E. I. M. M., & Shousha, M. A. (1988). Effect of low-dose irradiation on soybean protein solubility, trypsin inhibitor activity, and protein patterns separated by polyacrylamide gel electrophoresis. Journal of Agricultural and Food Chemistry, 36(4), 810–813. https://doi.org/10.1021/jf00082a035.

Alanber, M. N., Alharbi, N. S., & Khaled, J. M. (2019). Evaluation of multidrug-resistant Bacillus strains causing public health risks in powdered infant milk formulas. Journal of Infection and public Health. https://doi.org/10.1016/j.jiph.2019.11.013.

Arroyo, C., Dorozko, A., Gaston, E., O'Sullivan, M., Whyte, P., & Lyng, J. G. (2017). Light based technologies for microbial inactivation of liquids, bead surfaces and powdered infant formula. Food Microbiology, 67, 49–57. https://doi.org/10.1016/j.fm.2017.06. 001.

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. Nature, 181(4617), 1199–1200. https://doi.org/10.1038/1811199a0.

Botsoglou, N. A., Fletouris, D. J., Papageorgiou, G. E., Vassilopoulos, V. N., Mantis, A. J., & Trakatellis, A. G. (1994). Rapid, sensitive, and specific thiobarbituric acid method for measuring lipid peroxidation in animal tissue, food, and feedstuff samples. Journal of Agricultural and Food Chemistry, 42(9), 1931–1937. https://doi.org/10.1021/ jf00045a019.

Cheng, Y., Wei, H., Sun, R., Tian, Z., & Zheng, X. (2016). Rapid method for protein quantitation by Bradford assay after elimination of the interference of polysorbate 80. Analytical Biochemistry, 494, 37–39. https://doi.org/10.1016/j.ab.2015.10.013.

Chiasson, F., Borsa, J., Ouattara, B., & Lacroix, M. (2004). Radiosensitization of Escherichia coli and Salmonella Typhi in ground beef. Journal of Food Protection, 67(6), 1157–1162. https://doi.org/10.4315/0362-028x-67.6.1157.

Cho, T. J., Kim, H. W., Kim, N. H., Park, S.m., Kwon, J. I., Kim, Y. J., et al. (2018). New insights into the thermophilic spore-formers in powdered infant formula: Implications of changes in microbial composition during manufacture. Food Control, 92, 464–470. https://doi.org/10.1016/j.foodcont.2018.05.036.

Davies, K. J. (1987). Protein damage and degradation by oxygen radicals. I. general aspects. Journal of Biological Chemistry, 262(20), 9895–9901 PMID: 3036875.

Dickson, J. S. (2001). Radiation inactivation of microorganisms. In Ricardo A. Molins (Ed.). Food irradiation-principles and applications (pp. 23–35). New York: Wiley Interscience.

Dionísio, A. P., Gomes, R. T., & Oetterer, M. (2009). Ionizing radiation effects on food vitamins: A review. Brazilian Archives of Biology and Technology, 52, 1267–1278. https://doi.org/10.1590/S1516-89132009000500026.

Dogbevi, M., Vachon, C., & Lacroix, M. (2000). Effect of gamma irradiation on the microbiological quality and on the functional properties of proteins in dry red kidney beans (Phaseolus vulgaris). Radiation Physics and Chemistry - RADIAT PHYS CHEM, 57, 265–268. https://doi.org/10.1016/S0969-806X(99)00442-9.

Fiester, S., Helfinstine, S., Redfearn, J., Uribe, R., & Woolverton, C. (2012). Electron beam irradiation dose dependently damages the Bacillus spore coat and spore membrane. International journal of microbiology, 2012. https://doi.org/10.1155/2012/579593.

González-Olivares, L. G., Añorve-Morga, J., Castañeda-Ovando, A., Contreras-López, E., & Jaimez-Ordaz, J. (2014). Peptide separation of commercial fermented milk during refrigerated storage. Food Science and Technology (Campinas), 34(4), 674–679. https://doi.org/10.1590/1678-457x.6415.

Ham, J. S., Jeong, S. G., Lee, S. G., Han, G. S., Jang, A., Yoo, Y. M., et al. (2009). Quality of irradiated plain Yogurt during storage at different temperatures. Asian-Australasian Journal of Animal Sciences, 22(2), 289–295. https://doi.org/10.5713/ajas.2009. 80329.

Hassan, A. B., Mahmoud, N. S., Elmamoun, K., Adiamo, O. Q., & Ahmed, I. A. M. (2018). Effects of gamma irradiation on the protein characteristics and functional properties of sesame (Sesamum indicum L.) seeds. Radiation Physics and Chemistry, 144, 85–91. https://doi.org/10.1016/j.radphyschem.2017.11.020.

Hossain, F., Follett, P., Vu, K. D., Salmieri, S., Senoussi, C., & Lacroix, M. (2014). Radiosensitization of Aspergillus Niger and Penicillium chrysogenum using basil essential oil and ionizing radiation for food decontamination. Food Control, 45, 156–162. https://doi.org/10.1016/j.foodcont.2014.04.022.

Lacroix, M. (2012). Irradiation. In D-W Sun (Ed.). Handbook of food safety engineering (pp. 497–515). Oxford, UK: Blackwell.

Lacroix, M., Ayari, S., Dussault, D., Turgis, M., Salmieri, S., Perlette, T., et al. (2013). Irradiation in combined treatments and food safety. Journal of Radioanalytical and Nuclear Chemistry, 296(2), 1065–1069. https://doi.org/10.1007/s10967-012-2041-2.

Lacroix, M., Bernard, L., Jobin, M., Milot, S., & Gagnon, M. (1990). Effect of irradiation on the biochemical and organoleptic changes during the ripening of papaya and mango fruits. International Journal of Radiation Applications and Instrumentation - Part C: Radiation Physics and Chemistry, 35(1), 296–300. https://doi.org/10.1016/1359- 0197(90)90105-Q.

Lacroix, M., & Ouattara, B. (2000). Combined industrial processes with irradiation to assure innocuity and preservation of food products — a review. Food Research International, 33(9), 719–724. https://doi.org/10.1016/S0963-9969(00)00085-5.

Maherani, B., Khlifi, M. A., Salmieri, S., & Lacroix, M. (2019). Microemulsion-based biopreservatives and γ-irradiation as combined treatments to provide healthy and safe orange juice. Journal of Food Processing and Preservation, 43(4), e13909. https:// doi.org/10.1111/jfpp.13909.

Mahrour, A., Caillet, S., Nketsa-Tabiri, J., & Lacroix, M. (2003). Microbial and sensory quality of marinated and irradiated chicken. Journal of Food Protection, 66(11), 2156–2159. https://doi.org/10.4315/0362-028X-66.11.2156.

Monk, J. D., Beuchat, L. R., & Doyle, M. P. (1995). Irradiation inactivation of food-Borne microorganisms. Journal of Food Protection, 58(2), 197–208. https://doi.org/10. 4315/0362-028x-58.2.197.

Odueke, O. B., Chadd, S. A., Baines, R. N., Farag, K. W., & Jansson, J. (2018). Effects of gamma irradiation on the shelf-life of a dairy-like product. Radiation Physics and Chemistry, 143, 63–71. https://doi.org/10.1016/j.radphyschem.2017.09.013.

Osman, A. M. A., Hassan, A. B., Osman, G. A. M., Mohammed, N., Rushdi, M. A. H., Diab, E. E., et al. (2014). Effects of gamma irradiation and/or cooking on nutritional quality of faba bean (Vicia faba L.) cultivars seeds. Journal of Food Science & Technology, 51(8), 1554–1560. https://doi.org/10.1007/s13197-012-0662-7.

Richmond, M. L., Harte, B. R., Gray, J. I., & Stine, C. M. (1987). Determination of sugars in Yogurt and microbiological media by high performance liquid chromatography during processing and subsequent Storage 1. Journal of Dairy Science, 70(6), 1140–1147. https://doi.org/10.3168/jds.S0022-0302(87)80124-8.

Roberts, P. B. (2016). Food irradiation: Standards, regulations and world-wide trade. Radiation Physics and Chemistry, 129, 30–34. https://doi.org/10.1016/j. radphyschem.2016.06.005.

Romeu-Nadal, M., Morera-Pons, S., Castellote, A. I., & López-Sabater, M. C. (2006). Rapid high-performance liquid chromatographic method for Vitamin C determination in human milk versus an enzymatic method. Journal of Chromatography B, 830(1), 41–46. https://doi.org/10.1016/j.jchromb.2005.10.018.

Saroj, S. D., Hajare, S., Shashidhar, R., Dhokane, V., Sharma, A., & Bandekar, J. R. (2007). Radiation processing for elimination of Salmonella Typhimurium from inoculated seeds used for sprout making in India and effect of irradiation on germination of seeds. Journal of Food Protection, 70(8), 1961–1965. https://doi.org/10.4315/0362- 028X-70.8.1961.

Shih, F. F., & Kalmar, A. D. (1987). SDS-catalyzed deamidation of oilseed proteins. Journal of Agricultural and Food Chemistry, 35(5), 672–675. https://doi.org/10.1021/ jf00077a009.

Sohn, S. H., Jang, A., Kim, J. K., Song, H. P., Kim, J. H., Lee, M., et al. (2009). Reduction of irradiation off-odor and lipid oxidation in ground beef by α-tocopherol addition and the use of a charcoal pack. Radiation Physics and Chemistry, 78(2), 141–146. https://doi.org/10.1016/j.radphyschem.2008.09.003.

Song, W.-J., Sung, H.-J., Kim, S.-Y., Kim, K.-P., Ryu, S., & Kang, D.-H. (2014). Inactivation of Escherichia coli O157: H7 and Salmonella typhimurium in black pepper and red pepper by gamma irradiation. International Journal of Food Microbiology, 172, 125–129. https://doi.org/10.1016/j.ijfoodmicro.2013.11.017.

Stefanova, R., Vasilev, N. V., & Spassov, S. L. (2010). Irradiation of food, current legislation framework, and detection of irradiated foods, Vol. 3https://doi.org/10.1007/s12161- 009-9118-8.

Tesfai, A., Beamer, S. K., Matak, K. E., & Jaczynski, J. (2014). Effect of electron beam on chemical changes of nutrients in infant formula. Food Chemistry, 149, 208–214. https://doi.org/10.1016/j.foodchem.2013.10.110.

Wang, J., & Chao, Y. (2003). Effect of 60Co irradiation on drying characteristics of apple. Journal of Food Engineering, 56(4), 347–351. https://doi.org/10.1016/S0260- 8774(02)00160-7.

Wazed, M. A., Ismail, M., & Farid, M. (2020). Pasteurized ready-to-feed (RTF) infant formula fortified with lactoferrin: A potential niche product. Journal of Food Engineering, 273, 1–10. https://doi.org/10.1016/j.jfoodeng.2019.109810.

Zarei, H., Bahreinipour, M., Eskandari, K., MousaviZarandi, S.-A., & Ardestani, S. K. (2017). Spectroscopic study of gamma irradiation effect on the molecular structure of bovine serum albumin. Vacuum, 136, 91–96. https://doi.org/10.1016/j.vacuum. 2016.11.029.

Zelic, B., & Nesek, B. (2006). Mathematical modeling of size exclusion chromatography. Engineering in Life Science, 6(2), 163–169. https://doi.org/10.1002/elsc.200620903.

CHAPITRE 3:
EFFECT OF ϒ-IRRADIATION AND FOOD ADDITIVES ON THE MICROBIAL INACTIVATION OF FOODBORNE PATHOGENS IN INFANT FORMULA

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Contribution des auteurs

Valérie Robichaud a réalisé les manipulations et la rédaction de l'article.

Leila Bagheri et Stéphane Salmieri ont participé à la correction de l'article.

Blanca Aguilar-Uscanga : Co-directrice du projet.

Mathieu Millette : Co-directeur du projet.

Monique Lacroix : Directrice de recherche, responsable scientifique et coordinatrice du projet de recherche, a participé à la planification des expériences et à la correction de l'article.

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Résumé

Le but de cette étude est d'évaluer l'effet de l'irradiation γ sur l'inactivation microbienne de certains agents pathogènes d'origine alimentaire (*B. cereus*, *L. monocytogenes*, *S. aureus*, *E. coli* O157: H7 et *S.* Typhimurium) en association avec 2,5% de citrate de sodium, 0,5% de carbonate de sodium et 0,75% d'acide citrique comme additifs alimentaires dans les préparations pour nourrissons (IF) congelées et en poudre. L'étude a démontré que l'irradiation était plus efficace contre les agents pathogènes dans les IF congelées que les poudres. Un traitement combiné avec du carbonate de sodium a induit une radiosensibilisation élevée (RS) dans les IF en poudre et congelés contre tous les agents pathogènes, par rapport aux autres additifs qui ont induit un effet de RS plus faible. Contrairement à d'autres agents pathogènes, *B. cereus* qui est sporulante était plus radiosensibilisée dans l'IF en poudre par le carbonate de sodium, le citrate de sodium et l'acide citrique, avec des valeurs de radiosensibilité allant jusqu'à 4,1. *Escherichia coli* était fortement radiosensibilisée en présence de carbonate de sodium et de citrate de sodium dans l'IF congelée avec des valeurs allant jusqu'à 2,4. Cette étude a démontré que l'utilisation d'additifs alimentaires - principalement du carbonate de sodium - en combinaison avec l'irradiation y peut être un moyen rentable de garantir la sécurité du produit IF.

Mots clés: Préparation pour nourrissons, décontamination, irradiation, additif alimentaire, radiosensibilisation

Abstract

The aim of this study was to assess the effect of γ-irradiation on the microbial inactivation of selected foodborne pathogens (*Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, Escherichia coli* 0157:H7*, and Salmonella* Typhimurium) in combination with 2.5% sodium citrate, 0.5% sodium carbonate and 0.75% citric acid as food additives in frozen and powdered infant formula (IF). The study demonstrated that γ-irradiation alone was more efficient against pathogens in frozen IF. A hurdle technology with sodium carbonate induced a high radiosensitization in powdered and frozen IF against all pathogens, compared to other additives that induced a lower radiosensitization effect. Contrarily to other pathogens, spore-producing *B. cereus* was more radiosensitized in powdered IF by carbonate, citrate, and citric acid, with radiosensitivity values up to 4.1. *E. coli* was strongly radiosensitized in presence of carbonate and citrate in frozen IF with values up to 2.4. This study demonstrated that the use of food additives – mainly sodium carbonate – in combination with γ-irradiation can be a good way to reduce the time of irradiation treatment to assure the safety of the IF product.

Keywords: Infant formula, Decontamination, γ-Irradiation, Food additive, Radiosensitization

1. Introduction

Powdered infant formula (IF) is the best alternative to breast milk and provides adequate nutrition to the newborns. It is convenient for handling, preservation and is easy-to-use. However, clinical cases of foodborne illnesses have been associated with this product because of poor standards of manufacturing and sterilization (Losio et al., 2018). Industries are constantly looking to increase food safety without increasing the production cost. Decontamination technologies such as γ-irradiation would be an interesting option as a non-thermal, versatile and efficient method to inactivate bacteria and extend the shelf-life of food products (Lacroix et al., 2013).

Infantile infectious diseases associated with IF are often related to *Cronobacter sakazakii*, *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus* and different serovars of *Salmonella* (Arroyo et al., 2017; Cho et al., 2019). Sporulated bacteria are a challenge to eliminate in food because they are highly resistant and require high irradiation doses (Dauphin, Newton, Rasmussen, Meyer, & Bowen, 2008). It has been established that the combination of irradiation and natural additives can induce radiosensitization of food pathogens (Caillet, Millette, Turgis, Salmieri, & Lacroix, 2006). An increased radiation sensitivity of the target microorganisms, particularly spore-forming bacteria, would result in lower doses required for lethality, which contributes to increase food safety while being cost-effective and time-effective.

Sodium citrate, sodium carbonate and citric acid are among accepted food additive in IF permitted by the Codex Alimentarius (Join FAO/WHO Codex Alimentarius Commission, 1995). They have been used as stabilizers, acidity regulators and food additives. The concentrations used in the present study were added according to the minimal inhibitory concentrations (MIC) obtained in a preliminary study.

More recently, some additives have been used in combination with γ-irradiation and heat-treatment to improve the inactivation of foodborne pathogens (Lacroix, Caillet, & Shareck, 2009; Lenzi, Lucchesi, Medico, Burgán, & Krüger, 2016; Mahmoud, Mohamed, Botros, & Sabri, 2011; Negrón-Mendoza & Ramos-Bernal, 2015; Tuner & Korkmaz, 2010).

The objective of this study was to evaluate the effect of sodium citrate, sodium carbonate and citric acid as food additives and radiosensitizers in IF, in order to reduce the dose of γ-irradiation needed to eliminate common foodborne pathogens (*B. cereus*, *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7, and *S.* Typhimurium) in the powdered and frozen solution of IF.

2. Materials and methods

2.1. Materials

Tryptic soy broth (TSB), Tryptic soy agar (TSA), and peptone water were purchased from Alpha Biosciences Inc. (Baltimore, MD, USA). We used sterile IF in a can. The product was then frozen/spray-dried under sterile conditions. All chemicals were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). Commercial IF was provided from a local store in Laval, QC, Canada.

2.2. Preparation of bacterial culture, samples, additive and inoculation procedures

E. coli O157: H7 (EDL 993; ATCC 43895), *S. aureus* (ATCC 29213), *S.* Typhimurium (ATCC SL1344), *L. monocytogenes* (HPB 2812 serovar 1/2a) and *B. cereus* (ATCC 14579) were maintained at −80 °C in TSB containing glycerol (10% v/v). Before each experiment, stock cultures were propagated through 2 consecutive 24-h growth cycles in TSB at 37 °C to obtain working cultures of approximately 10^8 CFU/mL for all target bacteria except for *B. cereus* to obtain 10^6 CFU/mL. The working cultures were obtained through centrifuging a certain volume of 24-h cultures, followed by washing the collected pellets twice with 0.85% (w/v) of sterile saline solution. The powdered IF was then inoculated with the selected pathogens and thoroughly mixed. For the frozen solution samples, the powdered IF was dissolved (14.5% w/v) according to the manufacturer's instructions in Milli-Q water prior to inoculation. Additives were added to obtain a final reconstituted concentration of 0.5% (w/v) sodium carbonate, 2.5% sodium citrate or 0.75% citric acid. All tubes containing inoculated samples were double sealed under a normal atmosphere. Frozen samples were kept at −20 °C and powdered samples were kept at 4 °C for 24 h before irradiation. Some studies have shown a higher D_{10} (*i.e.* a lower radiosensitivity) at low temperatures for *E. coli*, S. *aureus* and *L. monocytogenes* in meat (Thayer & Boyd, 1995, 2001). Therefore, one of the objectives of this study was to evaluate the effect of freezing on the radiosensitivity of the target pathogens in the IF.

2.3. γ-Irradiation treatment

Irradiation (9.172 kGy/h) was done in a UC-15A irradiator (Nordion Inc., Kanata, ON, Canada) equipped with a ^{60}Cobalt source. Dry ice was inserted in the radiator to keep the samples frozen. The different doses of irradiation (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10 kGy) were applied to determine D_{10} values. D_{10} is defined as the radiation dose needed to destroy one log cycle (90%) of the target microorganism. D_{10} values and relative sensitivity were calculated based on the reciprocal of the linear regression of bacterial populations (log CFU/g) plotted against irradiation doses (Hossain et al., 2014).

The relative radiation sensitivity (RS) that was obtained by the equation below:

$$\text{RS (Control/Teatment)} = \frac{\text{D10(KGy, for control)}}{\text{D10(KGy, for treatment)}}$$

The irradiator used in this study was certified by the National Institute of Standards and Technology (Gaithersburg, Md.). The dose rate was established using a correction for decays of the source. Amber Oersoex 3042D (Atomic Energy Research Establishment, Harwell, Oxfordshire, UK) was used to validate the doses distributions.

2.4. Microbiological analysis

Frozen samples were thawed and immediately diluted in sterile peptone water (0.1%). Each tube (250 mg) of powdered IF was reconstituted (14.5% v/v) in sterile peptone water (0.1%). For each sample, serial dilutions were prepared in peptone water (0.1% w/v). A volume of 20 µL of appropriate dilution was plated on TSA and incubated at 37 °C for 24 h following the method of Herigstad (Herigstad, Hamilton, & Heersink, 2001).

3. Results and discussion

3.1. Inactivation of bacteria using γ-irradiation

The results in Table 1 demonstrate a significant ($p \leq 0.05$) higher bacterial sensitivity trend in frozen IF than in powdered IF. It is observed that *E. coli*, *L. monocytogenes* and *S.* Typhimurium were respectively 1.5, 1.6 and 2 times more sensitive to irradiation in frozen IF. The sporulated bacteria *B. cereus* had the highest difference ($p \leq 0.05$) in sensitivity with a relative sensitivity of 3.0 in frozen IF compared to the powdered product, whereas no impact on *S. aureus* was observed ($p > 0.05$). A possible explanation for the increased bacterial sensitivity in the frozen formula is associated with the effects of oxygen availability, water content and food matrix rather than the influence of temperature (Ben Miloud Yahia et al., 2018; Robichaud, Bagheri, Aguilar-Uscanga, Millette, & Lacroix, 2020). Indeed, some studies have shown a higher D_{10} (*i.e.* a lower radiosensitivity) at low temperatures for *E. coli*, S. *aureus* and *L. monocytogenes* in meat (Thayer & Boyd, 1995, 2001). On the other hand, higher water activity and water content are associated with an increased radiosensitivity because of the greater production of water-derived free radicals (Harrell, Djonov, Fellabaum, & Volarevic, 2018).

By comparing bacterial resistance in powdered IF, *B. cereus* was the most resistant bacteria (D_{10} of 2.49 kGy), followed by *S.* Typhimurium (D_{10} of 0.97 kGy), known as being relatively insensitive to

irradiation among Gram-negative bacteria (Monk, Beuchat, & Doyle, 1995) and *L. monocytogenes* (D_{10} of 0.92 kGy), *E. coli* (0.64 kGy) and *S. aureus* (0.46 kGy). By comparing frozen to powdered IF, results show that the frozen state affected especially the most resistant bacteria after irradiation, with an increase in sensitivity of *B. cereus* (3.0), *S.* Typhimurium (2.0), and *L. monocytogenes* (1.6) compared to powdered IF. Hence, these strains could be particularly prone to indirect γ-irradiation consequences such as oxidative damages that are exhibited in frozen IF. Furthermore, the higher sensitivity of *B. cereus* in frozen IF might be explained by the diminution in spore production due to the inoculation procedure. The incorporation of the bacteria in a dry environment such as powdered IF is known to induce sporulation at a higher rate than in a nutritive aqueous media (Tetz & Tetz, 2017). Consequently, a greater abundance and resistance of vegetative cells in powdered IF would explain the radical difference in D_{10} values (2.49 kGy in powdered state *vs* 0.82 kGy in a frozen state).

3.2. Inactivation of bacteria using γ-irradiation combined with sodium carbonate

The effects of adding sodium carbonate on the bacterial radiosensitivity in IF are presented in Table 2. Under the powdered IF form, results suggest that *S. aureus* and *L. monocytogenes* were not significantly sensitized ($p > 0.05$) in the presence of sodium carbonate, with similar D_{10} values (0.45–0.46 kGy for *S. aureus* and 0.92–0.94 kGy for *L. monocytogenes*) and therefore a radiosensitivity of 1.0. On the other hand, *S.* Typhimurium, *E. coli* and *B. cereus* showed higher radiosensitivity in presence of carbonate with respective RS of 1.3, 2.0 and 4.1, suggesting that the most drastic sensitivity values were obtained for *E. coli* and *B. cereus* (2.0 and 4.1 respectively). For the frozen samples, all strains were significantly different ($p \leq 0.05$) from their respective control. Minor RS effects were observed for *L. monocytogenes and B. cereus*, with relative RS of 1.3 and 1.2 respectively. Besides, *S.* Typhimurium, *S. aureus* and *E. coli* respectively showed a RS of 1.5, 1.8 and 2.4 compared to their control, suggesting they were the most radiosensitive bacteria. Sodium carbonate is a "Generally Recognized As Safe" (GRAS) ingredient and is commonly used in the processed food industry without known impact on health. However, a limit of 2000 mg/kg (0.2% w/w) is accepted in IF according to the Codex Alimentarius. Sodium carbonate is a moderate strength base used as an acidity regulator, anticaking agent, emulsifier, leavening agent, stabilizer, flavoring agent and thickener. It is also used as a disinfectant for citrus to reduce mold by more than 50%. Furthermore, a radiosensitivity of indigenous microbial flora and spores was also observed in apples and peaches when irradiation treatment was done in combination with sodium carbonate at concentrations between 0.5 and 3% (w/v) (Mahmoud et al., 2011; Temur & Tiryaki, 2014). Hence, our results suggest that *E. coli* is very sensitive to sodium carbonate both in powdered and frozen IF. According to Jarvis, Fields, Adamovich, Arthurs, and Russell (2001), under an alkaline environment, part of sodium carbonate is present under anionic CO_3^{2-} form, which is a reactive moiety that forms insoluble divalent metal ion

complexes, compromising the function of enzymes and the outer membrane of Gram-negative bacteria. Moreover, some studies have shown that this chemical is able to inhibit or at least interfere in the germination of different spore-forming bacteria at 0.1% w/v (Cheung, So, & Sun, 1998; Hachisuka, Kato, & Asano, 1956). This is the combined effect of the alkalinization of the medium (pH > 7.8) that tends to inhibit the germination of spores, and the effect of the carbonate itself that induces structural alterations of keratin-like proteins in the spores (Cheung et al., 1998). Thus, sodium carbonate 0.5% can be considered as a good radiosensitizer in both frozen and powdered forms of IF against all tested strains, with a higher radiosensitization effect in frozen IF, especially against *E. coli*, *S. aureus* and *S.* Typhimurium and a typical higher radiosensitization effect in powdered IF against *B. cereus*.

3.3. Inactivation of bacteria using γ-irradiation combined with sodium citrate

The effects of adding sodium citrate on the bacterial radiosensitization and RS in IF are presented in Table 3. Results showed that in powdered IF, the addition of sodium citrate led to an important RS of 2.6 against *B. cereus* and a low RS of 1.2 against *E. coli*. Indeed, the addition of citrate induced a significant decrease of D_{10} value from 2.49 to 0.96 kGy ($p \leq 0.05$) to inhibit *B. cereus* whereas *E. coli* was slightly sensitized with a significant decrease of D_{10} value from 0.64 to 0.52 kGy ($p \leq 0.05$). Other bacteria were not significantly affected by sodium citrate ($p > 0.05$). In frozen IF, *E. coli* and *B. cereus* both showed a higher RS of 1.4 in presence of citrate with a significant decrease of D_{10} values ($p \leq 0.05$) compared to the other bacteria. Sodium citrate is a weak base that has a sour taste. It is used as a preservative, antioxidant, acidity regulator, emulsifier and stabilizer. It is an affordable compound and is regulated as a GRAS ingredient when used according to the Good Manufacturing Practices (GMP) in food processing. Its antimicrobial and antioxidant effects were studied on refrigerated sliced salmon (2.5% w/v) (Sallam, 2007). It was also shown *in vitro* to have antimicrobial properties against Gram-positive bacteria such as *S. aureus*, and *S. epidermidis* at a minimal inhibitory concentration (MIC) lower than 1.56% (w/v), and against Shiga toxin-producing *E. coli* at 2.5% (w/v) (Lee, Cesario, Owens, Shanbrom, & Thrupp, 2002; Lenzi et al., 2016). In our study, the results showed that the only Gram-positive bacterium affected by sodium citrate was *B. cereus* in both powdered and frozen IF. Some studies demonstrated that 0.5% sodium citrate in combination with heat treatment was able to increase the spore destruction of *B. cereus* in skim milk (González, López, Mazas, González, & Bernardo, 1997; Mazas, López, Martínez, Bernardo, & Martin, 1999; Shehata & Collins, 1972). Thus, our results show that sodium citrate 0.5% in both frozen and powdered IF presents a good potential as a radiosensitizer against *B. cereus* and *E. coli*.

3.4. Inactivation of bacteria using γ-irradiation combined with citric acid

The effects of adding citric acid on the bacterial radiosensitization in IF are presented in Table 4. Relatively to powdered IF, results show that *B. cereus* showed the highest RS (2.1) in presence of citric acid with a decrease of D_{10} value from 2.49 to 1.20 kGy ($p \leq 0.05$). All other bacteria were not affected by the presence of citric acid. In frozen IF, citric acid did not generate any radiosensitivity effect against all bacteria as no significant difference ($p > 0.05$) was observed in the D_{10} values, with low RS in a range of 0.9–1.2. Citric acid is a GRAS ingredient and is used in a wide variety of foodstuff in industries as an acidity regulator, an emulsifier, and a stabilizer. It is a weak organic acid that can provide sour taste to food. *In vitro*, it can inhibit some types of *Shigella* in concentrations between 0.5 and 2% (Kim, Kim, & Oh, 2013; Olaimat et al., 2017). In general, citric acid tends to have antimicrobial properties at lower pH, but without effect towards *E.coli, S. aureus or L. monocytogenes* at neutral pH (Hawkins, 2014). It was also shown to have heat sensitizing properties on beef carcasses (Scott et al., 2015). To our best knowledge, citric acid was never tested in combination with γ-irradiation, but it was effective to prevent the germination of *Bacillus* spores *in vitro* and *in situ* alone or in combination with mild heat treatments or electrolyzed water (Palop, Marco, Raso, Sala, & Condón, 1997; Palop, Raso, Condón, & Sala, 1996; Park, Guo, Rahman, Ahn, & Oh, 2009). By comparing these results with those obtained in presence of sodium carbonate or sodium citrate, citric acid 0.75% was assessed as a good sensitizer against *B. cereus* only in powdered IF. However, it demonstrated no radiosensitization efficiency against other bacteria and it was also ineffective against all bacteria in frozen IF.

3.5. Inactivation of bacteria using γ-irradiation combined with other additives

Other food additives with promising antioxidant, antimicrobial or radiosensitizing properties were tested such as ascorbic acid, ascorbyl palmitate, β-carotene and tocopheryl phosphate − a water-soluble derivative of α-tocopherol − with concentrations from 0.002 to 2% (w/w). No radiosensitization effect was found for these compounds against the selected pathogens in either powdered or frozen solution of IF (data not shown). Ascorbyl palmitate, β-carotene and α-tocopherol have been shown to provide very effective antioxidant protection and potentially demonstrated a synergistic antimicrobial effect with γ-irradiation in beef patties at a concentration of 0.02% (w/w) (Lee, Yook, Kim, Lee, & Byun, 1999). Although the antioxidant properties of these compounds are now widely accepted, to our knowledge, no study was able to reproduce their antibacterial results in food (Cort, 1974, 1982). Based on the fact these compounds are highly antioxidant, their antiradical activity may protect not only the IF matrix but also the bacteria by scavenging free radicals generated by γ-irradiation, as reported previously (Oussalah, Caillet, Salmieri, Saucier, & Lacroix, 2004). The Ascorbic acid (0.5% w/w) was shown to induce radiosensitization in beef

patties while stabilizing the coloring of beef undergoing irradiation (Giroux et al., 2001; Ouattara, Giroux, Smoragiewicz, Saucier, & Lacroix, 2002). Its antimicrobial activity was also demonstrated *in vitro* on pathogenic bacteria *E. coli* and *Klebsiella pneumoniae* at a concentration of 1% (w/v) (Verghese, Mathew, & David, 2017). Ascorbic acid is known to work by competing with mineral bonding sites, altering cell surface, acting as a barrier for oxygen availability, and lowering the pH in aqueous media (Rawal, 1978; Tajkarimi & Ibrahim, 2011). In this study, the milk was irradiated in powdered and frozen form. Thus, it is possible that these conditions may have inhibited or inactivated the antimicrobial action of ascorbic acid.

4. Conclusion

When treated by γ-irradiation alone, all tested pathogens (*B. cereus*, *L. monocytogenes*, *S. aureus*, *E. coli* O157: H7 and *S.* Typhimurium) were more affected in the frozen form of IF rather than in its powdered form, with a higher radiosensitization effect against *B. cereus*, *S.* Typhimurium and *L. monocytogenes*. The combination of γ-irradiation with 0.5% sodium carbonate was the most effective treatment to induce radiosensitization. All the tested bacteria had a higher radiosensitization when exposed to sodium carbonate either in powdered or frozen solution, whereas only *E. coli* and *B. cereus* were sensitive to sodium citrate. Citric acid was only effective against *B. cereus* in powdered IF but induced no radiosensitization in the frozen products. Ascorbic acid, ascorbyl palmitate, β-carotene and tocopheryl phosphate generated no radiosensitization effect in our study. As a result, the combination of sodium carbonate and γ-irradiation is a promising treatment to induce radiosensitization against a broad spectrum of IF pathogens in frozen products. Overall, this study showed that the incorporation of GRAS food additives to IF in combination with γ-irradiation can be a good way to ensure better safety of the product with a lower time of treatment.

Declaration of competing interest

I confirm that there is no conflict of interest in this study.

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List of tables

Table 1. D_{10} (kGy), required irradiation dose to reach 6-log reduction and relative radiation sensitivity (RS) values of *B. cereus*, *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *S.* Typhimurium in frozen and powdered IF.

Bacteria	D_{10} (kGy)		RS (powdered/frozen)
	Powdered	Frozen	
E. coli	0.64 ± 0.04b	0.44 ± 0.09a	1.5
S. aureus	0.46 ± 0.08a	0.42 ± 0.02a	1.1
***S.* Typhimurium**	0.97 ± 0.07b	0.50 ± 0.10a	2.0
L. monocytogenes	0.92 ± 0.15b	0.52 ± 0.10a	1.6
B. cereus	2.49 ± 0.13b	0.82 ± 0.06a	3.0

Data are expressed as mean ± standard deviation for triplicate tests (n = 3). Means with different lowercase letters within a row are significantly different ($p \leq 0.05$).

Table 2. D_{10} (kGy) and relative radiation sensitivity (RS) values of *B. cereus*, *L. monocytogenes*, *S. aureus*, *E.coli* O157:H7 and *S.* Typhimurium in frozen and powdered IF with 0.5% w/v of sodium carbonate.

Bacteria	D_{10} (kGy) Control (without additive)		D_{10} (kGy) 0.5% sodium carbonate		RS (control/treatment)	
	Powdered	**Frozen**	**Powdered**	**Frozen**	**Powdered**	**Frozen**
E. coli	0.64 ± 0.04b	0.44 ± 0.09B	0.32 ± 0.02a	0.18 ± 0.02A	2.0	2.4
S. aureus	0.46 ± 0.08a	0.42 ± 0.02B	0.45 ± 0.06a	0.23 ± 0.05A	1.0	1.8
***S.* Typhimurium**	0.97 ± 0.07b	0.50 ± 0.10B	0.76 ± 0.08a	0.33 ± 0.04A	1.3	1.5
L. monocytogenes	0.92 ± 0.15a	0.52 ± 0.10B	0.94 ± 0.06a	0.40 ± 0.04A	1.0	1.3
B. cereus	2.49 ± 0.13b	0.82 ± 0.06B	0.61 ± 0.13a	0.67 ± 0.10A	4.1	1.2

Data are expressed as mean ± standard deviation for triplicate tests (n = 3). For powdered IF, means with different lowercase letters within a row (control *vs* sodium carbonate) are significantly different ($p \leq 0.05$). For frozen IF, means with different uppercase letters within a row (control *vs* sodium carbonate) are significantly different ($p \leq 0.05$).

Table 3. D_{10} (kGy) and relative radiation sensitivity (RS) values of *B. cereus*, *L. monocytogenes*, *S. aureus*, *E.coli* O157:H7 and *S.* Typhimurium in frozen and powdered IF with 2.5% w/v of sodium citrate.

Bacteria	D_{10} (kGy) Control (without additive)		D_{10} (kGy) 2.5% sodium citrate		RS (control/treatment)	
	Powdered	**Frozen**	**Powdered**	**Frozen**	**Powdered**	**Frozen**
E. coli	0.64 ± 0.04b	0.44 ± 0.09B	0.52 ± 0.02a	0.31 ± 0.04A	1.2	1.4
S. aureus	0.46 ± 0.08a	0.42 ± 0.02A	0.46 ± 0.01a	0.38 ± 0.10A	1.0	1.1
***S.* Typhimurium**	0.97 ± 0.07a	0.50 ± 0.10A	0.91 ± 0.02a	0.42 ± 0.09A	1.1	1.2
L. monocytogenes	0.92 ± 0.15a	0.52 ± 0.10A	0.92 ± 0.05a	0.45 ± 0.15A	1.0	1.1
B. cereus	2.49 ± 0.13b	0.82 ± 0.06B	0.96 ± 0.12a	0.58 ± 0.07A	2.6	1.4

Data are expressed as mean ± standard deviation for triplicate tests (n = 3). For powdered IF, means with different lowercase letters within a row (control *vs* sodium carbonate) are significantly different ($p \leq 0.05$). For frozen IF, means with different uppercase letters within a row (control *vs* sodium carbonate) are significantly different ($p \leq 0.05$).

Table 4. D_{10} (kGy) and relative radiation sensitivity (RS) values of *B. cereus*, *L. monocytogenes*, *S. aureus*, *E.coli* O157:H7 and *S.* Typhimurium in frozen and powdered IF with 0.75% w/v of citric acid.

Bacteria	D_{10} (kGy) Control (without additive)		D_{10} (kGy) 0.75% citric acid		RS (control/treatment)	
	Powdered	**Frozen**	**Powdered**	**Frozen**	**Powdered**	**Frozen**
E. coli	0.64 ± 0.04a	0.44 ± 0.09A	0.63 ± 0.10a	0.46 ± 0.08A	1.0	1.0
S. aureus	0.46 ± 0.08a	0.42 ± 0.02A	0.50 ± 0.10a	0.36 ± 0.05A	0.9	1.2
***S.* Typhimurium**	0.97 ± 0.07a	0.50 ± 0.10A	1.09 ± 0.09a	0.52 ± 0.05A	0.9	1.0
L. monocytogenes	0.92 ± 0.15a	0.52 ± 0.10A	0.97 ± 0.05a	0.55 ± 0.09A	1.0	0.9
B. cereus	2.49 ± 0.13b	0.82 ± 0.06A	1.20 ± 0.13a	0.84 ± 0.11A	2.1	1.0

Data are expressed as mean ± standard deviation for triplicate tests (n = 3). For powdered IF, means with different lowercase letters within a row (control *vs* sodium carbonate) are significantly different ($p \leq 0.05$). For frozen IF, means with different uppercase letters within a row (control *vs* sodium carbonate) are significantly different ($p \leq 0.05$).

References

Arroyo, C., Dorozko, A., Gaston, E., O'Sullivan, M., Whyte, P., & Lyng, J. G. (2017). Light based technologies for microbial inactivation of liquids, bead surfaces and powdered infant formula. *Food Microbiology*, *67*, 49–57.

Ben Miloud Yahia, N., Ghorbal, S. K. B., Maalej, L., Chatti, A., Elmay, A., Chihib, N.-E., & Landoulsi, A. (2018). Effect of temperature and gamma radiation on *salmonella* hadar biofilm production on different food contact surfaces. *Journal of Food Quality*, *2018*, 1–6.

Caillet, S., Millette, M., Turgis, M., Salmieri, S., & Lacroix, M. (2006). Influence of antimicrobial compounds and modified atmosphere packaging on radiation sensitivity of Listeria monocytogenes present in ready-to-use carrots (Daucus carota). *Journal of Food Protection*, *69*, 221–227.

Cheung, H. Y., So, C. W., & Sun, S. Q. (1998). Interfering mechanism of sodium bicarbonate on spore germination of Bacillus stearothermophilus. *Journal of Applied Microbiology*, *84*, 619–626.

Cho, T. J., Hwang, J. Y., Kim, H. W., Kim, Y. K., Kwon, J. I., Kim, Y. J., Lee, K. W., Kim, S. A., & Rhee, M. S. (2019). Underestimated risks of infantile infectious disease from the Caregiver's typical handling practices of infant f
ormula. *Scientific Reports*, *9*, 9799.

Cort, W. M. (1974). Antioxidant activity of tocopherols, ascorbyl palmitate, and ascorbic acid and their mode of action. *Journal of the American Oil Chemists' Society*, *51*, 321–325.

Cort, W. M. (1982). Antioxidant properties of ascorbic acid in foods. In P. A. Seib & B. M. Tolbert (Eds.), *Ascorbic acid: chemistry, metabolism, and uses* (Vol. 200, pp. 533–550). Washington DC: American Chemical Society.

Dauphin, L. A., Newton, B. R., Rasmussen, M. V., Meyer, R. F., & Bowen, M. D. (2008). Gamma irradiation can be used to inactivate Bacillus anthracis spores without compromising the sensitivity of diagnostic assays. *Applied and Environmental Microbiology*, *74*, 4427–4433.

Giroux, M., Ouattara, B., Yefsah, R., Smoragiewicz, W., Saucier, L., & Lacroix, M. (2001). Combined effect of ascorbic acid and gamma irradiation on microbial and sensorial characteristics of beef patties during refrigerated storage. *Journal of Agricultural and Food Chemistry*, *49*, 919–925.

González, I., López, M., Mazas, M., González, J., & Bernardo, A. N. A. (1997). Thermal resistance of bacillus cereus spores as affected by additives in the recovery medium. *Journal of Food Safety*, *17*, 1–12.

Hachisuka, Y., Kato, N., & Asano, N. (1956). Inhibitory effect of sodium bicarbonate and sodium carbonate on spore germination of Bacillus subtilis. *Journal of Bacteriology*, *71*, 250–251.

Harrell, C. R., Djonov, V., Fellabaum, C., & Volarevic, V. (2018). Risks of using sterilization by gamma radiation: the other side of the coin. *International Journal of Medical Sciences*, *15*, 274–279.

Hawkins, S. G. (2014). Antimicrobial activity of cinnamic acid, citric acid, cinnamaldehyde, and levulinic acid against foodborne pathogens.

Herigstad, B., Hamilton, M., & Heersink, J. (2001). How to optimize the drop plate method for enumerating bacteria. *Journal of Microbiological Methods*, *44*, 121–129.

Hossain, F., Follett, P., Vu, K. D., Salmieri, S., Senoussi, C., & Lacroix, M. (2014). Radiosensitization of Aspergillus niger and Penicillium chrysogenum using basil essential oil and ionizing radiation for food decontamination. *Food control*, *45*, 156–162.

In, Y.-W., Kim, J.-J., Kim, H.-J., & Oh, S.-W. (2013). Antimicrobial Activities of Acetic Acid, Citric Acid and Lactic Acid against *Shigella* Species. *Journal of food safety*, *33*, 79–85.

Jarvis, G. N., Fields, M. W., Adamovich, D. A., Arthurs, C. E., & Russell, J. B. (2001). The mechanism of carbonate killing of Escherichia coli. *Letters in Applied Microbiology*, *33*, 196–200.

Joint FAO/WHO Codex Alimentarius Commission. (1995). Codex Alimentarius: Norme générale pour les additifs alimentaires. World Health Organization: Food and Agriculture Organization of the United Nations, Pub. L. No. CODEX STAN 192 - 1995 (2018).
Lacroix, M., Caillet, S., & Shareck, F. (2009). Bacterial radiosensitization by using radiation processing in combination with essential oil: Mechanism of action. *Radiation Physics and Chemistry*, *78*, 567–570.
Lacroix, M., Samia, A., Dominic, D., Mélanie, T., Stéphane, S., Takala, P., & Khanh, V. D. (2013). Irradiation in combined treatments and food safety. *Journal of Radioanalytical and Nuclear Chemistry*, *296*, 1065–1069.
Lee, J. W., Yook, H. S., Kim, S. A., Lee, K. H., & Byun, M. W. (1999). Effects of antioxidants and gamma irradiation on the shelf life of beef patties. *Journal of Food Protection*, *62*, 619–624.
Lee, Y., Cesario, T., Owens, J., Shanbrom, E., & Thrupp, L. D. (2002). Antibacterial activity of citrate and acetate. *Nutrition*, *18*, 665–666.
Lenzi, L. J., Lucchesi, P. M. A., Medico, L., Burgán, J., & Krüger, A. (2016). Effect of the Food Additives Sodium Citrate and Disodium Phosphate on Shiga Toxin-Producing Escherichia coli and Production of stx-Phages and Shiga toxin. *Frontiers in microbiology*, *7*, 992.
Losio, M. N., Pavoni, E., Finazzi, G., Agostoni, C., Daminelli, P., Dalzini, E., Varisco, G., & Cinotti, S. (2018). Preparation of powdered infant formula: could product's safety be improved? *Journal of Pediatric Gastroenterology and Nutrition*, *67*, 543–546.
Mahmoud, G. A., Mohamed, G. A., Botros, H. W., & Sabri, M. A. (2011). Combined effect of gamma radiation, hot water and sodium bicarbonate on quality and chemical constituents of stored mandarin fruits. *Archives Of Phytopathology And Plant Protection*, *44*, 1115–1127.
Mazas, M., López, M., Martínez, S., Bernardo, A., & Martin, R. (1999). Heat resistance of Bacillus cereus spores: effects of milk constituents and stabilizing additives. *Journal of Food Protection*, *62*, 410–413.
Monk, J. D., Beuchat, L. R., & Doyle, M. P. (1995). Irradiation Inactivation of Food-Borne Microorganisms. *Journal of Food Protection*, *58*, 197–208.
Negrón-Mendoza, A., & Ramos-Bernal, S. (2015). Gamma irradiation of isocitric and citric acid in aqueous solution: Relevance in prebiotic chemistry (Vol. 1671, p. 020012). Presented at the RADIATION PHYSICS: XI International Symposium on Radiation Physics, AIP Publishing LLC.
Olaimat, A. N., Al-Holy, M. A., Abu-Ghoush, M. H., Osaili, T. M., Al-Nabulsi, A. A., & Rasco, B. A. (2017). Inhibition of Shigella sonnei and Shigella flexneri in Hummus Using Citric Acid and Garlic Extract. *Journal of Food Science*, *82*, 1908–1915.
Ouattara, B., Giroux, M., Smoragiewicz, W., Saucier, L., & Lacroix, M. (2002). Combined effect of gamma irradiation, ascorbic acid, and edible coating on the improvement of microbial and biochemical characteristics of ground beef. *Journal of Food Protection*, *65*, 981–987.
Oussalah, M., Caillet, S., Salmieri, S., Saucier, L., & Lacroix, M. (2004). Antimicrobial and antioxidant effects of milk protein-based film containing essential oils for the preservation of whole beef muscle. *Journal of Agricultural and Food Chemistry*, 52. 5598–5605.
Palop, A, Marco, A., Raso, J., Sala, F. J., & Condón, S. (1997). Survival of heated Bacillus coagulans spores in a medium acidified with lactic or citric acid. *International Journal of Food Microbiology*, *38*, 25–30.
Palop, A., Raso, J., Condón, S., & Sala, F. J. (1996). Heat Resistance of Bacillus subtilis and Bacillus coagulans : Effect of Sporulation Temperature in Foods With Various Acidulants. *Journal of Food Protection*, *59*, 487–492.
Park, Y. B., Guo, J. Y., Rahman, S. M. E., Ahn, J., & Oh, D.-H. (2009). Synergistic effect of electrolyzed water and citric Acid against bacillus cereus cells and spores on cereal grains. *Journal of Food Science*, *74*, M185–9.

Rawal, B. D. (1978). Bactericidal action of ascorbic acid on Pseudomonas aeruginosa: alteration of cell surface as a possible mechanism. *Chemotherapy*, *24*, 166–171.
Robichaud, V., Bagheri, L., Aguilar-Uscanga, B. R., Millette, M., & Lacroix, M. (2020). Effect of ɣ-irradiation on the microbial inactivation, nutritional value, and antioxidant activities of infant formula. *LWT*, *125*, 109211.
Sallam, K. I. (2007). Antimicrobial and antioxidant effects of sodium acetate, sodium lactate, and sodium citrate in refrigerated sliced salmon. *Food control*, *18*, 566–575.
Scott, B. R., Yang, X., Georgnaras, I., Delmore, R. J., Woerner, D. R., Adler, J. M., & Belk, K. E. (2015). Antimicrobial Efficacy of a Lactic Acid and Citric Acid Blend against Shiga Toxin-Producing Escherichia coli, Salmonella, and Nonpathogenic Escherichia coli Biotype I on Inoculated Prerigor Beef Carcass Surface Tissue. *Journal of Food Protection*, *78*, 2136–2142.
Shehata, T. E., & Collins, E. B. (1972). Sporulation and heat resistance of psychrophilic strains of bacillus. *Journal of Dairy Science*, *55*, 1405–1409.
Tajkarimi, M., & Ibrahim, S. A. (2011). Antimicrobial activity of ascorbic acid alone or in combination with lactic acid on Escherichia coli O157:H7 in laboratory medium and carrot juice. *Food control*, *22*, 801–804.
Temur, C., & Tiryaki, O. (2012). Combination of irradiation and sodium carbonate to control postharvest Penicillium decay of apples. *The Journal of Turkish Phytopathology*.
Tetz, G., & Tetz, V. (2017). Introducing the sporobiota and sporobiome. *Gut Pathogens*, *9*, 38.
Thayer, D. W., & Boyd, G. (1995). Radiation Sensitivity of Listeria monocytogenes on Beef as Affected by Temperature. *Journal of Food Science*, *60*, 237–240.
Thayer, D. W., & Boyd, G. (2001). Effect of irradiation temperature on inactivation of Escherichia coli O157:H7 and Staphylococcus aureus. *Journal of Food Protection*, *64*, 1624–1626.
Tuner, H., & Korkmaz, M. (2010). Effects of gamma radiation on solid trisodium citrate dihydrate: radical kinetics, radiosensitivity and dosimetry. *Radiation and Environmental Biophysics*, *49*, 723–729.
Verghese, R., Mathew, S., & David, A. (2017). Antimicrobial activity of Vitamin C demonstrated on uropathogenic Escherichia coli and Klebsiella pneumoniae. *Journal of Current Research in Scientific Medicine*, *3*, 88.

CHAPITRE 4:
IMPACT OF GAMMA IRRADIATION OR HEAT PASTEURISATION TREATMENT ON NUTRITIONAL AND IMMUNOLOGICAL PROPERTIES OF HUMAN MILK

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Contribution des auteurs

Valérie Robichaud a réalisé les manipulations et la rédaction de l'article.

Blanca Aguilar-Uscanga : Co-directrice du projet, a fourni le lait maternel et une partie des analyses nutritionnelles.

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Monique Lacroix : Directrice de recherche, responsable scientifique et coordinatrice du projet de recherche, a participé à la planification des expériences et à la correction de l'article.

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Résumé

La préservation de la qualité nutritionnelle (protéines, lactose, gras, vitamine C), des immunoglobulines, des propriétés de la lactoferrine ainsi que la qualité microbiologique du lait maternel a été comparée avant et après séchage par atomisation. Les résultats ont montré que l'atomisation du lait maternel ne modifiait pas significativement la valeur nutritionnelle en protéines, lactose et lipides ($p > 0,05$). La rétention des immunoglobulines varie entre 70% et 82% pour les IgA, IgG et IgM. Par la suite, le lait atomisé a été décontaminé par deux méthodes soit l' irradiation γ du lait atomisé en poudre qui a été comparé à la décontamination du lait congelé soit par irradiation ou soit par la pasteurisation Holder du lait liquide.

Les résultats du traitement de 5 kGy par irradiation a entraîné une élimination de 99,7% de la flore aérobique totale dans le lait atomisé en poudre et dans le lait congelé, alors que le lait liquide traité par pasteurisation Holder montrait environ 95,1% d'élimination.

Les micronutriments, les macronutriments ainsi que certains composés bioactifs clés (lysozyme, amylase) présents dans le lait maternel atomisé n'ont pas été affecté par l'irradiation gamma et ce, même à une dose de 10 kGy. Le traitement par irradiation à 10 kGy n'a pas modifié le potentiel antioxydant, cependant, une augmentation de plus de 40 µg/g de malondialdéhyde a été observée. La pasteurisation Holder n'a montré aucune modification des propriétés nutritionnelles et antioxydantes.

Ces résultats suggèrent que l'irradiation gamma du lait atomisé est une excellente alternative à la pasteurisation Holder pour entreposer le lait maternel et assurer la sécurité du produit avec une altération nutritionnelle et immunologique minimale du produit.

1. Abstract

The preservation of the nutritional quality (proteins, lactose, fat, vitamin C), immunoglobulins, lactoferrin as and the microbiological quality of human milk was compared before and after spray drying. The results showed that the atomization of human milk did not significantly change the nutritional value in term of protein, lactose and fat content ($p > 0.05$). The retention of immunoglobulins showed a retention from 70% - 82% for IgA, IgG and IgM. Thereafter, the atomized milk was decontaminated by two methods: gamma irradiation of frozen and powdered milk and Holder pasteurization of liquid milk.

The results showed that a dose of 5 kGy irradiation treatment resulted in 99.7% destruction of the total aerobic flora in the powdered atomized milk and in the frozen milk, while the liquid milk processed by Holder pasteurization showed approximately 95.1%. elimination.

Micronutrients, macronutrients as well as some key bioactive compounds (lysozyme, amylase) present in atomized human milk were not affected by γ-irradiation even at a dose of 10 kGy. Treatment with irradiation at 10 kGy did not alter the antioxidant potential, however, an increase of more than 40 µg / g of malondialdehyde was observed. Holder pasteurization showed no modification of nutritional, enzymatic or antioxidant properties.

These results suggest that γ-irradiation of atomized milk is an excellent alternative to Holder pasteurization for storing breast milk and ensuring product safety with minimal nutritional and immunological damage to the product.

2. Introduction

It is accepted that human milk (HM) is a unique food that has exceptional properties for the development and recovery of preterm babies. Unfortunately, there are many reasons why these newborns cannot benefit from their mother's milk (illnesses, incapacity to express, etc.) (Kim and Unger, 2010). Infant formula (IF) has been widely used as a replacement from HM. Despite IF being rather similar to HM regarding macronutrients, it is far from offering the immunological and biological complexity of the specie-specific HM. Medically, it is shown that HM-fed preterm babies have less infections, a lower rate of necrotizing enterocolitis and a healthier gut microbiota less prone to pathogenic colonization (Kim and Unger, 2010). To provide the best nutrition to newborns with a precarious health, Human Milk Banks (HMB) have been set up to provide hospitals with the best quality HM.

HMB are based on eager mothers to give their extra milk on a philanthropic basis. The donors are taught to collect their milk in a sanitary way through a pump, and the milk is immediately frozen. The donations are treated as a human body substance and processed as a food substance under the Human Milk Banking Association of North America guidelines. To prevent transmission of viruses or bacteria to the recipients, mothers are rigorously screened and microbial quality of the milk is monitored before and after pasteurization (Kim and Unger, 2010).

Unfortunately, commonly used methods for preservation and decontamination (freeze / thaw cycles, Holder pasteurization) can alter nutritional and immunological qualities. They are not suited for long term conservation and often fail to eliminate spore-forming bacteria (Vieira *et al.*, 2011). A study of 261 samples of breast milk donations in 17 different countries showed that 52% of samples contained between 0.3 and 10 spore-forming bacteria *Bacillus cereus* per gram (Becker *et al.,* 1994). HMB reject every donation that comes positive to *B. cereus*, positive to any pathogen or contains more than 10^4 CFU/mL (Holsinger, Rajkowski and Stabel, 1997; Kim and Unger, 2010; Rigourd *et al.*, 2018).

Some studies found that long-term storage of frozen HM can have an effect on the nutritional quality (Kim *et al.*, 2019; Schlotterer and Perrin, 2018). Aside for a significant decrease in total fat, an increased lipolysis and a diminution in bactericidal capacities, overall nutrients and bioactive components of HM seems to be retained over a 3-6 months period at -20°C (Kim *et al.*, 2019; Schlotterer and Perrin, 2018). Freezing of the donations is the common method for processing of HM in HMB. However, conservation of HM at this temperature can be a challenge in underdeveloped sub-equatorial countries due to storage and transportation issues. As an alternative, conversion of HM in powder by accessible technologies could be an interesting option to facilitate long-term storage of HM with almost no impact on its nutritional and immunological properties (Lozano, Castellote, Montes *et al.*, 2014). Spray-drying (SD) and freeze-drying are reliable methods to produce a stable powder from milk (Castro-Albarrán *et al.*, 2016). SD is used in the food industry and

pharmaceutics for heat-sensitive compounds and bioactive compounds (Celli, Ghanem and Brooks, 2015). SD is simple and fast and can work for large volumes of homogenic or heterogenic fluids with a very wide range of consistency. The production cost is often lower than for freeze-drying. It provides a better redispersibility in aqueous solution and a very low water-activity, resulting in a higher shelf-life (Arpagaus *et al.,* 2018). Briefly, the solution to be atomized passes trough a thin needle with a stream of hot gaz to obtain droplets. The solvent evaporates and cools the particles, keeping the temperature low enough to avoid molecule damages. It produces a powder dry enough (5% water) to obtain a very stable product. Depending on the food matrix and the type of particles that are wanted in the final product, a temperature from 110 °C to 190°C can be used. For example, low-temperature SD (< 140°C) creates wider particles whereas high-temperature SD are more porous. It can also affect solubility and the shape of the particle (Yu *et al.,*2011). Long-term preservation of HM in a powdered form by SD was previously assed by Arreola *et al.* (2018). They showed that the milk can be stored up to six weeks at room temperature, away from humidity, oxygen and light with almost no alteration of the product. Even thought it is an effective method to preserve enzymes and nutriments, a decrease in the content of immunoglobulins such as IgA of approximately 25% was observed (Castro-Albarrán *et al.*, 2016).

To ensure milk safety, a decontamination process is essential in a context of HMB. Holder pasteurisation is a low-temperature decontamination method for liquids (62,5°C, 30 min) in order to inactivate 100% of pathogenic bacteria and 99,99% of total microorganisms. This treatment can also inactivate certain viruses but is mostly ineffective on sporulated bacteria (Cacho *et al.*, 2017; Arreola *et al.*, 2018). Most macronutrients and micronutrients are preserved but up to 13% of protein are denaturated, resulting in total loss of certain enzymes (lipase, alkaline phosphatase) and significant reduction of biologically active compounds (vitamins, hormones, growth factors, enzymes, lactoferrin, immunoglobulins) (Hema-Québec, 2020; Peila *et al.*, 2016; Arreola *et al.*, 2018).

γ-irradiation presents many advantages over the conventional Holder pasteurization method. First, it is a cold-pasteurisation or sterilisation process, which greatly reduces the risks of denaturing proteins and damaging immunological analyte compared to a heat-involved method. Secondly, this process can be performed on solid milk (frozen or powdered), which eliminate the freeze/thawing/dissolving cycles (Vieira *et al.*, 2011). Finally, a high dose of irradiation could eliminate vegetative and sporulate bacteria, while Holder pasteurization doesn't affect bacterial spores (Dauphin *et al.*, 2008; Lima *et al.*, 2017).

γ-irradiation has been approved by the United Nations since 1981 as a conservation treatment. This technology is used on all types of products: fresh, dry or frozen. A dose from 2-10 kGy is commonly used to decontaminate most of fresh foods without compromising nutritional and sensory quality, however, it is known that under appropriate conditions, nutritional value of the food is generally unaffected by γ-irradiation,

even at 45 kGy (Roberts, 2016). γ-irradiation penetrates matter without macroscopically modifying it and causes damages to DNA. Depending of the dose and the irradiation conditions, irradiation can create a partial degradation of proteins and polysaccharides and oxidation of unsaturated lipids. Irradiation can also create damages to the bacterial external structure and their genetic material, which causes their inactivation (Szczawinska, 2017). In addition, γ-irradiation interferes with available water, which creates free radicals that are toxic to bacteria. The resistance of microorganisms to irradiation depends on the food matrix and many parameters such as the concentration of bacteria, the temperature, the humidity, the atmosphere conditions and others (Szczawinska, 2017). Vitamins are known to be more sensitive depending on the nature of the food and the nature of the vitamins (Woodside, 2015). Finally, even if the irradiation process generates ROS (reactive oxygen species), the antioxidant value of the product does not necessarily decrease. It seems to depend of the irradiation doses, the food matrix and the nutrient composition (lipids, proteins and antioxidant properties) (Kavitha *et al.*, 2015; Kim *et al.*, 2009).

The aim of this study is to first evaluate the effect of the spray-drying treatment on nutritional quality of human milk. Thereafter, γ-irradiation was compared to Holder pasteurization to assess the microbiological and nutritional quality after the respective decontamination treatment in powdered and liquid frozen spray-dried human milk.

3. Materials and methods

3.1 Materials

Tryptic soy broth (TSB), Tryptic soy agar (TSA), and peptone water were purchased from Alpha Biosciences Inc. (Baltimore, MD, USA). All chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Pierce BCA protein assay kit was obtained from Thermo scientific (Montreal, QC, Canada). *B. cereus* (ATCC 14579), *L. monocytogenes* (HPB 2812 serovar 1/2a), *S. aureus* (ATCC 2921), *E. coli* O157:H7 (EDL 993), and *S.* Typhimurium (SL1344, INRS-IAF) were maintained at −80°C in TSB containing glycerol (10% v/v).

3.2 Collection of human milk

Human milk was obtained from Hema-Quebec and from the Human Bank Milk of the Hospital Civil of Guadalajara, Mexico, where the study was approved by the Ethical Research Committee of the respective institution. All donations were pooled together and sampled with a sterile manual pump. The donors were in good health, not smoking, not taking drugs incompatible with breastfeeding and free from alcohol. Volunteer donors provided a written agreement about the donation of excess human milk.

3.3 Spray-drying of human milk

High-temperature spray-drying was done in a Niro-Atomizer pilot unit with conical base (Model 209/S, Soeborg, Denmark) fed with a Watson Marlow1 (Model 503U) peristaltic pump and a nozzle atomizer (three bar pressure). The air inlet temperature was set at 150 °C with a feed rate of 1 mL / min.

3.4 Lactoferrin purification and analysis

3.4.1 Lactoferrin purification from spray-dried human milk

The lactoferrin purification was carried out according to Conesa *et al.* (2008). Briefly, 5 g of SDHM (spray-dried human milk) was resuspended in phosphate buffer (0.02 M NaH_2PO_4, 0.4 M NaCl, 0.02% (v / v) Tween 20, pH 7.4). The milk was centrifuged at 2500 xg for 30 min at 4 °C in order to separate the fat. The supernatant was incubated overnight with agitation at 4 °C with 5 mL of the SP-Sepharose column for cation-exchange chromatography (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). The column was washed with 130 mL of phosphate buffer (0.02 M NaH_2PO_4, 0.4 M NaCl, 0.02% Tween 20, pH 7.4) to elute the unbound proteins. The OD at 280nm and 465nm was determined along the washings to monitor the elution of unbound proteins until OD was lower than 0.2. The column was then washed with elution buffer (0.02 M NaH2PO4, 1 M NaCl, pH 7.4) and 5mL fractions were collected and analysed. The reconstituted HM and the fractions containing lactoferrin obtained in the chromatographic isolation were analysed by SDS-PAGE using a 12% polyacrylamide gel.

3.4.2 Antiradical activity of purified lactoferrin

The antiradical properties of lactoferrin present in spray-dried milk was evaluated on the different lactoferrin (LF) fractions using α, α-diphenyl-β-picrylhydrazyl (DPPH) and FRAP (Ferricreducing / antioxidant power) methods. First, a volume of 1 mL of 0.25 mM DPPH solubilized in methanol was mixed with 1 mL of the lactoferrin fraction and incubated at room temperature. After 20 min, the mixture was centrifuged 8000 x g for 3 min and the OD of the supernatant was read at 517 nm (Letchoumy and Mohan, 2008).

For the FRAP method, 200 µl of freshly prepared reagent (1 mL of 10 mM TPTZ, 2.5 mL of 20 mM $FeCl_3.6H_2O$, 2.4 mL H_2O and 10 mL of 300 mM sodium acetate, pH 3.6) were mixed with 10 µl of sample in a microplate. The absorbance values of the samples and controls were measured at the wavelength of 593 nm after 10 minutes of incubation at room temperature (Joubran, Mackie and Lesmes, 2013).

3.4.3 Well diffusion assay

In order to verify the effect of the treatment on the bioactivity of lactoferrin, the well diffusion assay was performed with purified lactoferrin from spray-dried milk. Sterile Tryptic Soy Agar (TSA) at 55 °C is

inoculated with one of the 5 different 24h cultures (*S. aureus* (ATCC 2921), *Escherichia coli* O157:H7, *Listeria monocytogenes* HPB 2812 serovar 1/2a, *Salmonella* Typhimurium (SL1344) or *Bacillus cereus* ATCC 14579) to obtain a concentration of 10^6 CFU/mL of pathogenic bacteria. A volume of 25 mL of the inoculated liquid agar (Tryptic Soy Agar) is poured into petri dishes and let to solidify at room temperature. A 1 cm diameter well was punched aseptically and a volume of 100µl of antibiotic, buffer or lactoferrin solution is pipetted into the well. The petri dish is then incubated at 37 °C for 24h. The zone of inhibition is then measured.

The antibiotic for *S. aureus* and *L. monocytogenes* was 10 µg of gentamicin, and 25 µg of ampicillin for *E. coli*, *S.* Typhimurium and *B. cereus.* The lactoferrin concentration tested were 0, 0.25, 0.5, 1 and 1.5 mg.

3.5 Decontamination methods and microbiological analysis of spray-dried human milk

SDHM powder was let to sit in an uncontrolled environment at room temperature with normal humidity in a sterile container for several weeks to develop microbial flora.

The Holder pasteurization treatment was done according to Peila *et al.* (2016). The spray – dried HM samples were solubilised in sterile Milli-Q water 10% (w/v) and were heated in a hot water bath at 62,5 °C for 30 min and shortly kept on ice until analysis.

The γ-irradiation treatment (9.172 kGy/h) was done in a UC-15 A irradiator (Nordion Inc., Kanata, ON, Canada) equipped with a $Cobalt^{60}$ source in aliquots of 5 mL for liquids or 2 g for powdered human milk. Dry ice was added in the irradiator to keep the samples frozen. The dosages applied for the study were 0 (control), 3, 5 and 10 kGy.

For the microbiological analysis, samples were diluted in sterile peptone water (0.1%). An amount of 100 µl of appropriate dilution was plated on Tryptic Soy Agar and incubated at 37°C for 24 h. For selective counting, selective media were used: tryptic soy agar for total aerobic flora, MacConkey agar for coliforms, de Man, Rogosa and Sharpe agar for lactic acid bacteria and potato dextrose agar for yeast and molds. All incubations were performed at 37°C for 24 h.

3.6 Determination of nutritional quality of human milk after spray-drying

Analyzes of nutrients (proteins, lactose, fat) were performed before and after spray-drying. Lactose was quantified using the DNS method (Miller, 1959), proteins were measured by Lowry's method (Lowry *et al.*, 1951), and fat was extracted using Folch's method (Floch, 1957). Immunoglobulin quantification was carried out by the nephelometry technique, which is based on the quantification of the scattered light by antigen-antibody complexes formed during immunoprecipitation in liquid phase (Montagne, Laroche and Bessou,

1992). Finally, water activity on SDHM was analysed with an AquaLab Pre Water Activity Meter (Decagon Devices).

3.7 Determination of nutritional and immunological quality of human milk after radiation and pasteurization treatments

3.7.1 Determination of lactose and vitamin C

Lactose content was measured using HPLC according to Richmond *et al.* (1987). Briefly the samples were prepared by adding an equal part of trichloroacetic acid (TCA) 12% (w/v) into liquid HM. The samples were then centrifuged (Sorvall® Instrument, Du Pond, USA) at 10 000 xg for 20 min at 4°C to remove casein and fat. The supernatant was then filtered through 0.2 µm PVDF syringe filter (Sarstedt AG & Co. KG) and stored at −20°C prior to use. A ZORBAX carbohydrate column (4.6 × 250 mm, 5 µm particle size, pore size 70 Å) and an Agilent 1260 series HPLC system (Agilent Technologies, Palo Alto, California, USA) was used under isocratic conditions. Mobile phase was Milli-Q water at a flow rate of 1.5 mL/min and volume of injection was 20 µL. The temperature of the reflective index detector and column was set at 50 °C, and 80°C, respectively. Data acquisition and processing was performed by Chemstation v. 2.0 (Agilent). Lactose solution (0, 1, 10, 20, 30, 40, 50 mg/mL) was used to prepare a standard curve.

Vitamine C samples were prepared by adding an equal part of 0.56% (v/v) phosphoric acid into the liquid HM (Romeunadal *et al.*, 2006). The samples were then centrifuged and the supernatant was filtered (0.2µm PVDF syringe filter) and immediately injected (20µl) into the HPLC. Analysis was done using an Agilent 1260 system, equipped with a DAD detector. A Poroshell 120 EC-C18 (4.6 × 50 mm, 2.7 µm particle size, pore size120 Å) (Agilent, California, USA) was used for quantification under isocratic conditions at room temperature. The mobile phase consisted of 95% solution A (40mM monobasic phosphate buffer, pH = 2.5) and 5% solution B (60% methanol and 40% acetonitrile). The flow rate was 1 mL/min and detection was performed at 243nm. Ascorbic acid was used to obtain the standard curve (0, 5, 10, 15, 20, 25 and 30 µM).

3.7.2 Protein content

Protein concentration of the samples was determined using Bradford protein assay kit (Thermo scientific, Montreal, Canada) according to the manufacturer's specifications (Cheng *et al.*, 2016). Bovine serum albumin (BSA, 20 − 2000 µg/mL) was used as a control and absorbance was read at 562 nm using a UV-VIS spectrophotometer (S-3100 Scinco, Triad Scientific, North Carolina, USA).

3.7.3 Antiradical activity of HM

Briefly, 250 µL of liquid HM was added to the 1 mL of 60 µM DPPH methanolic solution (Kedare and Singh, 2011). The mixture was left to incubate with agitation for 60 min in the dark at room temperature. The absorbance of the samples was determined at 517 nm by a UV-VIS spectrophotometer (S-3100 Scinco). Ascorbic acid (250 µL) and dimethyl sulfoxide (DMSO) (10%) were used respectively for positive and negative control, respectively. Radical scavenging activity was calculated using the following equation: radical scavenging activity (%) = $(DO_{control} - DO_{sample})/DO_{control} \times 100$.

3.7.4 Thiobarbituric acid-reactive substances (TBARS)

TBARS content in HM was measured according to the method described by Botsoglou and Fletouris (1994) with some modifications. Briefly, 1mL of the reconstituted sample was mixed with 8mL of 5% TCA and 5mL of butylated hydroxyl anisole (0.8% in hexane). The mixture was mixed using a high sheer homogenizer (Ultra-Turrax T25, Montreal, Canada) at 18 000 rpm for 30s followed by centrifugation at 3 000 xg for 3 min. For each sample, the top hexane layer was removed and 5% TCA was added to obtain a total volume of 10mL in each tube. Subsequently, 2.5 mL of each tube was mixed with 1.5 mL of 0.8% thiobarbituric acid and incubated at 70°C for 30 min. The solution was cooled and analysed by spectrophotometry against a blank reaction mixture. The 3rd derivative spectrum was calculated and plotted against a MDA calibration curve (0−2 µg / mL).

3.7.5 Amylase and Lysozyme activity

Amylase activity in human milk was assessed according to the manufacturer's protocol of the Amylase Activity Assay Kit (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Lyzozyme activity was determined according to Shugar (1952) with some modifications. A suspension of *Micrococcus lysodeikticus* in 50 mM Potassium Phosphate Buffer, pH 6.24 was prepared to obtain a OD of 0.7 at 450nm in a 1 cm^2 cuvette. A volume of 2.5mL of this suspension was mixed with 0.1mL of the enzyme solution and optical density (DO) was recorded every 30 seconds for 3 minutes. The maximum linear rate of 5 points was taken to make the unit calculation with the following formula:

Units/mL enzyme = ((ΔA_{450}/min sample - ΔA_{450}/min Blank) x (dilution factor)) / (0.001 x V), where 0.001 is the change in absorbance (ΔA_{450}) as per the Unit Definition and V is the volume (mL) of sample. From these results, the concentration is calculated using the following formula: Units/mg solid = (units/mL enzyme) / (mg solid/mL enzyme).

3.8 Sec-HPLC analysis of lactoferrin fractions and human milk's peptide

Milk samples were prepared by adding an equal part of TCA 12% (w/v) to the liquid HM. The mixture was centrifuged 10,000g at 4°C for 10 min to remove casein and fat. The supernatant was filtered using 0.2 µm PVDF syringe filter (González-Olivares *et al.*, 2014). The samples were stored at −20°C until being analyzed. Purified lactoferrin samples were filtered 0.2µm and directly injected in the column. A Biosep-SEC 2000 column (300 × 7.8 mm, 5 mm particle size, pore size 145 Å) from Phenomenex (Torrance, ON, Canada) connected to an Agilent 1260 series HPLC system was used. Mobile phase was a phosphate buffer solution (150mM, pH 6.8) and detection was done by a diode array detector at 280 nm. The analysis was performed at room temperature at a flow rate of 1mL/min for 16min with a volume of injection of 20µl. A mathematical modeling of the size exclusion chromatography was used to estimate the molecular weight of the peptides (Zelic and Nesek, 2006). Bovine thyroglobulin (670 KDa), gamma globulin (IgA, 300 KDa), IgG (150 KDa), ovalbumin (44 KDa), myoglobin (17 KDa) and uridine (244 Da) were used as standards (Phenomenex).

4. Results and discussion

4.1 Nutritional and immunological values of spray-dried human milk

Results in Table 1 showed the content of protein, lactose and fat in SDHM and show no significant difference ($p > 0.05$) before and after the spray-drying of milk and both have similar values observed by Michaelsen *et al.* (1990) and Wojcik *et al.* (2009). The ascorbic acid levels were below the detection limit of 15ppm in all samples. According to Francis, Rogers, Brewer, Dickton and Pardini (2008), a mean ascorbic acid level of 42 ppm in freshly expressed milk and 20 ppm in pasteurised donor human milk is normally observed. Previous studies showed a 15 to 36% decrease of ascorbic acid after Holder Pasteurization (HoP) treatment (Moltó-Puigmartí *et al.,* 2011; Peila *et al.*, 2016; Picaud and Buffin, 2017; Wesolowska *et al.*, 2019). However, this vitamin is also known to be very sensitive to freezing, refrigeration, handling and to several other treatments (Schlotterer and Perrin, 2018). Since ascorbic acid is an essential vitamin that is not stored or produces by the human body, a deficiency of this nutriment could be problematic and induce scurvy (Francis *et al.*, 2008). The SDHM should therefore undergo more studies on sensitive micronutrients to assess if supplementation would be needed to ensure an optimal product.

Water activity is a fundamental concept to prevent microbial proliferation and stability of dehydrated food. Normally, a water activity (aw) < 0.4 limits microbial growth (Feeney *et al.*, 2014). In this study, the aw of the powder observed was 0.21, which is comparable to commercial IF where the aw is around 0.2 (Feeney *et al.*, 2014). The spray drying method in this study makes it possible to obtain a powder with a suitable water activity (aw) without the need for an additional drying step.

The results presented in Table 2 shows that the spray-drying method retained 82% of IgA, 78% of IgG and 70% of IgM. This is partly in agreement with the study of Castro-Albarrán *et al.* (2016) who obtained respectively 62%, 88% and 67% retention of IgA IgG and IgM after SD treatment. Overall, the results suggest that SD is an effective and non-invasive technique for the preservation of immunoglobulins.

4.2 Lactoferrin isolation and evaluation of its antioxidant and antimicrobial properties

Lactoferrin from HM was isolated by cation-exchange chromatography to assess the effect of SD on the antimicrobial and antioxidant properties. The fractions were analysed for identification and purity by electrophoresis by SDS-PAGE (Fig. 1) and SEC-HPLC (Fig. 2). The fractions 1 to 26 correspond to the elution on the unbound proteins to the cation-exchange column, while fraction 26 to 30 contain the purified lactoferrin (fig. 1a). According to the SDS-Page (3 to 6) and HPLC analysis, lactoferrin (LF) is identified with a main band of 80 kDa and the purity is maximal at 95% for the fraction 29. The HPLC analysis of the purified lactoferrin shows a single peak at 11.2, whereas the whole milk presents no peak at this elution time (Fig. 2). Lactoferrin in HM is normally found under dimer and other oligomer and under our conditions, lactoferrin is present under monomeric form (Persson, Lund, Forsman, Chatterton and Akesson, 2010).

Purified lactoferrin from SDHM from the fractions 27, 28 and 29 were found to be respectively 6.22, 40.95 and 0.81mg/mL. The estimated LF concentration in the milk powder is 48mg LF/g in SDHM, which corresponds to a concentration of 4,85mg/mL when solubilised in water. It is a normal concentration in human transitional milk (Czosnykowska-Łukacka *et al.*, 2019). Lactoferrin seems to be effectively preserved by spray-drying and has been tested for its antioxidant and antimicrobial capacities in order to assess the preservation of its properties. The antioxidant properties of the whole milk were found to be 33% for DPPH assay and 1,25 mM Trolox-equivalent in FRAP assay. Both results are lower than found by Cloetens, Panee and Åkesson (2013) and Zarban *et al.* (2009), which could be related with the destruction of the ascorbic acid during spray-drying process (Table 1). The antiradical property of the purified LF, showed that the most concentrated fraction (40.95mg/mL) presents 26% radical scavenging capacity in the DPPH assay, and no fraction demonstrated antioxidant power for the FRAP experiment. In the present study, the purified LF showed significant lower antioxidant activities as compared to literature (Cloetens, Panee and Åkesson, 2013; Zarban *et al.*, 2009). This could be due to the purification process. However, it is to be noted that a good part of the protective capacities of LF have been found in peptides derived from its proteolysis during digestion (Giansanti et al., 2016).

The antimicrobial properties of the purified human lactoferrin was assessed by well diffusion assay and the results are presented in Table 4. Results showed that none of the concentrations tested showed antibacterial effect on the spore-forming bacteria *B. cereus*. The four other pathogenic bacteria showed some inhibition. The most sensitive bacteria were *E. coli* and *L. monocytogenes*, with a very clear complete inhibition up to 3 mm around the well in presence of 1.5mg of LF. *S. aureus* and *S.* Typhimurium showed a slightly higher resistance. Bacteriostatic and the bacteriocidic properties of LF have been studies against various species of pathogenic bacteria, viruses, yeasts and parasites. Among these bacteria, *L. monocytogenes, S. aureus,* certain *Bacillus* strains and some serovar of S. Typhimurium have also been listed to be sensitive to LF. However, *B. cereus* was not recognized to be sensitive to LF (Pierce, Legrand and Mazurier, 2009). In any case, it is observed that purified LF form SDHM has kept its antimicrobial properties.

4.3 Microbiological quality of powdered and frozen human milk after spray drying

The microbial viability was determined in HM samples before and after SD process to determine the presence of aerobic mesophilic bacteria, total coliforms, molds and yeasts. The results in Table 5 shows that the HM before SD treatment contained between 4 and 6 log CFU/mL of bacteria such as cocci and bacilli. However, after the spray drying process, no viable microorganisms were found (data not shown). HM normally contains a natural microbiota (eg. *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Lactobacillus*) that varies with each donor depending on their diet, their age and the sanitary conditions that breast milk was extracted (Rodríguez, Jiménez and Merino, 2008). Even if SD is not meant to inactivate bacteria, the heat treatment involved in the process can result in microbial inactivation (Alvarenga *et al.*, 2018). According to Thompson, Harmon and Stine (1977), skim milk containing 10^6 UFC/mL of *Bacillus subtilis, Micrococcus flavus*, or *Escherichia coli* had a surviving rate after spray-drying ranging between 0.02 and 28%, with a better survival rate for lower drying temperature (Thompson, Harmon and Stine, 1978).

4.4 Microbiological quality of powdered and frozen human milk after Spray drying

SDHM samples were treated with a cold and a heated pasteurization methods for microbiological analysis purposes. The milk powder contained 2.65log CFU/mL of yeast and molds, 2.5 log CFU/mL lactic bacteria, 2.5 log CFU/mL of total aerobic flora and 0.67 log CFU/mL for total coliforms before the decontamination treatments. Frozen or powdered samples were treated by irradiation at doses of 0, 3, 5 or 10 kGy or treated by Holder pasteurization (HoP) (62.5 °C, 30min). HoP and 3kGy of γ-irradiation were sufficient to eliminate all coliforms in both milk forms and greatly reduced total aerobic flora. HoP group had significantly higher level of yeast/molds, lactic bacteria and total aerobic flora ($p \leq 0.05$) as compared to all irradiated samples. HoP inactivated 95.1% of the total aerobic flora, whereas 3kGy eliminated over 99% of this microorganism

group. At 5kGy, all lactic acid bacteria, total aerobic flora (>99.7% reduction) and coliforms were completely inactivated and only 0.33 CFU/mL of yeast and mold remained. At 10 kGy, all microorganisms were inactivated. Overall, yeast and molds are the hardest to deactivate in the contaminated HM. Those microorganisms are known to be more resistant to irradiation due to their complexity and their physical structure. Bacterial and fungal spores are also very resistant to irradiation and heat treatments because their stable configuration and their small DNA (Calado, Venâncio and Abrunhosa, 2014; Monk, Beuchat and Doyle, 1995). It is accepted that Holder pasteurization should eliminate over 99% of saprophyte bacteria in HM. The HoP treatment applied in this study was less efficient to eliminate microorganisms as compared HoP on untreated human milk (Capriati *et al.*, 2019; Landers and Updegrove, 2010; Rodríguez *et al.*, 2018). Similar results were obtained by Pacheco *et al.* (2019). This study has permitted to conclude that a higher temperature of pasteurization (85º C, 5min) was more suited in combination with SD. A possible explanation is that the metabolism of lactic acid bacteria could be altered after SD, making them more resistant (Huang *et al.*, 2017). To our best knowledge, no study was done until now to evaluate the bacterial resistance to heat-decontamination subsequent to atomization. However, our results showed that γ-irradiation showed a more potent decontamination method when used in combination with SD than HoP. Moreover, γ-irradiation treatment has shown to inactivate bacillus spores, which is a concern in HM banking, while HoP is found to be ineffective (Dauphin *et al.*, 2008; De Lara *et al.*, 2002; Lewin *et al.*, 2019). In a previous study, it was shown that ɣ-irradiation effectively suppressed the vegetative bacteria and spores of *B. cereus*, with respective D_{10} values in powdered and frozen infant formula of 0.77 and 0.44 kGy for vegetative bacteria and 6.13 and 2.37kGy for spores (Robichaud *et al.*, 2020). Therefore, an irradiation dose of 5kGy would meet the criteria to effectively decontaminate frozen and powdered HM with the very important advantage of inactivating the spore-forming bacteria *Bacillus cereus.*

4.5 Effect of γ-radiation and HoP treatment on the nutritional and antioxidant properties of HM

The effect of HoP and γ-irradiation on protein, lactose, antioxidant activity, autoxidation of lipids and lysozyme and amylase activities are presented in Table 6. Results showed that proteins and lactose were not significantly altered by any of the treatment tested ($p > 0.05$). These results are consistent with previous studies in various food matrices. Irradiation of peanut butter and soy beans showed a great retention of protein (El-Rawas *et al.*, 2012; Hafez *et al.*, 1985). Irradiation of yogurt and infant formula (liquid frozen and powdered) showed no impact on lactose and protein content (Ham *et al.,* 2008; Robichaud *et al.*, 2020). Those studies concluded that γ-irradiation is effective to extend shelf-life without compromising the nutritional or sensory quality.

Antioxidant properties were not significantly affected by any treatment tested ($p > 0.05$). This is in contradiction with our previous study in infant formula where the liquid frozen IF was found to lose both ascorbic acid and antioxidant capacities after treatment with 5kGy and 10kGy of γ-irradiation (Robichaud *et al.*, 2020). This could be explained by the differences in the food matrix. In the present study, spray-dried human milk contained undetectable amount of ascorbic acid. Then, an indicator of lipid oxidation in ɣ-irradiated samples was measured by a TBARS assay of malondialdehyde (MDA) formation after irradiation and HoP. As expected, there were no change in MDA quantification for the HoP treated HM (Peila *et al.*, 2016). In the frozen and powdered samples, no significant change was found after irradiation treatment > 5 kGy, but a significant increase of TBARS from 0.17 to 0.66 µg/g was measured at 10 kGy as compared to the control value. In infant formula, a similar effect was observed after γ-irradiation with a dose-dependent increase from 0.41 to 1.09 µg/g of MDA content from 0 to 10kGy (Robichaud *et al.*, 2020). It is known that ionizing radiation may initiate auto-oxidation of fat-containing food by producing oxygen radicals. This can result in formation of lipid oxides depending of the food matrix (Stefanova, Vasilev and Spassov, 2010). Finally, the enzymatic activity of lysozyme and amylase in HM was assessed after irradiation and HoP. Results showed no impact of irradiation treatment on these enzymes. However, lysozyme activity in HoP-treated HM was decreased significantly ($p \leq 0.05$) as found formerly in literature (Kim and Unger, 2010; Peila *et al.*, 2016). Peila *et al.* (2016) also states that amylase could be partially destroyed by HoP treatment. On the contrary, irradiation has shown to preserve the integrity of many enzymes such as lysozyme and amylase in human saliva at 3.5kGy (Ruhl *et al.*, 2011). At small doses, irradiation effectively kill bacteria, but leaves most protein unaffected due to their size (Ruhl *et al.*, 2011).

The protein profile of frozen and powdered HM after hot and cold pasteurisations is presented in Figure 3. Results showed that in the powdered HM, no significant difference in irradiated samples as compared to the control not irradiated for all fractions was observed ($p > 0.05$). In the liquid frozen samples, HM treated with 5kGy and 10kGy showed similar peptide profiles and are significantly different from the control and HoP treated samples ($p \leq 0.05$). The protein with a molecular weight of >300kDa and total peptide groups are respectively 15% and 12% lower for the irradiated frozen samples, and 26 and 21% lower for HoP as compared to the control respectively. It is accepted that HoP affects several milk components, such as of IgA, IgM, IgG, lactoferrin, although it is difficult to quantify a degradation degree (Vieira *et al.*, 2011). Irradiation in an aqueous food matrix at doses > 5kGy is known to induce degradation in relation with the greater production of oxide radicals. This could be prevented by the addition of antioxidant compounds such as ascorbic acid (Saloua *et al.*, 2011). Our results show that HoP is the treatment that presents the most modifications on the peptide profile, followed by frozen HM, whereas powdered irradiated samples present no changes. The smaller fractions present no modifications in all treatments compared to the control. Since

most of the important proteins for bioactive properties are below 80 kDa, it is possible to conclude that changes in the peptide profile will have little impact on the biological function of breast milk (Lönnerdal, 2013).

5. Conclusion

The nutritional and immunological value of HM after SD showed that the resulting product has equivalent amount of protein, fat and lactose compare to the original HM, with an excellent stability due to low a_w and has no bacterial contamination. The milk has a good retention of immunoglobulins but a total loss in ascorbic acid was observed. The purified lactoferrin retained its antimicrobial against pathogenic bacteria and its radical-scavenging properties, demonstrating that the lactoferrin from SDHM is still active. γ-irradiation of SDHM in powdered and liquid frozen form showed some advantages compared to the current HoP heat standard decontamination method. Decontamination of HM was obtained at a dose of 5 kGy. Proteins, lactose, some key enzymes and antioxidant activities were found to be intact even after a 10 kGy radiation dose, however, lipid peroxidation was increased by a factor of 3.7 as compared to the control. Overall, the peptide content seems to be more stable when treated by a cold-pasteurisation method *i.e.* γ-irradiation and no significant difference ($P > 0.05$) was found when the HM was decontaminated in its liquid frozen form or in powder.

6. Acknowledgments

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7. Conflict of Interests

There is no conflict of interest.

List of tables

List of figures

Table 1. Protein, lactose, fat and ascorbic acid content and water activity level of liquid and spray-dried human milk compared to literature.

	Liquid human milk	**Spray-dried human milk**	**Literature of donor milk samples[1]** 415 samples g/100mL	**Literature of donor milk samples[2]** 2553 samples
Protein	1.65 ± 0.59	1.53± 0.45	1.2 ± 0.5	1.0 ± 0.4
Lactose	7.36 ± 0.94	6.25 ± 0.69	7.8 ± 1.8	7.0 ± 0.6
Fat	2.46 ± 0.46	2.13 ± 0.37	3.2 ± 2.0	5.4 ± 3.6
Vitamin C	NA	>0.0015	NA	NA
Aw	NA	0.21± 0.15	NA	NA

Different letters in rows indicate significant differences at $P \leq 0.05$ between liquid human milk and spray-dried human milk.

References : 1.Wojcik *et al.* (2009) 2.Michaelsen *et al.* (1990)

Table 2. Immunoglobulin content (mg/mL) and % retention in liquid human milk before and after spray-drying.

Immunoglobulins	**Liquid human milk (mg/mL)**	**Spray-dried human milk (mg/mL)**	**% retention**
IgA	1.29 ± 0.58	1.06 ± 0.36	82
IgG	0.73 ± 0.10	0.57 ± 0.58	78
IgM	1.56 ± 0.46	1.09 ± 0.47	70

Table 3. Assessment of antioxidant capacities by DPPH and FRAP methods of purified lactoferrin fractions by cation-exchange chromatography of spray-dried human milk.

Sample	Milk before purification	Fraction 27	Fraction 28	Fraction 29
DPPH activity (%)	33 ± 9a	0 ± 0b	26 ± 7a	0 ± 0b
Frap activity (Trolox equivalent mM)	1.25 ± 0.33a	0 ± 0.00b	0 ± 0.00b	0 ± 0.00b

Different letters in rows indicate significant differences at $P \leq 0.05$.

Table 4. Antimicrobial activity (mm) of purified lactoferrin from human milk by well diffusion assay.

Bacteria	Positive control (antibiotics)	Complete inhibition (mm)	Partial inhibition (mm)
Escherichia coli	30a	3 ± 1b	4 ± 2b
Staphylococcus aureus	28a	2 ± 1b	3 ± 2b
Salmonella Typhimurium	35a	>1 ± 1b	2 ± 1b
Listeria monocytogenes	30a	3 ± 2b	0 ± 0b
Bacillus cereus	15a	0 ± 0b	0 ± 0 b

Diameter (D) in mm is calculated with the following formula: D(mm) = (Diameter – Well Diameter). Data represents the mean between 3 replicates for a concentration of 1.5 mg of purified lactoferrin from human milk. Different letters in rows indicate significant differences at $P \leq 0.05$.

Table 5. The effect of ɣ-irradiation compared to Holder pasteurization on the microbiological content of liquid frozen and powdered spray-dried human milk.

Treatment	Yeast and molds (Log CFU/mL)		Lactic bacteria (Log CFU/mL)		Total coliforms (Log CFU/mL)		Aerobic total flora (Log CFU/mL)		% Elimination of total aerobic flora	
	Powder	Frozen	Powder	Frozen	Powder	Frozen	Powder	Frozen	Powder	Frozen
Control 0 kGy	2.64 ± 0.24a		2.51 ± 0.19a		0.67 ± 0.52a		2.50 ± 0.19a		NA	
HoP	1.74 ± 0.23b		0.76 ± 0.83b		< LM b		1.19 ± 0.60b		95.1%	
3 kGy	0.63 ± 0.71c	0.50 ± 0.55c	0.25 ± 0.60bc	0.17 ± 0.41c	< LM b	< LM b	0.17 ± 0.41c	0.17 ± 0.41c	99.5%	99.5%
5 kGy	0.33 ± 0.52d	0.17 ± 0.41d	< LM c	< LM c	< LM b	< LM b	< LM c	< LM c	>99.7%	>99.7%
10 kGy	< LM d	< LM d	< LM c	< LM c	< LM b	< LM b	< LM c	< LM c	>99.7%	>99.7%

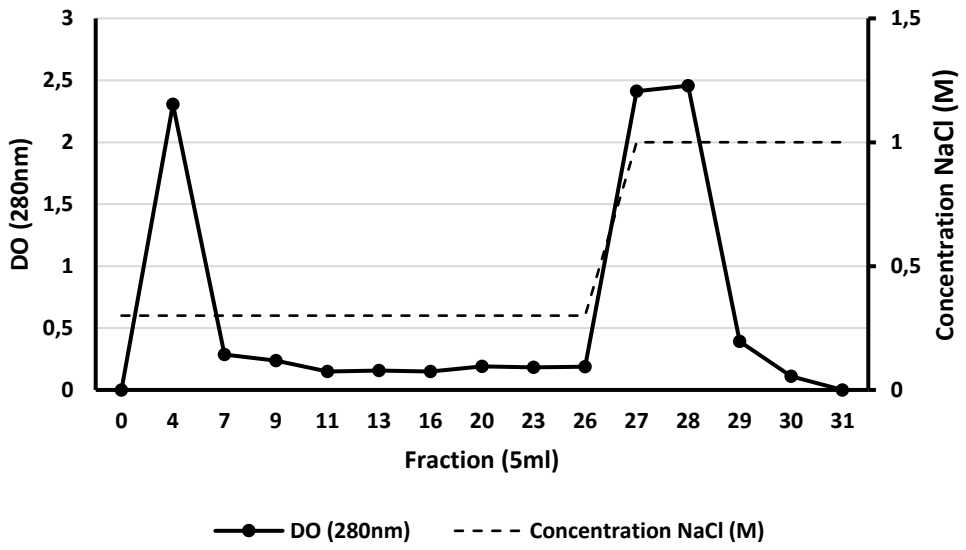
Different letters in rows and columns between the same microbial type indicate significant differences at $p \leq 0.05$. No significant difference ($p > 0.05$) was found between powder and frozen for all conditions. HoP means Holder Pasteurization; < LM means that the data was under the limit of detection.

Table 6. The effect of ɣ-irradiation dose on protein, lactose, antioxidant activity, autoxidation of lipids and lysozyme and amylase activities of liquid frozen and powdered spray-dried human milk.

Compound	Powder			Frozen			HoP
	0kGy	5kGy	10kGy	0kGy	5kGy	10kGy	
Protein (g/100mL)	1.14 ± 0.03[a]	1.17 ± 0.03[a]	1.08 ± 0.07[a]	1.11 ± 0.03[a]	1.08 ± 0.03[a]	1.11 ± 0.03[a]	1.04 ± 0.06[a]
Lactose (g/100mL)	7.6 ± 0.1[a]	8.1 ± 0.1[a]	7.8 ± 0.4[a]	7.6 ± 0.4 [a]	7.3 ± 0.1 [a]	7.7 ± 0.1 [a]	8.1 ± 0.2 [a]
DPPH (% Antioxidant)	62 ± 9[a]	59 ± 7[a]	61 ± 6[a]	63 ± 1[a]	66 ± 3[a]	62 ± 1[a]	68 ± 8[a]
TBARS (µg/g)	0.17 ± 0.02[a]	0.27 ± 0.04[a]	0.66 ± 0.10[b]	0.21 ± 0.01[a]	0.33 ± 0.13[a]	0.75 ± 0.06[b]	0.17 ± 0.02[a]
Lysozyme (U/mL)	1383 ± 141[b]	1339 ± 58[b]	1261 ± 110[b]	1338 ± 117[b]	1247 ± 227[b]	1315 ± 183[b]	1220 ± 35[a]
Amylase (U/mL)	1591 ± 120[a]	1393 ± 164[a]	1464 ± 90[a]	1559 ± 118[a]	1393 ± 125[a]	1442 ± 106[a]	1531 ± 138[a]

A mean followed by a different letter from the respective control 0 kGy (untreated) in the same row indicates significant difference at $p \leq 0.05$. HoP means Holder Pasteurization.

a)



b)

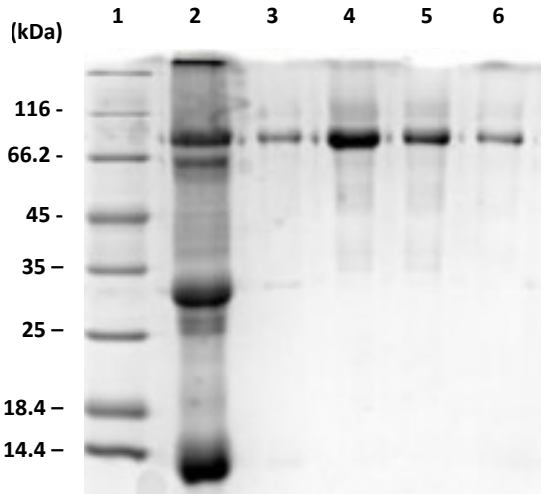


Figure 1. a) Cation exchange chromatography on SP-Sepharose of the human spray-dried milk. The flow rate was 3 mL/min and the volume of the fractions was 5 mL b) SDS-Page of chromatography fractions: (1) molecular weight marker, (2) human milk before chromatography, (3-6) Chromatography fractions 27 to 30 which corresponds to lactoferrin.

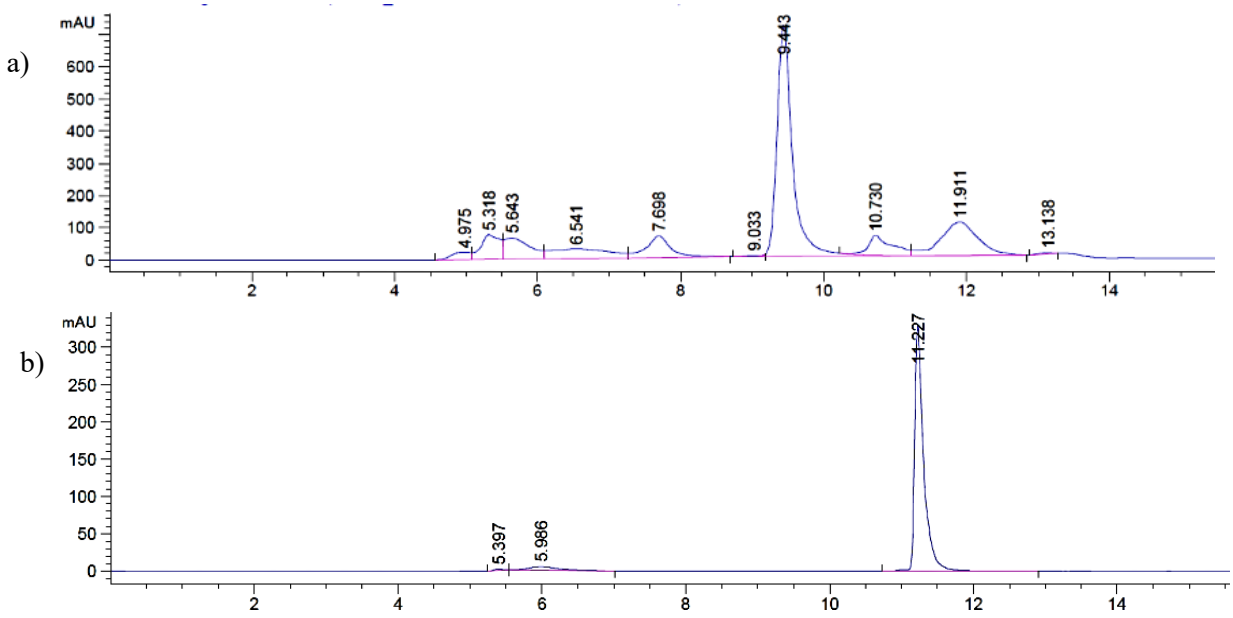
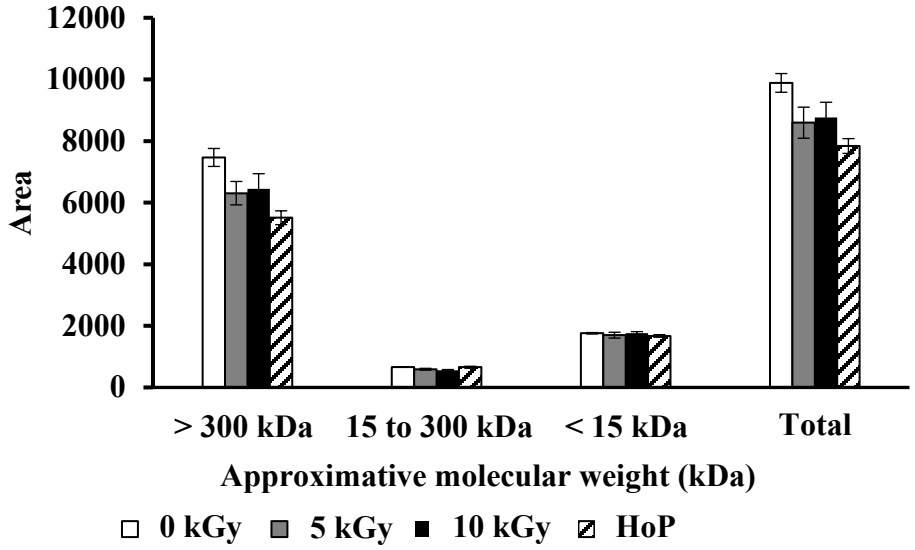


Figure 2. a) SEC-HPLC spectrum of spray-dried human milk b) SEC-HPLC spectrum of lactoferrin fraction # 29 purified from spray-dried human milk.

a)



b)

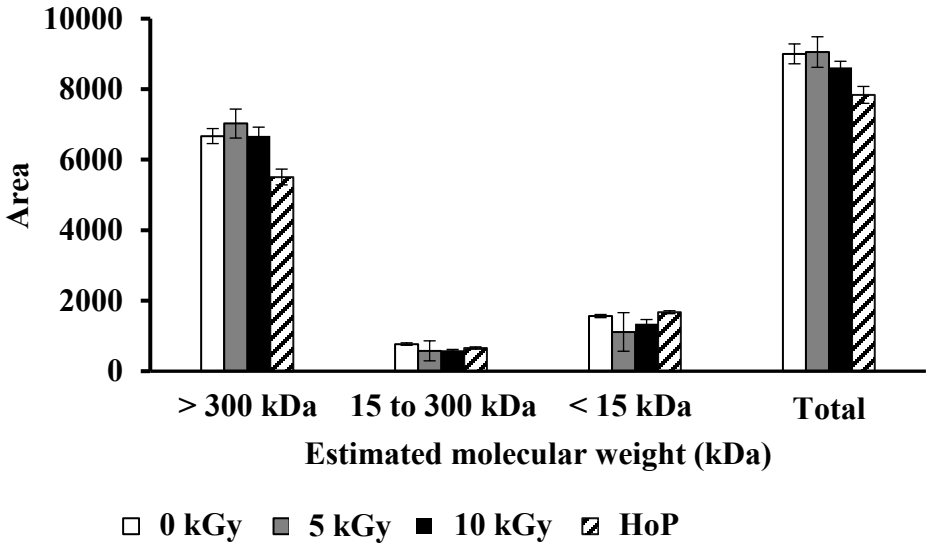


Figure 3. Effect of different irradiation doses (0, 5, and 10 kGy) and HoP treatment on the peptide size profiles in spray-dried powdered **(a)** and frozen **(b)** HM.

8. References

Alvarenga, V. O., Campagnollo, F. B., Pia, A. K. R., Conceição, D. A., Abud, Y., Sant'Anna, C., Hubinger, MD, Sant'Ana, A. S. (2018). Quantifying the Responses of Three Bacillus cereus Strains in Isothermal Conditions and During Spray Drying of Different Carrier Agents. *Frontiers in microbiology*, *9*, 1113. doi:10.3389/fmicb.2018.01113

Arpagaus, C., Collenberg, A., Rütti, D., Assadpour, E. and Jafari, S. M. (2018). Nano spray drying for encapsulation of pharmaceuticals. *International Journal of Pharmaceutics*, *546*(1-2), 194-214. doi:10.1016/j.ijpharm.2018.05.037

Becker, H., Schaller, G., von Wiese, W. and Terplan, G. (1994). Bacillus cereus in infant foods and dried milk products. *International Journal of Food Microbiology*, *23*(1), 1-15. doi:10.1016/0168-1605(94)90218-6

Botsoglou, N. A., Fletouris, D. J., Papageorgiou, G. E., Vassilopoulos, V. N., Mantis, A. J., & Trakatellis, A. G. (1994). Rapid, sensitive, and specific thiobarbituric acid method for measuring lipid peroxidation in animal tissue, food, and feedstuff samples. *Journal of Agricultural and Food Chemistry*, *42*(9), 1931–1937. doi:10.1021/jf00045a019

Cacho, N. T., Harrison, N. A., Parker, L. A., Padgett, K. A., Lemas, D. J., Marcial, G. E., Lorca, G. L. (2017). Personalization of the Microbiota of Donor Human Milk with Mother's Own Milk. *Frontiers in microbiology*, *8*, 1470. doi:10.3389/fmicb.2017.01470

Calado, T., Venâncio, A. and Abrunhosa, L. (2014). Irradiation for mold and mycotoxin control: A review. *Comprehensive Reviews in Food Science and Food Safety*, *13*(5), 1049-1061. doi:10.1111/1541-4337.12095

Capriati, T., Goffredo, B. M., Argentieri, M., De Vivo, L., Bernaschi, P., Cairoli, S., Diamanti, A. (2019). A modified holder pasteurization method for donor human milk: preliminary data. *Nutrients*, *11*(5). doi:10.3390/nu11051139

Castro-Albarrán, J., Aguilar-Uscanga, B. R., Calon, F., St-Amour, I., Solís-Pacheco, J., Saucier, L. and Ratti, C. (2016). Spray and freeze drying of human milk on the retention of immunoglobulins (IgA, IgG, IgM). *Drying Technology*, *34*(15), 1801-1809. doi:10.1080/07373937.2016.1141781

Celli, G. B., Ghanem, A. and Brooks, M. S.-L. (2015). Bioactive encapsulated powders for functional foods—a review of methods and current limitations. *Food and Bioprocess Technology*, *8*(9), 1825-1837. doi:10.1007/s11947-015-1559-z

Cheng, Y., Wei, H., Sun, R., Tian, Z. and Zheng, X. (2016). Rapid method for protein quantitation by Bradford assay after elimination of the interference of polysorbate 80. *Analytical Biochemistry*, *494*, 37-39. doi:10.1016/j.ab.2015.10.013

Cloetens, L., Panee, J. and Åkesson, B. (2013). The antioxidant capacity of milk--the application of different methods in vitro and in vivo. *Cellular and molecular biology (Noisy-le-Grand, France)*, *59*(1), 43-57.

Conesa, C., Sánchez, L., Rota, C., Pérez, M.-D., Calvo, M., Farnaud, S. and Evans, R. W. (2008). Isolation of lactoferrin from milk of different species: calorimetric and antimicrobial studies. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, *150*(1), 131-139. doi:10.1016/j.cbpb.2008.02.005

Czosnykowska-Łukacka, M., Orczyk-Pawiłowicz, M., Broers, B. and Królak-Olejnik, B. (2019). Lactoferrin in human milk of prolonged lactation. *Nutrients*, *11*(10). doi:10.3390/nu11102350

Dauphin, L. A., Newton, B. R., Rasmussen, M. V., Meyer, R. F. and Bowen, M. D. (2008). Gamma irradiation can be used to inactivate Bacillus anthracis spores without compromising the sensitivity of diagnostic assays. *Applied and Environmental Microbiology*, *74*(14), 4427-4433. doi:10.1128/AEM.00557-08

De Lara, J., Fernández, P. S., Periago, P. M. and Palop, A. (2002). Irradiation of spores of Bacillus cereus and Bacillus subtilis with electron beams. *Innovative Food Science & Emerging Technologies*, *3*(4), 379-384. doi:10.1016/S1466-8564(02)00053-X

El-Rawas, A., Hvizdzak, A., Davenport, M., Beamer, S., Jaczynski, J. and Matak, K. (2012). Effect of electron beam irradiation on quality indicators of peanut butter over a storage period. *Food chemistry*, *133*(1), 212-219. doi:10.1016/j.foodchem.2011.12.078

Feeney, A., Johnston, C. D., Govender, R., O'Mahony, J., Coffey, A. and Sleator, R. D. (2014). Analysis of the role of the Cronobacter sakazakii ProP homologues in osmotolerance. *Gut Pathogens*, *6*, 15. doi:10.1186/1757-4749-6-15

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of Biological Chemistry*, *226*(1), 497–509.

Francis, J., Rogers, K., Brewer, P., Dickton, D. and Pardini, R. (2008). Comparative analysis of ascorbic acid in human milk and infant formula using varied milk delivery systems. *International breastfeeding journal*, *3*, 19. doi:10.1186/1746-4358-3-19

Giansanti, F., Panella, G., Leboffe, L. and Antonini, G. (2016). Lactoferrin from Milk: Nutraceutical and Pharmacological Properties. *Pharmaceuticals (Basel, Switzerland)*, *9*(4). doi:10.3390/ph9040061

González-Olivares, L. G., Añorve-Morga, J., Castañeda-Ovando, A., Contreras-López, E. and Jaimez-Ordaz, J. (2014). Peptide separation of commercial fermented milk during refrigerated storage. *Food Science and Technology (Campinas)*, *34*(4), 674-679. doi:10.1590/1678-457X.6415

Hafez, Y. S., Mohamed, A. I., Singh, G., & Hewedy, F. M. (1985). Effects of gamma irradiation on proteins and fatty acids of soybean. *Journal of Food Science*, *50*(5), 1271–1274. doi:10.1111/j.1365-2621.1985.tb10459.x

Ham, J. S., Jeong, S. G., Lee, S. G., Han, G. S., Jang, A., Yoo, Y. M., Jo, C. (2009). Quality of Irradiated Plain Yogurt during Storage at Different Temperatures. *Asian-Australasian Journal of Animal Sciences*, *22*(2), 289–295. doi:10.5713/ajas.2009.80329

Hema-Québec. (2020, 2 March). ENCART' ' INFORMATIF - LAIT MATERNEL PASTEURISÉ . Repéré May 6, 2020, à https://www.hema-quebec.qc.ca/userfiles/file/media/francais/lait%20maternel/PUB-00031.pdf

Holsinger, V. H., Rajkowski, K. T. and Stabel, J. R. (1997). Milk pasteurisation and safety: a brief history and update. *Revue scientifique et technique (International Office of Epizootics)*, *16*(2), 441-451. doi:10.20506/rst.16.2.1037

Huang, S., Vignolles, M.-L., Chen, X. D., Le Loir, Y., Jan, G., Schuck, P. and Jeantet, R. (2017). Spray drying of probiotics and other food-grade bacteria: A review. *Trends in food science & technology*, *63*, 1-17. doi:10.1016/j.tifs.2017.02.007

Joubran, Y., Mackie, A. and Lesmes, U. (2013). Impact of the Maillard reaction on the antioxidant capacity of bovine lactoferrin. *Food chemistry*, *141*(4), 3796-3802. doi:10.1016/j.foodchem.2013.06.096

Kavitha, C., Kuna, A., Supraja, T., Sagar, S. B., Padmavathi, T. V. N. and Prabhakar, N. (2015). Effect of gamma irradiation on antioxidant properties of ber (Zizyphus mauritiana) fruit. *Journal of food science and technology*, *52*(5), 3123-3128. doi:10.1007/s13197-014-1359-x

Kedare, S. B. and Singh, R. P. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of food science and technology*, *48*(4), 412-422. doi:10.1007/s13197-011-0251-1

Kim, J. and Unger, S. (2010). Human milk banking. *Paediatrics & child health*, *15*(9), 595-602. doi:10.1093/pch/15.9.595

Kim, J.-H., Sung, N.-Y., Kwon, S.-K., Srinivasan, P., Song, B.-S., Choi, J.-I., Yoon, Y., Kim, JK., Byun, MW., Kim, MR., Lee, J.-W. (2009). Gamma-irradiation improves the color and antioxidant

properties of Chaga mushroom (Inonotus obliquus) extract. *Journal of medicinal food*, *12*(6), 1343-1347. doi:10.1089/jmf.2008.1281

Kim, M. H., Shim, K. S., Yi, D. Y., Lim, I. S., Chae, S. A., Yun, S. W., Lee, NM., Kim, SY., Kim, S. (2019). Macronutrient Analysis of Human Milk according to Storage and Processing in Korean Mother. *Pediatric gastroenterology, hepatology & nutrition*, *22*(3), 262-269. doi:10.5223/pghn.2019.22.3.262

Landers, S. and Updegrove, K. (2010). Bacteriological screening of donor human milk before and after Holder pasteurization. *Breastfeeding medicine : the official journal of the Academy of Breastfeeding Medicine*, *5*(3), 117-121. doi:10.1089/bfm.2009.0032

Letchoumy, P. V. and Mohan, K. C. (2008). In vitro antioxidative potential of lactoferrin and black tea polyphenols and protective effects in vivo on carcinogen activation, DNA damage, proliferation, invasion, and angiogenesis during experimental oral carcinogenesis. *Oncology Research,* 17(5), 193–203. doi:10.3727/096504008786111365

Lewin, A., Delage, G., Bernier, F. and Germain, M. (2019). Banked Human Milk and Quantitative Risk Assessment of Bacillus cereus Infection in Premature Infants: A Simulation Study. *The Canadian journal of infectious diseases & medical microbiology = Journal canadien des maladies infectieuses et de la microbiologie medicale / AMMI Canada*, *2019*, 6348281. doi:10.1155/2019/6348281

Lima, H. K., Wagner-Gillespie, M., Perrin, M. T. and Fogleman, A. D. (2017). Bacteria and Bioactivity in Holder Pasteurized and Shelf-Stable Human Milk Products. *Current Developments in Nutrition*, *1*(8), e001438. doi:10.3945/cdn.117.001438

Lönnerdal, B. (2013). Bioactive proteins in breast milk. *Journal of Paediatrics and Child Health*, *49 Suppl 1*, 1-7. doi:10.1111/jpc.12104

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, *193*(1), 265-275.

Lozano, B., Castellote, A. I., Montes, R. and López-Sabater, M. C. (2014). Vitamins, fatty acids, and antioxidant capacity stability during storage of freeze-dried human milk. *International Journal of Food Sciences and Nutrition*, *65*(6), 703-707. doi:10.3109/09637486.2014.917154

Michaelsen, K. F., Skafte, L., Badsberg, J. H. and Jørgensen, M. (1990). Variation in macronutrients in human bank milk: influencing factors and implications for human milk banking. *Journal of Pediatric Gastroenterology and Nutrition*, *11*(2), 229-239. doi:10.1097/00005176-199008000-00013

Miller, G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry*, *31*(3), 426-428. doi:10.1021/ac60147a030

Moltó-Puigmartí, C., Permanyer, M., Castellote, A. I. and López-Sabater, M. C. (2011). Effects of pasteurisation and high-pressure processing on vitamin C, tocopherols and fatty acids in mature human milk. *Food chemistry*, *124*(3), 697-702. doi:10.1016/j.foodchem.2010.05.079

Monk, J. D., Beuchat, L. R. and Doyle, M. P. (1995). Irradiation Inactivation of Food-Borne Microorganisms. *Journal of Food Protection*, *58*(2), 197-208. doi:10.4315/0362-028X-58.2.197

Montagne, P., Laroche, P. and Bessou, T. (1992). Measurement of eleven serum proteins by microparticle-enhanced nephelometric immunoassay. *European Journal of Clinical Chemistry and Clinical Biochemistry : Journal of the Forum of European Clinical Chemistry Societies,* 30(4), 217–222. doi:10.1515/cclm.1992.30.4.217

Pacheco, S., Arreola, R., Padilla, G., Morales, G., Preciado, M., Albarrán, C., Uscanga, A. (2019). Human Milk Powder an Alternative for Better Conservation and Healthier Use in the Banks of Human Milk. *CPQ nutrition, 3(2):* 01-12

Peila, C., Moro, G. E., Bertino, E., Cavallarin, L., Giribaldi, M., Giuliani, F., Coscia, A. (2016). The Effect of Holder Pasteurization on Nutrients and Biologically-Active Components in Donor Human Milk: A Review. *Nutrients*, *8*(8). doi:10.3390/nu8080477

Persson, B. A., Lund, M., Forsman, J., Chatterton, D. E. W. and Akesson, T. (2010). Molecular evidence of stereo-specific lactoferrin dimers in solution. *Biophysical Chemistry*, *151*(3), 187-189. doi:10.1016/j.bpc.2010.06.005

Picaud, J.-C. and Buffin, R. (2017). Human Milk-Treatment and Quality of Banked Human Milk. *Clinics in Perinatology*, *44*(1), 95-119. doi:10.1016/j.clp.2016.11.003

Pierce, A., Legrand, D. and Mazurier, J. (2009). [Lactoferrin: a multifunctional protein]. *Medecine Sciences*, *25*(4), 361-369. doi:10.1051/medsci/2009254361

Richmond, M. L., Harte, B. R., Gray, J. I. and Stine, C. M. (1987). Determination of sugars in yogurt and microbiological media by high performance liquid chromatography during processing and subsequent storage. *Journal of Dairy Science*, *70*(6), 1140-1147. doi:10.3168/jds.S0022-0302(87)80124-8

Rigourd, V., Barnier, J. P., Ferroni, A., Nicloux, M., Hachem, T., Magny, J. F., Bille, E. (2018). Recent actuality about Bacillus cereus and human milk bank: a new sensitive method for microbiological analysis of pasteurized milk. *European Journal of Clinical Microbiology & Infectious Diseases*, *37*(7), 1297-1303. doi:10.1007/s10096-018-3249-z

Roberts, P. B. (2016). Food irradiation: Standards, regulations and world-wide trade. *Radiation Physics and Chemistry*, *129*, 30-34. doi:10.1016/j.radphyschem.2016.06.005

Robichaud, V., Bagheri, L., Aguilar-Uscanga, B. R., Millette, M. and Lacroix, M. (2020). Effect of ɣ-irradiation on the microbial inactivation, nutritional value, and antioxidant activities of infant formula. *LWT*, *125*, 109211. doi:10.1016/j.lwt.2020.109211

Rodríguez AA., Lacroix, M., Raymundo Solís Pacheco, J., García Morales, E., Alfonso Gutiérrez Padilla, J., Angulo Castellanos, E., Rosa Aguilar Uscanga, B. (2018). Assessment of the Biological Activity in Human Milk Power Treated with Different Processes for Their Conservation. *Journal of Food and Nutrition Research*, *6*(5), 329-334. doi:10.12691/jfnr-6-5-8

Rodríguez, J. M., Jiménez, E. and Merino, V. (2008). Microbiota de la leche humana en condiciones fisiológicas. A*cta Pediatr Espanola.* 66 (2): 27-3

Romeunadal, M., Morerapons, S., Castellote, A. and Lopezsabater, M. (2006). Rapid high-performance liquid chromatographic method for Vitamin C determination in human milk versus an enzymatic method. *Journal of Chromatography B*, *830*(1), 41-46. doi:10.1016/j.jchromb.2005.10.018

Ruhl, S., Berlenbach, P., Langenfelder, S., Hörl, D., Lehn, N., Hiller, K.-A., Durchschlag, H. (2011). Integrity of proteins in human saliva after sterilization by gamma irradiation. *Applied and Environmental Microbiology*, *77*(3), 749-755. doi:10.1128/AEM.01374-10

Saloua, K. S., Salah, K., Nasreddine, B., Samia, A., Mouldi, S. and Ahmed, L. (2011). Sterilization and protection of protein in combinations of Camellia sinensis green tea extract and gamma irradiation. *International journal of biological macromolecules*, *48*(3), 452-458. doi:10.1016/j.ijbiomac.2011.01.003

Schlotterer, H. R. and Perrin, M. T. (2018). Effects of Refrigerated and Frozen Storage on Holder-Pasteurized Donor Human Milk: A Systematic Review. *Breastfeeding medicine : the official journal of the Academy of Breastfeeding Medicine*, *13*(7), 465-472. doi:10.1089/bfm.2018.0135

Shugar, D. (1952). The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. *Biochimica et Biophysica Acta*, *8*(3), 302-309. doi:10.1016/0006-3002(52)90045-0

Stefanova, R., Vasilev, N. V. and Spassov, S. L. (2010). Irradiation of food, current legislation framework, and detection of irradiated foods. *Food analytical methods*, *3*(3), 225-252. doi:10.1007/s12161-009-9118-8

Szczawinska, M. E. (2017). Application of ionizing radiation for control of salmonella in food. In M. Mares (dir.), *Current topics in salmonella and salmonellosis*, 14, 253-273. InTech. doi:10.5772/67408

Thompson, S. S., Harmon, L. G. and Stine, C. M. (1978). Survival of selected organisms during spray drying of skim milk and storage of nonfat dry milk 1. *Journal of Food Protection*, *41*(1), 16-19. doi:10.4315/0362-028X-41.1.16

Vieira, A. A., Soares, F. V. M., Pimenta, H. P., Abranches, A. D. and Moreira, M. E. L. (2011). Analysis of the influence of pasteurization, freezing/thawing, and offer processes on human milk's macronutrient concentrations. *Early Human Development*, *87*(8), 577-580. doi:10.1016/j.earlhumdev.2011.04.016

Wesolowska, A., Sinkiewicz-Darol, E., Barbarska, O., Bernatowicz-Lojko, U., Borszewska-Kornacka, M. K. and van Goudoever, J. B. (2019). Innovative techniques of processing human milk to preserve key components. *Nutrients*, *11*(5). doi:10.3390/nu11051169

Wojcik, K. Y., Rechtman, D. J., Lee, M. L., Montoya, A. and Medo, E. T. (2009). Macronutrient analysis of a nationwide sample of donor breast milk. *Journal of the American Dietetic Association*, *109*(1), 137-140. doi:10.1016/j.jada.2008.10.008

Woodside, J. V. (2015). Nutritional aspects of irradiated food. *Stewart Postharvest Review*, *11*(3), 1-6. doi:10.2212/spr.2015.3.2

Yu Huaning, Li Yunfei, Ardil Abdukerim, & Long Weiyun. (2011). Effects of low temperature spray drying conditions on physical properties of bovine colostrum powders. In *2011 Louisville, Kentucky, August 7 - August 10, 2011*. St. Joseph, MI: American Society of Agricultural and Biological Engineers Paper No. 11. doi:10.13031/2013.37355

Zarban, A., Taheri, F., Chahkandi, T., Sharifzadeh, G. and Khorashadizadeh, M. (2009). Antioxidant and radical scavenging activity of human colostrum, transitional and mature milk. *Journal of clinical biochemistry and nutrition*, *45*(2), 150-154. doi:10.3164/jcbn.08-233

Zelic, B. and Nesek, B. (2006). Mathematical modeling of size exclusion chromatography. *Engineering in life sciences*, *6*(2), 163-169. doi:10.1002/elsc.200620903

CHAPITRE 5:

ANTICANCER PROPERTIES AND PREVENTION OF METABOLIC SYNDROME OF PROBIOTIC-ENRICHED POWDERED HUMAN MILK

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Valérie Robichaud a réalisé les manipulations et la rédaction de l'article.

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Résumé

Objectifs: L'objectif de ce travail est de développer un aliment fonctionnel à base de lait maternel humain (HM) enrichi de la formulation probiotique Bio-K Plus^{MD} contenant 9 Log UFC/mL d'un mélange de *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R et *Lacticaseibacillus rhamnosus* CLR2 et d'évaluer *in vivo* ses propriétés anticancéreuses et son impact sur la prévention des facteurs de risque du syndrome métabolique.

Méthodes: Des études *in vitro* ont été menées sur les lignées cellulaires cancéreuses Hepa 1c1c7 et HT-29, et non-cancéreuse CHO-K1 pour évaluer les capacités antiprolifératives, apoptotiques et d'induire la quinone réductase par le lait humain (HM) et les probiotiques. L'étude *in vivo* a été menée sur 28 rats Wistar mâles nourris avec un régime riche en graisses. Les rats ont été gavés quotidiennement avec 0,5 mL de HM contenant, du HM contenant 9,5 Log UFC/mL de probiotiques ou de l'eau pendant 8 semaines. L'inflammation et le stress oxydatif ont été surveillés par des cytokines sanguines et par l'évaluation des dommages oxydatifs dans les reins. Le poids corporel et la glycémie ont été mesurés tout au long de l'étude.

Résultats: Les résultats *in vitro* ont montré que le HM avait des propriétés antiprolifératives et provoque l'apoptose des cellules tumorales intestinales. La quinone réductase était induite jusqu'à 1,8 fois dans la lignée cellulaire Hepa 1c1c7, ce qui démontre un potentiel chimiothérapeutique. L'étude chez le rat a montré que l'HM avec des probiotiques réduisait la prise de poids de 10% et avait des dommages oxydatifs rénaux réduits jusqu'à 97%. Les taux d'insuline dans le sang et de PAI-1 sont considérablement réduits de 22% ($p \leq 0,05$).

Conclusion: En conclusion, cette étude suggère que le développement d'un aliment fonctionnel à base de lait humain en poudre enrichi en probiotiques pourrait être utilisé pour moduler le taux d'inflammation et les dommages oxydatifs aux tissus induits par les maladies liées à l'obésité.

Mots-clés: lait humain, probiotiques, syndrome métabolique, stress oxydatif, antioxydant

ABSTRACT

Objectives: The aim of this work is to develop a functional food based on human milk (HM) enriched with a specific probiotic formulation comprised of *Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R and *Lacticaseibacillus rhamnosus* CLR2 and to evaluate *in vivo* its anticancer properties and its impact on the prevention of risk factors of metabolic syndrome.

Methods: *In vitro* studies were conducted on the Hepa 1c1c7 and HT-29 cancerous cell lines, and CHO-K1 normal cell line to assess the antiproliferative, apoptotic and quinone reductase induction properties of human milk (HM) and probiotics. The *in vivo* study was conducted with 28 male Wistar rats fed with high-fat diet. Rats were force-fed daily with 0.5 mL of HM, HM containing 9.5 CFU Log/mL of probiotics or water for 8 weeks. Inflammation and oxidative stress were monitored by blood cytokines and by the evaluation of oxidative damages in the kidneys. Body weight and blood glucose levels were recorded throughout the study.

Results: *In vitro* results showed that HM had antiproliferative properties and causes apoptosis of intestinal tumoral cells. Quinone reductase was induced up to 1.8x in Hepa 1c1c7 cell line, which demonstrates chemotherapeutic potential. The study in rats showed that HM enriched with probiotics reduced weight gain by 10% while renal oxidative damages were reduced by up to 97%. Blood insulin and Plasminogen Activator Inhibitor-1 (PAI-1) levels were significantly reduced by 22% ($p \leq 0.05$).

Conclusion: The study showed that human milk had antiproliferative and chemopreventive properties on cancer cell lines. The *in vivo* results suggest that the development of a functional food based on human milk powder enriched with a specific formulation of probiotics could be used to modulate the rate of inflammation and the oxidative damage to tissues thus reducing the risk of diseases related to obesity.

Keywords: Human milk, probiotics, metabolic syndrome, oxidative stress, antioxidant

INTRODUCTION

For the last 20 years, global health has been declining and the rate of malnutrition and obesity is increasing. These nutrition-related problems have multiple impacts on the health of the population such as metabolic syndrome and other chronic degenerative diseases (Saklayen, 2018).

Metabolic syndrome (MetS) is a cluster of conditions including abnormal blood glucose level, abdominal obesity, increased plasma LDL cholesterol and triglycerides, and elevated blood pressure. Taken together, these factors contribute to the acceleration of aging, functional decline and increase the risk of developing cardiovascular and chronic diseases such as Type 2 diabetes, stroke or atherosclerosis (Lalan *et al.*, 2018; Ogunsile *et al.*, 2019). MetS is an important factor in development, progression and mortality of many cancer types both in men and women. Moreover, diet has been related to almost 70 % of cancer incidence (Aggarwal, 2010).

To evaluate the effect of alimentation on health, different markers *in vitro* and *in vivo* are commonly used. *In vitro*, antiproliferative and an apoptosis assays on cancerous and non-cancerous cell lines are conducted to evaluate possible anticancer properties. The evaluation of the chemoprotective effect can be measured through the induction of nicotinamide adenine dinucleotide phosphate hydrogen: quinone reductase (NAD(P)H:QR), a cancer chemopreventive phase II detoxification enzyme. This protein is known to protect against toxic electrophilic metabolites directly involved in the first stage of carcinogenesis (Fortin *et al.*, 2017). *In vivo*, total antioxidant properties, protein and lipid oxidative damages (measured by PCO and MDA formation) and the induction of catalase, a detoxifying enzyme, can be measured to determine oxidative stress or low-grade inflammation in blood or tissue. In addition, some plasma inflammatory markers (TNF-a, IL-1B, PAI-1, PON1) are often quantified to track cell and tissue damages. TNF-a and IL-1B are pro-inflammatory cytokines and can result of cell injury. The protein PAI-1 is a marker for many inflammation-related diseases. Paraoxanase (PON1) is an antioxidant enzyme able to eliminate lipid peroxides. This enzyme is elevated in presence of oxidative stress (Chung *et al.*, 2018; Noeman, Hamooda and Baalash, 2011; Welty, Alfaddagh and Elajami, 2016).

Since obesity, low-grade inflammation and oxidative stress are known to be linked to cancer, the consumption of a diet containing agents that could prevents oxidative damages and induce anticancer enzymes may reduce the risk of cancer incidence (Marseglia *et al.*, 2014). Research is therefore turning to foods with hypolipemic, hypocholesterolemic, insulin sensitizing, anti-inflammatory and antioxidant properties. This includes beverages, fruits and herbs, prebiotics and probiotics that can induce a decrease of cardiovascular disease risks by targeting therapeutic drug of MetS. For example, grape seeds have shown a

decrease in blood pressure and lipid accumulation in humans (Cherniack, 2011). Weight management and inflammation linked to MetS would also be possible with probiotics (Mallappa *et al.*, 2012). Consequently, a balanced dietary pattern rich in functional foods could help in the prevention of risks factors of MetS and related conditions such as cancer (Hasler, 2002; Seki, Hendrie and Daire, 2019).

In this context, human milk (HM) is considered a functional food because of its content in macronutrients (proteins, lipids and carbohydrates), micronutrients (minerals and vitamins) and many immunological factors (hormones, growth factors, cytokines, enzymes) that together provide adequate nutrition to the newborn and even have a role in the prevention of overweight and obesity (Agostoni, Baselli and Mazzoni, 2013). In addition to anti-inflammatory, antioxidant and anti-infection properties (Ballard and Morrow, 2013), studies have shown possible anticancer properties due to the presence of several tumor suppressors components, such as Human Alpha-lactalbumin Made Lethal to Tumour cells (HAMLET) and cancer-fighting TNF-related apoptosis inducing ligand (TRAIL) (Davanzo *et al.*, 2013; Marcuzzi *et al.*, 2013; Mossberg *et al.*, 2010). It is also known that HM stimulates and shapes the development of the gut microbiota composition, contributes to the maturation of the immune system and helps protect infants from pathogens due to the presence of prebiotic fibers and beneficial bacteria (Thongaram *et al.*, 2017).

Probiotics are defined as live microorganisms which, when given in adequate amounts confer benefits to the health of consumers (Hill *et al.*, 2014). These bacteria are used in a variety of products in both children and adults, particularly in prevention and treatment of gastrointestinal disorders such as infectious diarrhea, necrotizing enterocolitis and irritable bowel syndrome (Hoyos, 1999; Kazemi *et al.*, 2019; Principi *et al*, 2018). Other potential benefits provided by probiotics are modulation of local and systemic immune responses, bioconversion of various dietary compounds into bioactive molecules and vitamin production (Chen *et al.*, 2015).

It has been shown that changes in the composition or diversity of the gut microbiota or an altered interaction between the intestinal microbiota and the host are linked to the development of MetS (Henao-Mejia *et al.*, 2012). Many studies have demonstrated that HM acts as a substrate for fermentation in the intestine, promoting the balance of the microbiota (Bode, 2009; Newburg and Morelli, 2015; Walker and Iyengar, 2015; Wall *et al.*, 2009). In addition, some probiotics have been shown to enhance lipid and glycemic markers in rodent models and clinical trials of obesity, type 2 diabetes mellitus and dyslipidemia (Kassaian *et al.,* 2018; Sun and Buys, 2016). Moreover, studies have already shown that infants fed with formula or HM supplemented with probiotics could significantly reduce incidence rates of gastrointestinal infections and upper respiratory tract infections, atopic dermatitis and colic (Bergmann *et al.*, 2014). In opposition, Repa *et al.* (2015) demonstrated that the synergistic effect would be specific between HM and

specific strains of probiotics, but not with a common commercial formulation. The creation of a functional food based on HM enriched with probiotics with proven nutritional and medicinal capacities could be considered to ameliorate metabolic parameters.

The aim of this study was to evaluate *in vitro* and *in vivo* the synergistic effect of HM in combination with probiotics (*Lactobacillus acidophilus* CL1285, *Lacticaseibacillus casei* LBC80R, *Lacticaseibacillus rhamnosus* CLR2) on anticancer properties and on the prevention of the development of metabolic syndrome (MetS) and other obesity-related diseases.

MATERIALS AND METHODS

Chemicals

Rat Adipokine Magnetic Bead Panel was purchased from MilliporeSigma (Sigma-Aldrich Canada, Oakville, ON, Canada). Methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), hydrogen peroxide (H_2O_2), triton X-100, dimethylsulfoxide (DMSO), trypsin, paraoxonase, paraoxon, 2,4-dinitrophénylhydrazine (DNPH), ethyl acetate, guanidine hydrochloride, flavine adenine dinucleotide (FAD; disodium salt hydrate), glucose-6-phosphate (G-6-P), nicotinamide adenine dinucleotide phosphate (NADP), menadione, glucose-6-phosphate dehydrogenase (G-6-PD), acetic acid and butanol were purchased from MilliporeSigma (Sigma-Aldrich Canada, Oakville, ON, Canada). Ethylenediaminetetraacetic acid (EDTA), Tris-HCl, Calcium chloride ($CaCl_2$), Sodium chloride (NaCl) and trichloroacetic acid (TCA), sodium dodecyl sulfate (SDS), Bovine Serum Albumin (BSA) and Pierce™ BCA Protein Assay Kit were purchased from Fisher Scientific (Ottawa, ON, Canada). Glucometer Accu-Chek Guide and bands were purchased from Uniprix Group (Montreal, QC, Canada).

Preparation of human milk and probiotics

The HM used for this study was collected from Hema-Québec and from Guadalajara Civil Hospital (Jalisco, Mexico), under informed consent from healthy donor mothers. This study was approved by the Ethical and Research Committee of the Civil Hospital “Fray Antonio Alcalde” in Guadalajara in February 2015 (HCG / CI-0127/15). HM was homogenized pooled and atomized in the Mini Spray-Dryer SD-Basic, Labplant (North Yorkshire, UK) at 160°C with a flow rate of 2 mL / min. The HM was homogenized in water (1% w/v) at 37°C in presence of 9.5 Log CFU/mL of probiotics (*L. acidophilus* CL1285, *L. casei LBC80R*, *L. rhamnosus* CLR2) from Bio-K Plus International Inc. (Laval, QC, Canada).

Free radical-scavenging activity

The antiradical properties of HM and probiotics was evaluated by their capacity to scavenge a stable radical, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich Chemical Co., St. Louis, MO, USA) according to Kedare and Singh (2011) with some modifications. Briefly, 1 mL of 80 µM DPPH previously dissolved in pure methanol was added to 250 µL of serial diluted HM and/or probiotics (5 to 100 mg/ml of HM and 10^5 to 10^{10} CFU/ml of probiotics). Each solution was mixed and kept at room temperature for 1 hour under agitation. Each tube was centrifuged 3min at 3000 × *g* at room temperature. Optical density of the supernatant was read at 517 nm using spectrophotometer model (Varian carry 1 spectrophotometer Canada Inc., Mississauga, ON, Canada). The blank consisted of 1.25 mL pure methanol whereas control consisted of 250 uL of the dilution buffer containing 1 mL of the DPPH solution. The inhibition percentage of free radical compounds (IP) was measured by the following equation:

IP (%) = ([*Control OD – Sample OD*] / *Control OD*) * 100

Cell lines and cells maintenance

The cancerous cell lines Hepa 1c1c7 ATCC CRL-2026 and HT-29 ATCC HTB-38, and the non-cancerous cell line CHO-K1 were purchased from American type culture collection (ATCC) (Manassas, VA, USA). Cell lines were cultured in 25 cm^2 cellular flasks (Corning, NY, USA) at 37ºC in a humidified incubator under an atmosphere of 5% CO_2 and 95% air. Hepa 1c1c7 and HT-29 cells were grown in complete DMEM (0.1% essential amino acids, 0.1% sodium pyruvate, 10% FBS). CHO-K1 cells (ATCC) (Manassas, VA, USA), were grown in Ham's F-12 media (20% FBS).

Antiproliferative assay

The antiproliferative activities of HM against cancerous (Hepa 1c1c7 and HT-29) and non-cancerous (CHO-K1) cell lines were assayed to assess chemopreventive effect and cell specificity by the ability of the metabolic active cells to cleave the tetrazolium salt to purple formazan crystals based on Vistica *et al.* (1991). Different cell lines were seeded at 2 x 10^4 cells/well in complete DMEM on a 96-well plate (200 µL/well) and were incubated for 24 h at 37°C under 5% CO_2. The media was removed using a multichannel micropipette and 100 µL of fresh DMEM containing 10 µL of sample previously serial diluted was added and microplate was then incubated for 48 h. Afterward, samples were removed using a multichannel micropipette and replaced with 90 µL of complete DMEM and 10 µL 5% (w/v) of bromure de 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium in Phosphate-buffered saline (PBS) followed by incubation for 4 h at 37°C under 5% CO_2 in the dark. Finally, the wells were carefully emptied using a

multichannel micropipette and replaced with 225 µL of DMSO containing 25 µL of Sorensen buffer containing 0.1 M glycine and 0.1 M NaCl at a pH of 10.5. The microplate was then read at 562 nm. The negative control and blank consisted of dilution buffer and media respectively. Growth inhibition was calculated as follows:

Cellular Growth Inhibition (%) = 100 – (([Sample OD] / Negative control OD) x 100)

Equations obtained by plotting the linear portion of growth inhibition versus increasing concentrations of samples were used to calculate concentrations that inhibit 50% of cellular growth (IC_{50} values).

NAD(P)H: QR assay

QR assay was done according to Fortin *et al.* (2017) with some modifications. Hepa 1c1c7 and HT-29 cells were seeded at a density of 2 x 10^3 cells/well in a 96-well plate using complete DMEM media and were incubated at 37°C in a humidified incubator under 5% CO_2 for 24h. Afterward, the media was removed using a multichannel micropipette and 10 µL of the serial diluted samples were added with 90 µL of media. The microplate was incubated for 48 h. Cells were washed with 200 µL dPBS solution thrice and 25 µL of 0.8% (w/v) digitonin was added to each well followed by a 20 min incubation. Then, samples of 10 µL were removed using a multichannel micropipette and used for total protein quantification. An amount of 5 µL of each well was mixed with 200 µL of complete reaction mixture (0.25 M Tris-HCl pH 7.0, 4.67% (w/v) BSA, 0.01% Tween-20, 5 M FAD, 1 mM glucose-6-phosphate, 3 µM NADP, 34.8 µM MTT, 50 µM menadione and 2 mU/µl glucose-phosphate dehydrogenase (Sigma Aldrich Chemical Co., St. Louis, MO, USA). OD measurement was done at 595 nm after 5 min of incubation at room temperature. A protein assay was conducted using Pierce BCA reagents following the manufacturer's instruction. Controls consisted of dilution solution whereas media was used as blank. Specific activity of QR was defined as nmol of blue formazan formed per mg protein per minute. Fold induction of QR was calculated as follows:

QR Fold Induction (treated on control) = Specific Activity of QR in Treated Group / Specific Activity of QR in Negative Control Group

Apoptosis assay

Annexin V-FITC/PI Dead Cell Apoptosis kit from Invitrogen (Burlington, ON, Canada) was used according to the manufacturer protocol to determine the mechanism involved in HM's induced cell death. HT-29 and Hepa 1c1c7 cells were seeded in a 6-well plate at 3 x 10^5 cells/well (3 mL/well) and incubated

as previously described for 24 h. Then, cells were incubated for 48 h at 37°C under 5% CO_2 in the presence of 80 ppm of HM and/or 9 $\log_{10}$ CFU/mL of probiotics or water as control in a final volume of 3 mL of media. Cells present in the supernatant were harvested by centrifugation at 500 × *g* for 10 min at 4°C. Adhered cells were treated with 1 mL of 1X trypsin-EDTA for 12 min at 37°C. Then, 2 mL of complete medium was added and cells were harvested by centrifugation as described previously. The cell-containing pellets (from the supernatant and the adhered cells) were washed twice with PBS containing 0.25% EDTA to avoid clumping and the apoptosis evaluation was performed by using Annexin V-FITC and Propidium Iodide (PI) double staining assays. Harvested cells were diluted in 1X binding buffer to obtain a concentration of 10^6 cells/mL and Annexin V-FITC/PI staining was performed according to the manufacturer's instructions with a total of 20,000 events by flow cytometry. Camptothecin was used as positive control and dilution buffer solution was used as negative control. An early event in apoptosis is the presence of phosphatidylserine on the outer cell membrane. Annexin V binds specifically to phosphatidylserine is used to detect apoptotic cells. Propidium Iodide is used to detect necrotic cells. Thus, this staining allows for the discrimination between viable (Annexin V -, PI -), apoptotic (Annexin V +, PI -) and necrotic cells (PI +). The percentage of annexin- and PI- positive cells out of 20,000 events was determined using flow cytometer.

Experimental design of the animal model

Rats were purchased from Charles River laboratories (Laval, Qc, Canada). The 28 male Wistar rats of 7 weeks old underwent a one-week period of acclimation. After a week, the 8 weeks old rats were randomly divided into 4 groups of 7 animals and fed *ad libidum* a specific diet purchased from Envigo (Montreal, Qc, Canada). Group 1 was fed low fat diet (LFD; 17% kcal from fat) while the others were fed high fat diet (HFD; 45% kcal from fat) as shown in table 1. The experimental design is schematised in Figure 1. **Group 1 (G1)** served as LFD control and received 0.5 mL water by gavage. **Group 2 (G2)** rats served as HFD control and received 0.5 mL water by gavage. **Group 3 (G3)** rats received 0.5 mL of HM. **Group 4 (G4)** rats received 0.5 mL of HM with probiotics to obtain 9.5 $\log_{10}$ CFU/mL (HM+Pb). Animals were monitored daily for general health and body weight was recorded every week for the duration of the study. Blood glucose was measured every two weeks. After eight weeks, the rats were sacrificed in a CO_2 chamber. The heart, kidneys and liver were collected and stored at -80°C for subsequent assays. Heart, liver and kidneys weight values (% bodyweight) were taken prior to freezing and calculated as follows: Organ weight (% bw) = organ weight (g) /body weight (g) × 100.

Sample preparation

Organs were collected from the rats and immediately weighted. They were stored at -80°C and cryo-homogenized in liquid nitrogen within the next weeks. Samples were kept at -80°C until analysis. After euthanasia, blood was removed from the heart and allowed to coagulate for 30 min at room temperature. Blood was then centrifuged and serum was stored at -80°C until analysis.

Determination of catalase activity in kidney

The induction of catalase, a detoxifying enzyme, can be measured to determine oxidative stress or low-grade inflammation in blood or tissue. Catalase activity in kidney was measured using a spectrophotometer (T60, PG Instruments LTD., Leicestershire, UK) by monitoring the decrease in the maximum absorbance of hydrogen peroxide at 240 nm and 25°C. Kidney sample (0.1 g)-was mixed with 500 µL of 50 mM sodium phosphate buffer (pH 7) and 1% (v/v) triton X-100. After, 2 µL of homogenized solution from kidney was mixed with 1.4 mL of 50 mM sodium phosphate buffer (pH 7) and 1% (v/v) triton X-100. The enzymatic reactions were initiated by adding a quantity of 3.5 µL of 30% H_2O_2. Absorbance at 240 nm was measured every 30 s for 3 min.

Paraoxonase 1 (PON1) Activity in serum

PON1 activity was determined to assess theinduction of this antioxidant enzyme due to oxidative stress in blood. The method was according to Richter and Furlong (1999), with modifications. Plasma PON1 activity was measured by the hydrolysis of paraoxon and release of *p*-nitrophenol. The assay mixture contained 1 µL of plasma in 200 µL buffer 1 (0.1 M Tris-HCl, pH 8.0, 2 mM $CaCl_2$ and 2 M NaCl) and buffer 2 (0.1 M Tris-HCl, pH 8.0, 1 mM EDTA and 2 M NaCl) incubated at 37°C for 5 min. Then, paraoxon was added so that the final solution concentration was 1.2 mM and incubated at 37 °C for 20 min. Immediately, 50 µL solution of 20 mM EDTA and 2% (v/v) TRIS base were added and read at 405 nm. $OD_{405\ nm}$ of buffer 2 was subtracted from absorbance of buffer 1 to eliminate non-PON1-mediated hydrolysis. The PON1 activity was expressed in µmoL paraoxon hydrolyzed/min/liter of serum.

PCO content in kidney

Protein carbonyl content (PCO) in kidney was measured according to Levine *et al.* (1990) with little modifications in order to determine oxidative damages to proteins. Kidney (100mg) was mixed in 0.5 mL of cold phosphate buffer pH 7.4 50mM and homogenized using a tissue homogenizer purchased from Fisher Scientific (Ottawa, ON, Canada) for 20s. The tube was centrifuged at 6000 × *g* for 10 min at room

temperature to remove cellular debris. The supernatant was collected and diluted 1:20 in phosphate buffer pH 7.4 50mM. The precipitation of the nucleic acids was accomplished by a 15 min incubation with 1% streptomycin (w/v) in water) to avoid interference (280/260 nm ratio must be greater than 1). Afterward, the tube was centrifuged 11 000 × *g* for 10 min and the supernatant was collected. Four-samples of 1 mL each were then transferred in 15 mL tubes. A quantity of 4 mL of 2.5M HCl or 10 mM 2,4-dinitrophénylhydrazine (DNPH) in 2.5 M HCl was added to the corresponding tube. One tube served as the blank containing only and received everything except DNPH. The tubes were incubated at room temperature for 1h under dark condition and vortexed for 10 s every 15 min. The tubes were centrifuged at 11 000 × *g* for 5 min and 5 mL of 20% TCA was added. Tubes were incubated on ice for 10 min and centrifuged 11 000 × *g* for 5 min at room temperature. Pellet was then resuspended in 4 mL 10% TCA, then centrifuged as described earlier. Pellet was then washed three times with 1:1 pure ethanol : ethyl acetate to eliminate DNPH and lipid contaminants. Finally, the pellet was dissolved in 2 mL of 6 M guanidine hydrochloride and absorbance was measured in quartz cuvette. The carbonyl content is calculated from the peak absorbance (355-390 nm) using an absorption coefficient of 22,000/M against the non-treated tube. The pellets derived from the 2.5 M HCl treated samples are dissolved in 6 M guanidine hydrochloride, and the proteins are quantified by reading the absorption at 280 mm.

Malondialdehyde content in kidney

Malondialdehyde (MDA) content was measured in kidney to assess oxidative damages in lipids according to the method described by Ohkawa *et al.* (1979) with little modifications. Briefly, 0.1g of kidney was mixed in 500 µl of cold phosphate buffer, pH 7.4 50mM and homogenized. One hundred µL of the kidney sample was mixed with 0.2 mL of 8.1% (w/v) sodium dodecyl sulfate, 1.5 mL of 20% acetic acid solution pH 3.5, 0.7 mL distilled water and 1.5 mL of 0.8% aqueous thiobarbituric acid (TBA). The mixture was homogenized and incubated at 95°C for 1h. The solution was then cooled down under tap water, then 1 mL of distilled water and 5 mL of *n*-butanol was added. The mixture was shaken vigorously using a tissue homogenizer (Fisher Scientific, Ottawa, ON, Canada) for 20s and centrifugated at 3000 xg at room temperature for 10 min. The *n*-butanol layer was removed and the absorbance spectrum was extracted against the negative control. Data were analysed by third-derivative spectrophotometry against a blank reaction mixture. The derivative data was plotted against a MDA calibration curve (0-2 µg/mL).

Serum biochemical factors evaluation

Milliplex Rat adipokine Magnetic beads panel was used to quantify these analytes: IL-1β, IL-6, insulin, leptin, PAI1, TNF-α. The assay was conducted using the manufacturer's instructions. The 96-plate was read with the Bio-Plex 200 (Bio-Rad, Mississauga, Ontario, Canada).

Statistical analysis

All measurements were done in triplicate (n=3) and for each replicate, three samples were analysed. Results are presented as average ± standard deviation. Results were analyzed by one-way analysis of variance (ANOVA) using IBM SPSS statistics software (IBM Corporation, Somers, NY, USA) and differences among treatments were analyzed with a post hoc Duncan's multiple-range test. Significance was considered at $P \leq 0.05$.

RESULTS AND DISCUSSION

Antioxidant potential of human milk enriched with probiotics

In vitro free radical scavenging activity evaluation is a standard method to assess and compare the antioxidant capacities of potential functional food (Puranik *et al.,* 2018). HM enriched with 9 $\log_{10}$ CFU/mL of Bio-K+ probiotics (HM+Pb) showed an antioxidant power of 62% compared to 49% and 26% for HM and probiotics alone respectively. These results showed that probiotics significantly increased HM antioxidant capacity by 13% ($p \leq 0.05$). The 50% Scavenging capacities (SC_{50}) of HM alone and (HM+Pb) were respectively 20 mg/mL and 15 mg/mL. Results showed that probiotics were able to potentialize SC_{50} of HM by 25% (Figure 2). It has been shown that antioxidant from nutrition, particularly from human milk, can help restore balance between oxidative stress and the ability of the cells to cope with radical damages by increasing antioxidant capacities to prevent cancer, liver and cardiovascular diseases (Talegawkar *et al.*, 2009; Jonscher *et al*., 2017). Multiple probiotics have been evaluated to establish the link between probiotics and reduction of oxidative damages *in vivo* (Pellegrino, 2016). The determination of the *in vitro* antioxidant activity of the specific probiotic formulation evaluated in this study shows that intracellular antioxidant enzymes of the probiotic are presents and able to potentialize HM antioxidant activity. According to Ramalho *et al*. (2019), probiotics *in vivo* could reduce oxidative stress related diseases in multiple ways such as metal ion chelating ability, the production of metabolites or by upregulation of antioxidant mechanism or downregulation of ROS production in tissues (Ramalho *et al.*, 2019).

In vitro effect of human milk on anticancer properties

The results of HM antiproliferation properties on human colorectal tumor cells (HT-29) and murine hepatoma cells (Hepa 1c1c7) are presented in table 2. The IC_{50} of the CHO-K1 non-cancerous cell line was not significantly different to HT-29 ($p > 0.05$) but significantly lower than Hepa 1c1c7 ($p \leq 0.05$), with respective IC_{50} of 1.7 ± 0.4, 2.1 ± 0.4 and 3.9 ± 1.0 mg/mL (Table 2). This indicates that the cytotoxic effect of HM was not specific to cancer cell lines, since the growth inhibition was similar between HT-29 and CHO-K1. To our knowledge, IC_{50} in HM has never been evaluated before, but an IC_{50} of 3.1 and 5.1 mg/mL in HCT 116 and MCF-7 cell line with camel milk was evaluated by Krishnankutty *et al.* (2018). According to this study, camel milk has shown to induce autophagic death in human colorectal and breast cancer cells. Few studies have been done on immunological factors in human milk for their specific anti-tumor effect, such as Human Alpha-lactalbumin Made Lethal to Tumor cells (HAMLET) or TNF-Related Apoptosis Inducing Ligand (TRAIL). HAMLET is a protein-lipid complex that was studied in many *in vivo* studies (Håkansson *et al.*,1995; Mérino *et al.*, 2007; Mossberg *et al.*, 2010; Svanborg *et al.*, 2003).This factor was

found to specifically induce apoptosis-like death in tumor cells without affecting the fully differentiated cells (Svanborg *et al.*, 2003). This protein seems to be activated by acidic pH in infant bowel, therefore conferring protection against childhood cancer (Håkansson *et al.*, 1995). Finally, TRAIL is a cytokine involved in the balance of apoptosis and proliferation. This factor is extremely abundant in HM and colostrum, and is specific to the cells lacking the antagonistic receptor, which is the case of many types of tumours (Davanzo *et al.*, 2013; Mérino *et al.*, 2007).

To assess the mechanism behind cell death by HM, Annexin V-FITC/PI double staining was performed to determine if apoptosis is involved. Based on our results shown in Figure 3, the induction of apoptosis in colorectal cancer cells might be responsible for the cell growth inhibitory effect. HM alone was able to significantly increase the percentage of apoptotic cells as compared to the negative control (56% and 24% respectively). The combination of probiotics and HM did not provide any additional effect. Our results showed that probiotics alone didn't induce apoptosis in HT-29 cell line and didn't significantly increase the apoptosis compare to the HM alone (17% and 64% respectively). Those results suggest that HM induces cytotoxicity in HT-29 cells via apoptosis rather than necrosis, based on the analysis of externalization of phosphatidylserine on the surface of the cell membrane using Annexin V-FITC and PI double staining ($p \leq 0.05$). Moreover, the probiotics tested does not present antiproliferative of cytotoxic effect at the chosen concentration 9 Log CFU/mL.

NAD(P)H: Quinone reductase analysis

Administration of 22 mL/day of HM for 4 weeks has led to a 2 folds induction of QR activity in skeletal muscle in rats (Trinchese *et al.*, 2018). Results of QR are presented in Table 2 and show an induction of 1.7-fold in the cancerous cell line HT-29, 1.8-fold in Hepa 1c1c7 cancerous cell line and 1.9-fold in non-cancerous cell line CHO-K1 at concentrations close to their IC_{50}. NAD(P)H Quinone reductase is a detoxifying, antioxidant enzyme and has a role to prevent tumors development by stabilizing anticancer protein p53 (Oh and Park, 2015). For this reason, components able to induce a 2-fold induction of QR were screened for this ability. A calculation was made to estimate the amount of food needed to be able to provide the physiological benefits taking in consideration bioavailability, distribution and clearance rates under normal biological conditions. It has been suggested that 33,333 units (one unit would be the amount needed to induce a 2-fold induction in Hepa 1c1c7 cells growing in 150 µL wells) per day would be sufficient for a compound to be chemopreventive (Kirlin *et al.*, 1999). In this study, one unit would correspond to about 0.3 mg of HM. Thus, an amount of 100 mL (or 10g of powdered HM) per day would be needed for an adult to observe the physiological anticancer properties.

Body and organ weight

The monitoring of the rats' body weight is showed in Table 3. The rats fed with a High Fat Diet (HFD) showed a significant higher body weight compared to the control Low Fat Diet (LFD) rats ($p \leq 0.05$) after only 2 weeks, respectively 441 ± 22g and 407 ± 14g. The weight gain increased in a constant linear pattern and the weight gain difference between the two groups increased after 8 weeks (Figure 5). The growth rate in HFD was significantly higher than LFD ($p \leq 0.05$), while HM and HM+ Pb showed similar weight gain. At the end of the study, LFD, HM and HM+Pb had significantly different weight gain as compared to the HFD diet group, showing respectively, 27%, 13% and 15% less than the HFD (Table 3).

Heart, liver and kidneys weight values (% bodyweight) are shown in Table 3. The increase in liver weight during HFD is a plausible first sign of fatty liver (Wolf, 1990). Results showed that the liver weight of HM and HM + Pb showed similar values but HM+Pb showed significantly lower weight values than HFD ($p \leq 0.05$) which shows a possible contribution of probiotic bacteria in the prevention of liver malfunction. In this study, HFD was not associated with a cardiac hypertrophy as shown by the similar heart weight between all diet (Table 3).

Leptin, glucose and insulin level

Results of plasma leptin, glucose and insulin levels are presented in Table 5. Results showed that the lowest plasma leptin level corresponds to the LFD group with a value of 963 pg/mL, which is also the group with the lowest weight intake (Table 3). There is no significant difference ($p > 0.05$) between leptin levels in HFD, HM and HM+PB groups showing values from 2,629 to 3,160 pg/mL. Leptin is an adipokine secreted by adipocytes proportionally to its mass. It maintains homeostasis in fat accumulation by regulating food intake, lipolysis and energy expenses. Inflammation can cause resistance in leptin, resulting in fat accumulation (Friedman, 2011). This could indicate that the process of diet induced leptin resistance is slowed down in HM and HM+PB groups.

Non fasting glucose was measured on a weekly basis. On baseline, the glucose of HFD, HM and HM+Pb were similar ($p > 0.05$) showing respective values of 7.8, 8.1 and 7.9 mmol/L of glucose (Figure 5). Circulating glucose declined throughout the study in all groups (Figure 5). However, all glucose measurements were within the normal range established in literature between 5 and 10mmol/L (Peterson *et al.*, 2015). A significant reduction was observed from all groups between week 0 to week 2. Glucose was then stable for the rest of the study for LFD and HM groups ($p > 0.05$), while HD and HM + PB continued to decrease until the end of the study ($p \leq 0.05$) (Table 5). Similar results are observed in mice and rats fed

with HFD by Winzell and Ahren (2004) and Orhan *et al.* (2017). They also observed an increase in insulin levels as compared to the control group. These results are consistent with our observations (Table 5, Figure 5). HFD and HM+Pb presented the most significative glucose reduction at week 8 with a difference of 1.1 and 1.5mM glucose compared to the baseline. These rats also had the highest insulin levels at week 8, respectively 1650 and 1501 pg/mL. On the other hand, LFD and HM had a very stable glucose level throughout the study with a diminution of 0.5mM glucose and had lower insulin levels of respectively 1353 and 1272pg/mL (Figure 5, Table 5). To measure the impact of HM and probiotics on the management of glucose levels and the prevention of diabetes from these observations, the calculation of insulin-to-glucose ratio was use to indirectly estimates insulin resistance. A higher ratio is associated with a better insulin sensitivity (Orhan *et al.*, 2017; Winzell and Ahrén, 2004). In our study, LFD and HM had a significantly higher ratio of 5.7 while HFD had a significantly lower ratio of 4.4 ($p \leq 0.05$). HM+PB group was not significantly different than all groups (ratio of 4.7). These results suggest that the administration of HM could slow the process of insulin resistance. The administration of probiotics with the HM seems to lower its protective effect.

Inflammation and metabolic syndrome markers in serum

The inflammation and metabolic syndrome markers are presented in Table 4. Control LFD group had a very low level of pro-inflammatory cytokines IL-1B and TNF-a, respectively 0.5 and 6.3 pg/mL. However, results showed no significant difference ($p > 0.05$) between all the groups after 8 weeks (data not shown). Normally, a low-grade inflammation is often observed in obese rats, which is correlated by elevated inflammatory cytokines and adipokines in tissue and blood, such as PAI-1, IFN-g, TNF-a, IL-1B and IL-6 (Francisqueti *et al.*, 2017; Tack *et al.*, 2012; Wu, Zhang *et al.*, 2016). Our results suggest that 8 weeks was not long enough to create inflammation. It is supported by a study of Francisqueti *et al.* (2017), in which the rats fed with a high-sugar/fat diet had a diminution of TNF-α and IL-6 at 12 weeks. It was proposed that it was due to a compensatory response in adipose tissue by anti-inflammatory macrophages. After 24 weeks, the animals presented an increase in the adiposity index, hypertrophy of the adipocytes and inflammation with high TNF-α levels.

Results in Table 5 shows that PAI-1 is significantly lower in HM+PB and LFD groups (14.5 pg/mL and 16.3pg/mL) and significantly lower than HFD group (18.8 pg/mL) ($p \leq 0.05$). The presence of PAI-1 has been shown to have a causal effect on obesity and insulin resistance and is also related to every factor of metabolic syndrome (inflammation, fat accumulation, adiponectin secretion, oxidative stress) (Alessi and Juhan-Vague, 2006; Ma *et al.*, 2004). It is also associated with an increased risk of developing

cardiovascular diseases and is a good indicator of general health (Alessi and Juhan-Vague, 2006). Thus, this indicates that HM+Pb administered daily can protect against obesity-related MetS factors.

Results obtained for the paraoxonase 1 (PON1) enzyme showed that it has a higher activity in HM group, even if the activity is not significantly different ($p > 0.05$), with a value of 168 µM as compared to values from 153 to 159 µM for the other groups. PON1 is an antioxidant enzyme present in tissues and serum. Low PON1 was previously found in diet-induced obese rats and is associated with oxidative stress-associated processes such as dyslipidemia, diabetes mellitus and atherosclerosis (Noeman *et al.*, 2011; Sentí *et al.*, 2003).

Oxidative stress in Kidneys

It has been shown that HFD induced obesity in rat modify many biochemical parameters in serum and organs such as liver, kidney and heart (Noeman *et al.*, 2011). The high level of oxidative damage proteins and lipids and modify expression of protective enzymes and disrupt biochemical path by damaging receptors and transport proteins (Noeman *et al.*, 2011). Stress parameters evaluated in this study showed that both MDA and PCO level in kidney were significantly higher in HFD group (Table 6). In HM and HM+Pb groups, MDA levels were significantly ($p \leq 0.05$) lower than LFD control group. Moreover, a 66% and 75% reduction of lipid damages compared to HFD group was respectively observed. These results show that HM alone has a very strong protective effect in rat kidney that is enhanced by the consumption of a specific formulation of probiotics. MDA measurements in tissues and urine is generally used as a biomarker for free radical-induced damages and endogenous lipid peroxidation (Aluwong *et al.*, 2013). Our study corroborated with Harisa, Taha, Khalil and Salem (2009) who showed that treatment with *L. acidophilus* alone or in combination with acarbose significantly decreased MDA concentration in diabetic rats.

The evaluation of the protein damages by quantification of PCO level showed that PCO level in HM group was 88 mmol/g as compared to 138 mmol/g in HFD representing a 38% of reduction. However, these values were not significantly different ($p > 0.05$). The combination of HM+Pb was able to significantly ($p \leq 0.05$) reduce up to 97% of oxidative damages to protein compared to HFD group. When compared with LFD, a 90% reduction was observed. It is known that HM is an important source of antioxidants, which were shown to reduce oxidative damages *in vivo* in rats for pathologies such as necrotizing enterocolitis (Aceti *et al.*, 2018). It was also shown that daily administration of specific strains of probiotics could decrease lipid peroxidation in humans, particularly on low density lipoprotein and 8-isoprostanes, and provide an optimized glutathione redox ratio (Mohammadi *et al.*, 2015). Results of Lamprecht *et al.* (2012) shows that probiotic supplementation for 14 weeks decreased PCO levels among athletes. It has been shown

that some probiotics can reduce oxidative stress by protecting the epithelial barrier and prevent endotoxemia which lead to a decrease level of pro-inflammatory factors such as IFN-α, TNF-β, IL-6 and IL-17, and associated with a reduction of immune cell infiltration and inflammation in numerous organs and tissues (Festi *et al.*, 2014; Núñez *et al*., 2015).

Catalase is a detoxifying enzyme that prevent oxidative damages by degrading peroxide in the cell. It is known to be induced in obese rats and reduced in cancer cells (Glorieux and Calderon, 2017; Maciejczyk *et al.*, 2018). The result of the kidney catalase activity presented in Table 6 showed that the level was not significantly affected between groups ($p > 0.05$). These results are similar to Ejtahed *et al.* (2012), where no significant difference ($p > 0.05$) of catalase activity were found in sixty-four patients who were administered probiotic yoghurt.

MetS can lead to tissue damages in kidney due to inflammation. In the present study, despite an increase in kidney weight in HM and HM + Pb groups, drastic diminution of 66% and 75% for lipid peroxidation and 36% and 97% in protein carbonylation has been measured in these groups, respectively (Table 6). It has already been showed that probiotics having a protective effect on rat kidney may also induced a slight increase in kidney weight (Sengul *et al.,* 2019; Wanchai *et al.*, 2018). According to Sengul *et al.* (2019), probiotics has been showed to significantly attenuate CP-induced nephrotoxicity in rats by reducing kidney oxidative stress and DNA damage by reducing the MDA level in the kidneys due to the potent anti-inflammatory and antioxidant effects of probiotics (Sengul *et al.*, 2019). In that case, Probiotic group had a ratio of kidney to body weight of 0.170 ± 0.003, compared to the control group 0.150 ± 0.004. A similar effect was reported about the probiotic *Lactobacillus paracasei* HII01 by Wanchai *et al.* (2018), where they suggested that probiotic supplementation confer an anti-inflammatory effect in the obese condition, although the mechanism is not clear. It has been shown that M2 macrophages in kidney can display a tissue repair role as well as anti-inflammatory effect (Guiteras, Flaquer and Cruzado, 2016). Further analysis would be needed to determine if the slight increase in kidney weight could be due to anti-inflammatory cell recruitment in the kidney creating a protective environment against MetS related oxidative stress.

CONCLUSION

This study investigated the potential of a new functional food made of HM enriched with a specific formulation of probiotics to prevent obesity related diseases and cancer. It was demonstrated *in vitro* potential anticancer properties by induction of apoptosis, protective enzyme NAD(P)H quinone reductase and antioxidant capacity. Enrichment of HM with that specific probiotic formulation also provided additional antioxidant properties. An 8 weeks study on rats fed with HFD was conducted to assess anti-

inflammatory and antioxidant properties of this functional food. The HFD group showed early factors of MetS after 8 weeks such as higher body weight and oxidative damages in kidney compared to the LFD group. Rats fed with a HFD supplemented with a daily intake of HM and probiotics weighed less at the end of the study and showed a reduction of lipid and protein oxidative damages compared to the control HFD group. Liver and heart to body weight ratio were also similar to LFD control group, although kidney weight was significantly higher. Rats who ingested HM had stable glucose levels while rats with HM+Pb had their values decrease throughout the study. The calculation of the insulin / glucose ratio suggested that HM could slow the process of insulin resistance compared to rats fed a HFD. On the other hand, the administration of probiotics seems to have little effect. A diminution in the serum markers of inflammation was not observed (TNF-a, IL-1B, PON1), apart from PAI-1 serum level, which can indicate a possible protective role against MetS. In summary, the study shows that the administration of HM and a specific formulation of probiotics as a functional food could reduce the weight gain, oxidative stress and inflammatory processes known to be associated with MetS.

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Table 1. Nutrient information on selected diets

Nutrient	% (g/g) Low fat diet (17% kcal from fat)	% (g/g) High fat diet (45% kcal from fat)
Protein	18.6	17.3
Carbohydrate	50.6	47.6
Fat	6.2	23.2
kcal/g	3.3	4.7

Table 2. Effect of human milk on the cellular proliferation of different cell lines and QR induction.

	HT-29	Hepa 1c1c7	CHO-K1
IC_{50} (mg/mL)	2.1 ± 0.4a	3.9 ± 1.0b	1.7 ± 0.4a
QR induction	1.7 ± 0.4a	1.8 ± 0.2a	1.9 ± 0.3a

Data within the same row with different letters are significantly different ($p \leq 0.05$). IC50: Concentration that inhibits 50% of the cellular growth. QR induction values are presented as the optimal concentration for each cell line (HT-29: 2mg/mL; Hepa 1c1c7: 3mg/mL; CHO-K1: 2mg/mL). Results are presented as average ± standard deviation of at least 3 independent experiments.

Table 3. Body weight gain and organ weight of rats on different diets for eight weeks.

	LFD	HFD	HM	HM+Pb
Bw gain (g)	322 ± 38a	441 ± 13b	382 ± 55c	376±43c
Weight gain rate (g/week)	39.0 ± 6.0a R^2 = 0.9860	50.2 ± 3.8b R^2 = 0.9914	45.9 ± 5.3ab R^2 = 0.9940	45.9 ± 3.9ab R^2 = 0.9837
Kidneys (% bw)	0.53 ± 0.08a	0.52 ± 0.05a	0.56 ± 0.08ab	0.63 ± 0.07b
Liver (% bw)	3.76 ± 0.63ab	4.04 ± 0.50a	3.71 ± 0.40ab	3.33 ± 0.63b
Heart (% bw)	0.29 ± 0.05a	0.29 ± 0.05a	0.31 ± 0.05a	0.32 ± 0.03a

The sample size was n = 6-7 per group. Means followed with different letters in the same row are significantly different ($P \leq 0.05$) (One-way Anova; Post-Hoc Duncan). BW, Body weight; LFD, Low fat diet; HFD, high-fat diet; HM, Human milk; Pb, Probiotics 9.5 Log CFU/day.

Table 4. Serum parameters after 8 weeks on different diets

	Control LFD	Control HFD	Human milk	Human milk enriched with probiotics
PAI-1 (pg/mL)	16.3 ± 3.2ab	18.8 ± 4.2a	17.34 ± 4.3ab	14.5 ± 2.6b
PON1 (μM/L)	159 ± 48a	159 ± 38a	168 ± 38a	153 ± 41a

Results (mean ± SD) followed with different letters in the same row are significantly different ($P \leq 0.05$) (One-way Anova; Post-Hoc Duncan). LFD, low fat diet; HFD, high-fat diet; Human milk enriched with 9.5 Log CFU/day; PAI-1, plasminogen activator inhibitor-1; PON1, Paraoxonase.

Table 5. Serum parameters for all groups

	LFD	HFD	HM	HM+Pb
Leptin (Week 8) (pg/mL)	963 ± 432a	2692 ± 867b	3160 ± 909b	2629 ± 802b
Insulin (Week 8) (pg/mL)	1353 ± 243a	1650 ± 319b	1272 ± 118a	1501 ± 256ab
Glucose (Week 8) (mmol/L)	6.7 ± 0.4ab	7.0 ± 0.3b	7.2 ± 0.3b	6.3 ± 0.4a
Ratio glucose/insulin (mmol/mL)/ (pg/mL)	5.7 ± 0.8a	4.4 ± 1.1b	5.7 ± 0.5a	4.7 ± 1.2ab

Results (mean ± SD) in the same row followed with different letters are significantly different ($P \leq 0.05$) One-way Anova; Post-Hoc Duncan). LFD, Low fat diet; HFD, high-fat diet; HM, Human milk; Pb, Probiotics 9.5 Log CFU/day.

Table 6. Levels of oxidative stress parameters (MDA, PCO and catalase) for all groups in the kidney tissue after eight weeks on different diet.

Biochemical marker in kidney	Control LFD	Control HFD	Human milk	Human milk enriched with probiotics
Catalase activity (U/wet weight)	926 ± 134a	842 ± 156a	881 ± 205a	848 ± 236a
MDA (ppm)	0.504 ± 0.067a	0.671 ± 0.153b	0.228 ± 0.168c	0.166 ± 0.156c
PCO (nmol/wet weight)	42 ± 30a	138 ± 33b	88 ± 30b	4 ± 6c

Means in the same row followed with different letters are significantly different ($P \leq 0.05$), and the results are expressed as mean ± SD (One-way Anova; Post-Hoc Duncan). BW, Body weight; LFD, Low fat diet; HFD, high-fat diet; HM, Human milk; Pb, Probiotics 9.5 Log CFU/day; MDA, malondialdehyde; PCO, Protein Carbonyl.

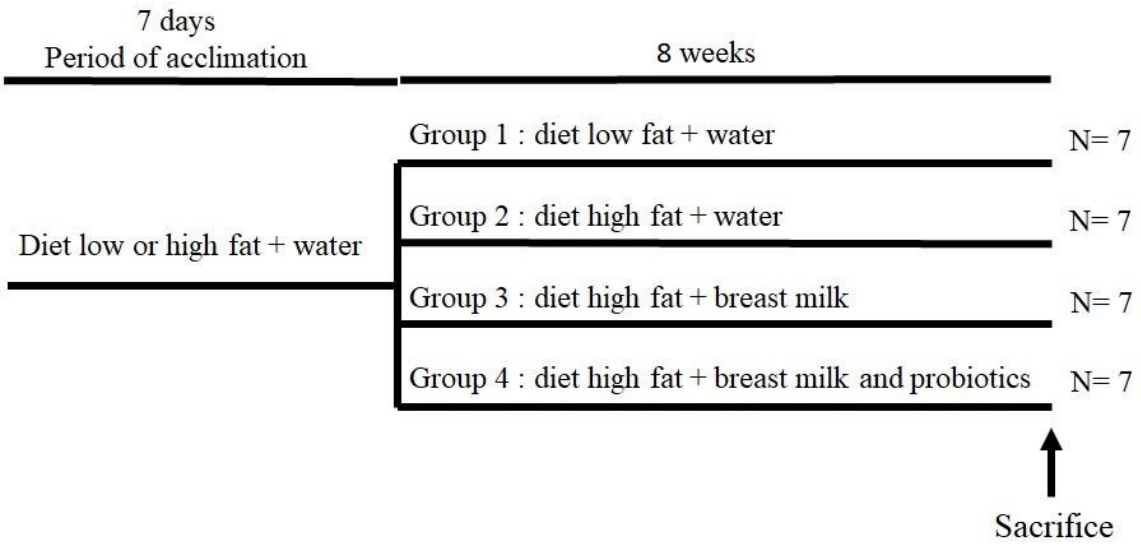

Figure 1. Representation of the experimental design.

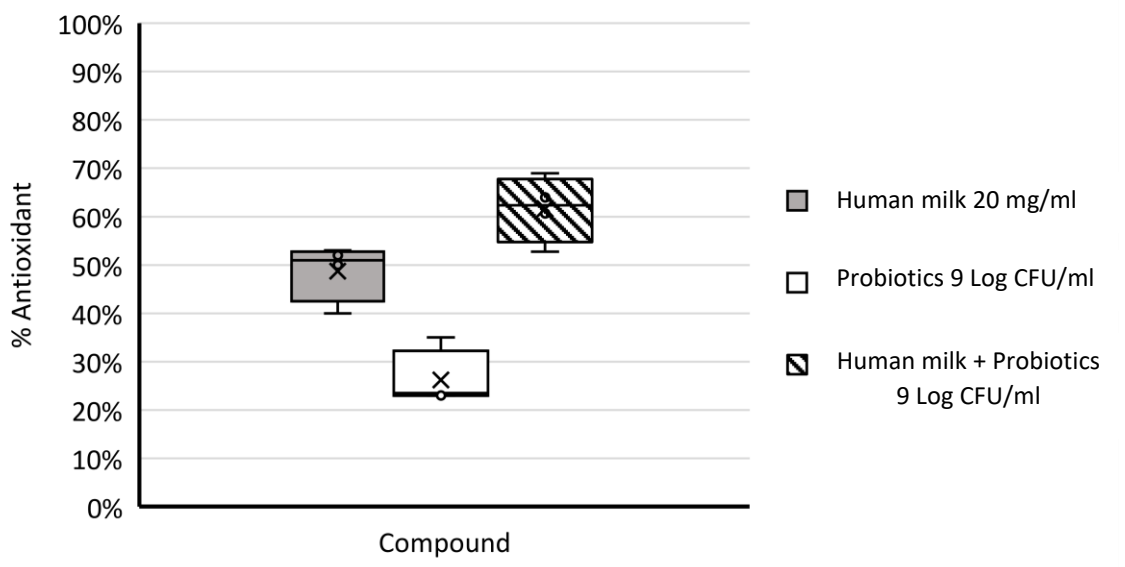

Figure 2. DPPH free radical scavenging activity of human milk 20mg/mL, Probiotics 9 Log CFU/mL and human milk 20 mg/mL enriched with probiotics 9 Log CFU/mL.

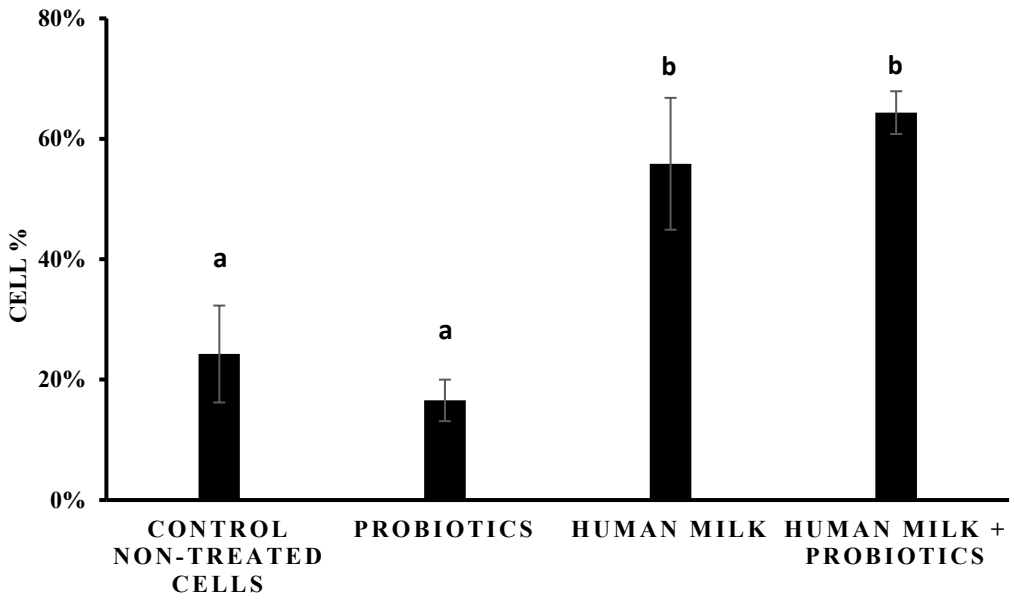

Figure 3. Effect of human milk on the percentage of apoptotic cells (Annexin V+ PI- cells) in human colorectal cancer HT-29 cells determined by flow cytometry using Annexin V-FITC and PI double staining method after 48 h of treatment in media. Water was added to the media for the negative control. Columns with different letters are significantly different ($p \leq 0.05$). Cell % represents the proportion of cells Annexin V positive and PI negative to staining out od 20 000 events.

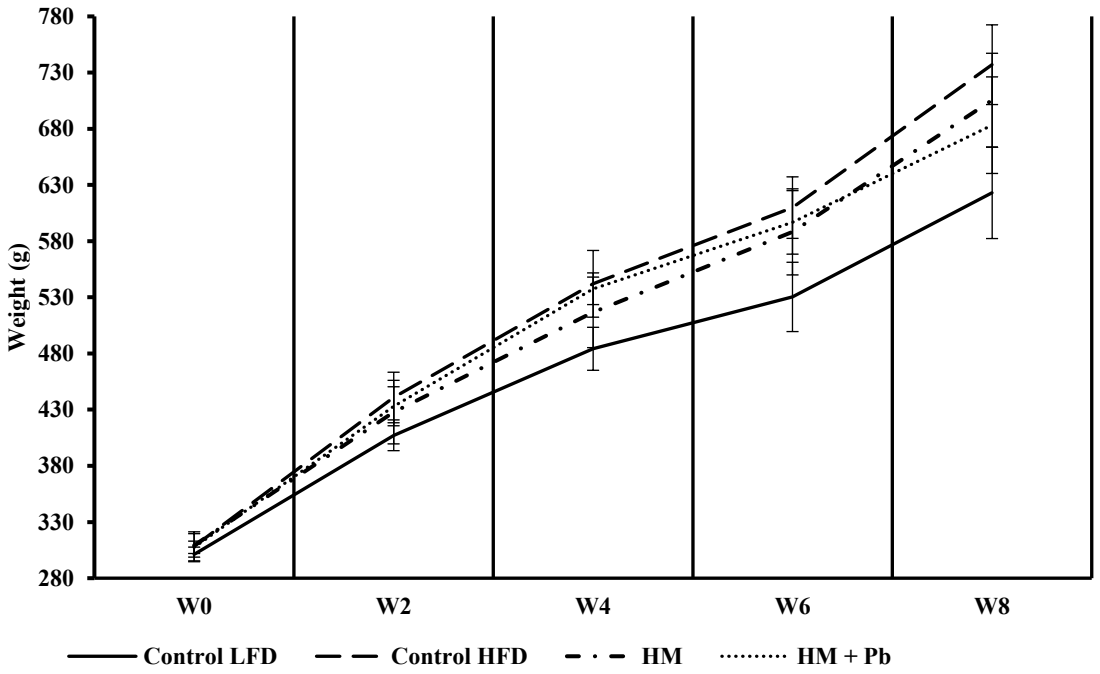

Figure 4. Cumulative weight gain of rats on different diets for eight weeks. Error bars indicate standard deviation. The sample size was n = 5-7 per group. Different letters indicate difference between groups ($p < 0.05$) (One-way Anova; Post-Hoc Duncan). LFD, Low fat diet; HFD, high-fat diet; HM, Human milk; Pb, Probiotics 9.5 Log CFU/day.

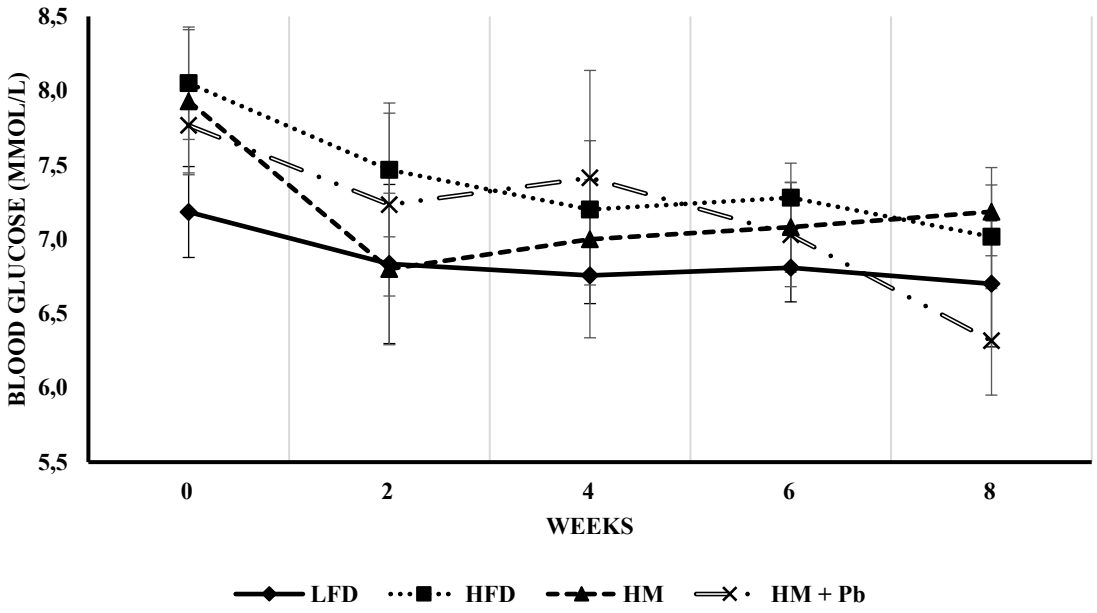

Figure 5. Non-fasting glucose level of rats on different diets for eight weeks. The sample size was n = 6-7 per group. LFD, Low fat diet; HFD, high-fat diet; HM, Human milk; Pb, Probiotics 9.5 Log CFU/day.

BIBLIOGRAPHY

Aceti, A., Beghetti, I., Martini, S., Faldella, G. and Corvaglia, L. (2018). Oxidative stress and necrotizing enterocolitis: pathogenetic mechanisms, opportunities for intervention, and role of human milk. *Oxidative medicine and cellular longevity*, *2018*, 7397659. doi:10.1155/2018/7397659

Aggarwal, B. B. (2010). Targeting inflammation-induced obesity and metabolic diseases by curcumin and other nutraceuticals. *Annual review of nutrition*, *30*, 173-199. doi:10.1146/annurev.nutr.012809.104755

Agostoni, C., Baselli, L. and Mazzoni, M. B. (2013). Early nutrition patterns and diseases of adulthood: a plausible link? *European Journal of Internal Medicine*, *24*(1), 5-10. doi:10.1016/j.ejim.2012.08.011

Alessi, M.-C. and Juhan-Vague, I. (2006). PAI-1 and the metabolic syndrome: links, causes, and consequences. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *26*(10), 2200-2207. doi:10.1161/01.ATV.0000242905.41404.68

Altunkaynak, M. E., Ozbek, E., Altunkaynak, B. Z., Can, I., Unal, D. and Unal, B. (2008). The effects of high-fat diet on the renal structure and morphometric parametric of kidneys in rats. *Journal of Anatomy*, *212*(6), 845-852. doi:10.1111/j.1469-7580.2008.00902.x

Aluwong, T., Kawu, M., Raji, M., Dzenda, T., Govwang, F., Sinkalu, V. and Ayo, J. (2013). Effect of yeast probiotic on growth, antioxidant enzyme activities and malondialdehyde concentration of broiler chickens. *Antioxidants (Basel, Switzerland)*, *2*(4), 326-339. doi:10.3390/antiox2040326

Antioxidant found in breast milk prevents liver disease, study finds. (s.d.). Repéré November 23, 2019, à https://www.medicalnewstoday.com/articles/315073.php

Ballard, O. and Morrow, A. L. (2013). Human milk composition: nutrients and bioactive factors. *Pediatric Clinics of North America*, *60*(1), 49-74. doi:10.1016/j.pcl.2012.10.002

Bergmann, H., Rodríguez, J. M., Salminen, S. and Szajewska, H. (2014). Probiotics in human milk and probiotic supplementation in infant nutrition: a workshop report. *The British Journal of Nutrition*, *112*(7), 1119-1128. doi:10.1017/S0007114514001949

Bode, L. (2009). Human milk oligosaccharides: prebiotics and beyond. *Nutrition Reviews*, *67 Suppl 2*, S183-91. doi:10.1111/j.1753-4887.2009.00239.x

Çelik, M. N. and Ünlü Söğüt, M. (2019). Probiotics improve chemerin levels and metabolic syndrome parameters in obese rats. *Balkan medical journal*, *36*(5), 270-275. doi:10.4274/balkanmedj.galenos.2019.2019.2.61

Chen, C., Zhao, G., Chen, W. and Guo, B. (2015). Metabolism of Fructooligosaccharides in Lactobacillus plantarum ST-III via Differential Gene Transcription and Alteration of Cell Membrane Fluidity. *Applied and Environmental Microbiology*, *81*(22), 7697-7707. doi:10.1128/AEM.02426-15

Cherniack, E. P. (2011). Polyphenols: planting the seeds of treatment for the metabolic syndrome. *Nutrition*, *27*(6), 617-623. doi:10.1016/j.nut.2010.10.013

Chung, A. P. Y. S., Gurtu, S., Chakravarthi, S., Moorthy, M. and Palanisamy, U. D. (2018). Geraniin Protects High-Fat Diet-Induced Oxidative Stress in Sprague Dawley Rats. *Frontiers in nutrition*, *5*, 17. doi:10.3389/fnut.2018.00017

Davanzo, R., Zauli, G., Monasta, L., Vecchi Brumatti, L., Abate, M. V., Ventura, G., Demarini, S. (2013). Human colostrum and breast milk contain high levels of TNF-related apoptosis-inducing ligand (TRAIL). *Journal of Human Lactation*, *29*(1), 23-25. doi:10.1177/0890334412441071

Dennis, J. M. and Witting, P. K. (2017). Protective role for antioxidants in acute kidney disease. *Nutrients*, *9*(7). doi:10.3390/nu9070718

Ejtahed, H. S., Mohtadi-Nia, J., Homayouni-Rad, A., Niafar, M., Asghari-Jafarabadi, M. and Mofid, V. (2012). Probiotic yogurt improves antioxidant status in type 2 diabetic patients. *Nutrition*, *28*(5), 539-543. doi:10.1016/j.nut.2011.08.013

Esposito, K., Chiodini, P., Colao, A., Lenzi, A. and Giugliano, D. (2012). Metabolic syndrome and risk of cancer: a systematic review and meta-analysis. *Diabetes Care*, *35*(11), 2402-2411. doi:10.2337/dc12-0336

Festi, D., Schiumerini, R., Eusebi, L. H., Marasco, G., Taddia, M. and Colecchia, A. (2014). Gut microbiota and metabolic syndrome. *World Journal of Gastroenterology*, *20*(43), 16079-16094. doi:10.3748/wjg.v20.i43.16079

Fortin, O., Aguilar-Uscanga, B., Vu, K. D., Salmieri, S., Zhao, J.-C. and Lacroix, M. (2017). Chemo-preventive Properties of Essential Oils Obtained from Boswellia carterii and B. sacra in Combination with Insoluble Î²-Glucan. *Journal of Nutraceuticals and Food Science*.

Francisqueti, F. V., Nascimento, A. F., Minatel, I. O., Dias, M. C., Luvizotto, R. de A. M., Berchieri-Ronchi, C., Corrêa, C. R. (2017). Metabolic syndrome and inflammation in adipose tissue occur at different times in animals submitted to a high-sugar/fat diet. *Journal of nutritional science*, *6*, e41. doi:10.1017/jns.2017.42

Friedman, J. M. (2011). Leptin and the regulation of body weigh. *The Keio Journal of Medicine*, *60*(1), 1-9.

Glorieux, C. and Calderon, P. B. (2017). Catalase, a remarkable enzyme: targeting the oldest antioxidant enzyme to find a new cancer treatment approach. *Biological Chemistry*, *398*(10), 1095-1108. doi:10.1515/hsz-2017-0131

Guiteras, R., Flaquer, M. and Cruzado, J. M. (2016). Macrophage in chronic kidney disease. *Clinical kidney journal*, *9*(6), 765-771. doi:10.1093/ckj/sfw096

Håkansson, A., Zhivotovsky, B., Orrenius, S., Sabharwal, H. and Svanborg, C. (1995). Apoptosis induced by a human milk protein. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(17), 8064-8068. doi:10.1073/pnas.92.17.8064

Harisa, G. I., Taha, E. I., Khalil, A. F. and Salem, M. M. (2009). Oral administration of *Lactobacillus acidophilus* restores nitric oxide level in diabetic rats. *Australian Journal of Basic and Applied Sciences*, 3(3): 2963-2969

Hasler, C. M. (2002). The cardiovascular effects of soy products. *The Journal of Cardiovascular Nursing*, *16*(4), 50-63; quiz 75.

Henao-Mejia, J., Elinav, E., Jin, C., Hao, L., Mehal, W. Z., Strowig, T., Flavell, R. A. (2012). Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature*, *482*(7384), 179-185. doi:10.1038/nature10809

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Sanders, M. E. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews. Gastroenterology & Hepatology*, *11*(8), 506-514. doi:10.1038/nrgastro.2014.66

Hoyos, A. B. (1999). Reduced incidence of necrotizing enterocolitis associated with enteral administration of Lactobacillus acidophilus and Bifidobacterium infantis to neonates in an intensive care unit. *International Journal of Infectious Diseases*, *3*(4), 197-202.

I. Puranik, S., B. Hiremat, M., B. Nerli, R. and C. Ghagane, S. (2018). Evaluation of *in vitro* Antioxidant and Anticancer Activity of *Tabernaemontana divaricata* Leaf Extracts Against T-24 Human Bladder Cancer Cell Lines. *International journal of cancer research*, *14*(2), 100-108. doi:10.3923/ijcr.2018.100.108

Kassaian, N., Feizi, A., Aminorroaya, A. and Amini, M. (2018). Probiotic and synbiotic supplementation could improve metabolic syndrome in prediabetic adults: A randomized controlled trial. *Diabetes & metabolic syndrome*. doi:10.1016/j.dsx.2018.07.016

Kazemi, A., Soltani, S., Ghorabi, S., Keshtkar, A., Daneshzad, E., Nasri, F. and Mazloomi, S. M. (2019). Effect of probiotic and synbiotic supplementation on inflammatory markers in health and disease status: A systematic review and meta-analysis of clinical trials. *Clinical Nutrition*. doi:10.1016/j.clnu.2019.04.004

Kedare, S. B. and Singh, R. P. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of food science and technology*, *48*(4), 412-422. doi:10.1007/s13197-011-0251-1

Kirlin, W. G., Cai, J., DeLong, M. J., Patten, E. J. and Jones, D. P. (1999). Dietary compounds that induce cancer preventive phase 2 enzymes activate apoptosis at comparable doses in HT29 colon carcinoma cells. *The Journal of Nutrition*, *129*(10), 1827-1835. doi:10.1093/jn/129.10.1827

Krishnankutty, R., Iskandarani, A., Therachiyil, L., Uddin, S., Azizi, F., Kulinski, M., Mohammad, R. M. (2018). Anticancer activity of camel milk via induction of autophagic death in human colorectal and breast cancer cells. *Asian Pacific Journal of Cancer Prevention*, *19*(12), 3501-3509. doi:10.31557/APJCP.2018.19.12.3501

Lalan, S., Jiang, S., Ng, D. K., Kupferman, F., Warady, B. A., Furth, S. and Mitsnefes, M. M. (2018). Cardiometabolic risk factors, metabolic syndrome, and chronic kidney disease progression in children. *The Journal of Pediatrics*, *202*, 163-170. doi:10.1016/j.jpeds.2018.06.007

Lamprecht, M., Bogner, S., Schippinger, G., Steinbauer, K., Fankhauser, F., Hallstroem, S., Greilberger, J. F. (2012). Probiotic supplementation affects markers of intestinal barrier, oxidation, and inflammation in trained men; a randomized, double-blinded, placebo-controlled trial. *Journal of the International Society of Sports Nutrition*, *9*(1), 45. doi:10.1186/1550-2783-9-45

Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A.-G., Stadtman, E. R. (1990). [49] Determination of carbonyl content in oxidatively modified proteins. In *Oxygen radicals in biological systems part B: oxygen radicals and antioxidants* (vol. 186, pp. 464-478). Elsevier. doi:10.1016/0076-6879(90)86141-H

Ma, L.-J., Mao, S.-L., Taylor, K. L., Kanjanabuch, T., Guan, Y., Zhang, Y., Fogo, A. B. (2004). Prevention of obesity and insulin resistance in mice lacking plasminogen activator inhibitor 1. *Diabetes*, *53*(2), 336-346. doi:10.2337/diabetes.53.2.336

Maciejczyk, M., Żebrowska, E., Zalewska, A. and Chabowski, A. (2018). Redox Balance, Antioxidant Defense, and Oxidative Damage in the Hypothalamus and Cerebral Cortex of Rats with High Fat Diet-Induced Insulin Resistance. *Oxidative medicine and cellular longevity*, *2018*, 6940515. doi:10.1155/2018/6940515

Mallappa, R. H., Rokana, N., Duary, R. K., Panwar, H., Batish, V. K. and Grover, S. (2012). Management of metabolic syndrome through probiotic and prebiotic interventions. *Indian journal of endocrinology and metabolism*, *16*(1), 20-27. doi:10.4103/2230-8210.91178

Marcuzzi, A., Vecchi Brumatti, L., Caruso, L., Copertino, M., Davanzo, R., Radillo, O., Monasta, L. (2013). Presence of IL-9 in paired samples of human colostrum and transitional milk. *Journal of Human Lactation*, *29*(1), 26-31. doi:10.1177/0890334412466958

Marseglia, L., Manti, S., D'Angelo, G., Nicotera, A., Parisi, E., Di Rosa, G., Arrigo, T. (2014). Oxidative stress in obesity: a critical component in human diseases. *International Journal of Molecular Sciences*, *16*(1), 378-400. doi:10.3390/ijms16010378

Mérino, D., Lalaoui, N., Morizot, A., Solary, E. and Micheau, O. (2007). TRAIL in cancer therapy: present and future challenges. *Expert Opinion on Therapeutic Targets*, *11*(10), 1299-1314. doi:10.1517/14728222.11.10.1299

Moghadam, A. R., Tutunchi, S., Namvaran-Abbas-Abad, A., Yazdi, M., Bonyadi, F., Mohajeri, D., Ghavami, S. (2015). Pre-administration of turmeric prevents methotrexate-induced liver toxicity and oxidative stress. *BMC complementary and alternative medicine*, *15*, 246. doi:10.1186/s12906-015-0773-6

Mohammadi, A. A., Jazayeri, S., Khosravi-Darani, K., Solati, Z., Mohammadpour, N., Asemi, Z., Eghtesadi, S. (2015). Effects of Probiotics on Biomarkers of Oxidative Stress and Inflammatory Factors in Petrochemical Workers: A Randomized, Double-blind, Placebo-controlled Trial. *International journal of preventive medicine*, *6*, 82. doi:10.4103/2008-7802.164146

Mossberg, A.-K., Puchades, M., Halskau, Ø., Baumann, A., Lanekoff, I., Chao, Y., Karlsson, R. (2010). HAMLET interacts with lipid membranes and perturbs their structure and integrity. *Plos One*, *5*(2), e9384. doi:10.1371/journal.pone.0009384

Newburg, D. S. and Morelli, L. (2015). Human milk and infant intestinal mucosal glycans guide succession of the neonatal intestinal microbiota. *Pediatric Research*, *77*(1-2), 115-120. doi:10.1038/pr.2014.178

Noeman, S. A., Hamooda, H. E. and Baalash, A. A. (2011). Biochemical study of oxidative stress markers in the liver, kidney and heart of high fat diet induced obesity in rats. *Diabetology & metabolic syndrome*, *3*(1), 17. doi:10.1186/1758-5996-3-17

Novotny Núñez, I., Maldonado Galdeano, C., de Moreno de LeBlanc, A. and Perdigón, G. (2015). Lactobacillus casei CRL 431 administration decreases inflammatory cytokines in a diet-induced obese mouse model. *Nutrition*, *31*(7-8), 1000-1007. doi:10.1016/j.nut.2015.02.006

Ogunsile, F. J., Bediako, S. M., Nelson, J., Cichowitz, C., Yu, T., Patrick Carroll, C., Lanzkron, S. (2019). Metabolic syndrome among adults living with sickle cell disease. *Blood Cells, Molecules & Diseases*, *74*, 25-29. doi:10.1016/j.bcmd.2018.10.005

Oh, E.-T. and Park, H. J. (2015). Implications of NQO1 in cancer therapy. *BMB Reports*, *48*(11), 609-617. doi:10.5483/bmbrep.2015.48.11.190

Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, *95*(2), 351–358. doi:10.1016/0003-2697(79)90738-3

Orhan, C., Şahin, N., Tuzcu, Z., Komorowski, J. R. and Şahin, K. (2017). Combined oral supplementation of chromium picolinate, docosahexaenoic acid, and boron enhances neuroprotection in rats fed a high-fat diet. *Turkish journal of medical sciences*, *47*(5), 1616-1625. doi:10.3906/sag-1701-54

Pellegrino, D. (2016). Antioxidants and cardiovascular risk factors. *Diseases (Basel, Switzerland)*, *4*(1). doi:10.3390/diseases4010011

Peterson, R. G., Jackson, C. V., Zimmerman, K., de Winter, W., Huebert, N. and Hansen, M. K. (2015). Characterization of the ZDSD rat: A translational model for the study of metabolic syndrome and type 2 diabetes. *Journal of diabetes research*, *2015*, 487816. doi:10.1155/2015/487816

Principi, N., Cozzali, R., Farinelli, E., Brusaferro, A. and Esposito, S. (2018). Gut dysbiosis and irritable bowel syndrome: The potential role of probiotics. *The Journal of Infection*, *76*(2), 111-120. doi:10.1016/j.jinf.2017.12.013

Ramalho, J. B., Soares, M. B., Spiazzi, C. C., Bicca, D. F., Soares, V. M., Pereira, J. G., Cibin, F. W. S. (2019). In Vitro Probiotic and Antioxidant Potential of Lactococcus lactis subsp. cremoris LL95 and Its Effect in Mice Behaviour. *Nutrients*, *11*(4). doi:10.3390/nu11040901

Repa, A., Thanhaeuser, M., Endress, D., Weber, M., Kreissl, A., Binder, C., Haiden, N. (2015). Probiotics (Lactobacillus acidophilus and Bifidobacterium infantis) prevent NEC in VLBW infants fed breast milk but not formula [corrected]. *Pediatric Research*, *77*(2), 381-388. doi:10.1038/pr.2014.192

Richter, R. J. and Furlong, C. E. (1999). Determination of paraoxonase (PON1) status requires more than genotyping. *Pharmacogenetics*, *9*(6), 745-753.

Saklayen, M. G. (2018). The global epidemic of the metabolic syndrome. *Current Hypertension Reports*, *20*(2), 12. doi:10.1007/s11906-018-0812-z

Seki, R. P., Hendrie, D. and Daire, J. (2019). Evaluation of policies for free maternal healthcare in low/middle-income countries: a scoping review protocol. *BMJ Open*, *9*(8), e031557. doi:10.1136/bmjopen-2019-031557

Sengul, E., Gelen, S. U., Yıldırım, S., Çelebi, F. and Çınar, A. (2019). Probiotic bacteria attenuates cisplatin-induced nephrotoxicity through modulation of oxidative stress, inflammation and apoptosis in rats. *Asian Pacific Journal of Tropical Biomedicine*.

Sentí, M., Tomás, M., Fitó, M., Weinbrenner, T., Covas, M.-I., Sala, J., Marrugat, J. (2003). Antioxidant paraoxonase 1 activity in the metabolic syndrome. *The Journal of Clinical Endocrinology and Metabolism*, *88*(11), 5422-5426. doi:10.1210/jc.2003-030648

Sun, J. and Buys, N. J. (2016). Glucose- and glycaemic factor-lowering effects of probiotics on diabetes: a meta-analysis of randomised placebo-controlled trials. *The British Journal of Nutrition*, *115*(7), 1167-1177. doi:10.1017/S0007114516000076

Svanborg, C., Agerstam, H., Aronson, A., Bjerkvig, R., Düringer, C., Fischer, W., Svensson, M. (2003). HAMLET kills tumor cells by an apoptosis-like mechanism--cellular, molecular, and therapeutic aspects. *Advances in cancer research*, *88*, 1-29.

Tack, C. J., Stienstra, R., Joosten, L. A. B. and Netea, M. G. (2012). Inflammation links excess fat to insulin resistance: the role of the interleukin-1 family. *Immunological Reviews*, *249*(1), 239-252. doi:10.1111/j.1600-065X.2012.01145.x

Talegawkar, S. A., Beretta, G., Yeum, K.-J., Johnson, E. J., Carithers, T. C., Taylor, H. A., Tucker, K. L. (2009). Total antioxidant performance is associated with diet and serum antioxidants in participants of the diet and physical activity substudy of the Jackson Heart Study. *The Journal of Nutrition*, *139*(10), 1964-1971. doi:10.3945/jn.109.107870

Thongaram, T., Hoeflinger, J. L., Chow, J. and Miller, M. J. (2017). Human milk oligosaccharide consumption by probiotic and human-associated bifidobacteria and lactobacilli. *Journal of Dairy Science*, *100*(10), 7825-7833. doi:10.3168/jds.2017-12753

Trinchese, G., Cavaliere, G., De Filippo, C., Aceto, S., Prisco, M., Chun, J. T., Mollica, M. P. (2018). Human milk and donkey milk, compared to cow milk, reduce inflammatory mediators and modulate glucose and lipid metabolism, acting on mitochondrial function and oleylethanolamide levels in rat skeletal muscle. *Frontiers in physiology*, *9*, 32. doi:10.3389/fphys.2018.00032

Vistica, D. T., Skehan, P., Scudiero, D., Monks, A., Pittman, A. and Boyd, M. R. (1991). Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production. *Cancer Research*, *51*(10), 2515-2520.

Walker, W. A. and Iyengar, R. S. (2015). Breast milk, microbiota, and intestinal immune homeostasis. *Pediatric Research*, *77*(1-2), 220-228. doi:10.1038/pr.2014.160

Wall, R., Ross, R. P., Ryan, C. A., Hussey, S., Murphy, B., Fitzgerald, G. F. and Stanton, C. (2009). Role of gut microbiota in early infant development. *Clinical medicine. Pediatrics*, *3*, 45-54.

Wanchai, K., Yasom, S., Tunapong, W., Chunchai, T., Eaimworawuthikul, S., Thiennimitr, P., Lungkaphin, A. (2018). Probiotic Lactobacillus paracasei HII01 protects rats against obese-insulin resistance-induced kidney injury and impaired renal organic anion transporter 3 function. *Clinical Science*, *132*(14), 1545-1563. doi:10.1042/CS20180148

Welty, F. K., Alfaddagh, A. and Elajami, T. K. (2016). Targeting inflammation in metabolic syndrome. *Translational Research*, *167*(1), 257-280. doi:10.1016/j.trsl.2015.06.017

Winzell, M. S. and Ahrén, B. (2004). The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. *Diabetes*, *53 Suppl 3*, S215-9. doi:10.2337/diabetes.53.suppl_3.s215

Wolf, D. C. (1990). Evaluation of the size, shape, and consistency of the liver. In H. K. Walker, W. D. Hall and J. W. Hurst (dir.), *Clinical methods: the history, physical, and laboratory examinations* (3rd ed.). Boston: Butterworths.

Wu, P., Zhang, F., Dai, Y., Han, L. and Chen, S. (2016). Serum TNF-α, GTH and MDA of high-fat diet-induced obesity and obesity resistant rats. *Saudi pharmaceutical journal : SPJ : the official publication of the Saudi Pharmaceutical Society*, *24*(3), 333-336. doi:10.1016/j.jsps.2016.04.011

CHAPITRE 6 : DISCUSSION ET CONCLUSION GÉNÉRALE

6.1 Discussion

Les mauvaises habitudes alimentaires et la sédentarité font des maladies chroniques et métaboliques le fléau du 21e siècle. Les aliments fonctionnels sont une alternative envisagée afin de promouvoir la santé de la population, particulièrement chez les personnes ayant un système immunitaire affaibli. L'objectif de cette étude est de développer et produire un aliment fonctionnel à base de lait maternel et de probiotiques. Pour ce faire, le premier objectif était d'évaluer l'effet de l'irradiation gamma comme méthode de décontamination pour l'utilisation sécuritaire du lait maternel chez des personne ayant un système immunitaire compromis. Le second objectif était d'évaluer *in vivo* et *in vitro* les propriétés antioxydantes et anticancer du lait maternel atomisé enrichi de probiotiques, ainsi que d'évaluer ses capacités *in vivo* potentielles à prévenir les facteurs de risque du syndrome métabolique.

6.1.1 Irradiation du lait maternel et du lait maternisé

Effet sur la qualité microbiologique

L'étude de l'irradiation sur la préparation pour nourrissons et le lait maternel a permis de démontrer la radiosensibilisation selon la forme physique (poudre ou liquide congelé) sur différents pathogènes alimentaires. En effet, l'irradiation gamma présente l'avantage de pouvoir passer à travers les emballages sans en modifier son efficacité. De plus, elle est applicable sur les produits solides, liquides, frais, congelés, sec, en poudre, etc. Pour notre étude, cela importe car le lait maternel pourrait être décontaminé avant ou après l'atomisation de ce dernier. En effet, la nature de chaque matrice alimentaire peut grandement influencer les doses d'irradiation requises pour obtenir le niveau de décontamination voulu. Particulièrement, la disponibilité en eau et la température d'irradiation peuvent grandement influencer l'efficacité de l'irradiation (Ben Miloud Yahia et al., 2018). Les tests préliminaires réalisés sur la préparation pour nourrisson suggèrent que certaines bactéries pathogènes d'origines alimentaires seraient plus susceptibles lorsqu'irradiées sous forme congelée, particulièrement *E. coli*, et *B. cereus.* De plus, *S.* Typhimurium et *L. monocytogenes* qui ne semblaient pas être affectés par l'état du lait alors que *S. aureus* était plus facilement inactivé dans le lait liquide congelé. Les résultats ont montré qu'une dose de 4.2 kGy permettait de diminuer d'au moins 6 Log d'UFC/mL toutes les bactéries pathogènes alimentaires testées nommées ci-haut, à l'exception de l'espèce sporulante *B. cereus*. Les spores étant beaucoup plus résistantes aux traitements de stérilisation et de décontamination. La présence de ces spores est un problème notable dans les banques de lait maternel et dans les industries de production de lait maternisé puisqu'elles résistent

plus fortement au traitement à la chaleur (Cacho et al., 2017; Froh, Vanderpool, & Spatz, 2018; Rigourd et al., 2018). L'irradiation gamma de la bactérie sporulante *B. cereus* dans la préparation pour nourrissons a été réalisée dans le lait congelé et en poudre. Les résultats d'irradiation ont montré que la dose est grandement dépendante du nombre de spores et du niveau de contamination. Ainsi, la dose nécessaire pour assurer une stérilité et la destruction de 6 Log UFC/mL de spores serait de 37 kGy pour la formulation en poudre et de 15 kGy pour l'irradiation du lait liquide congelé. En présence d'un mélange de spores et de bactéries végétatives, les résultats obtenus montrent que respectivement 15 kGy et 6 kGy serait suffisant pour stériliser le lait en poudre et congelé. Or, ces valeurs dépendent du ratio entre le nombre de cellules végétatives et de cellules sporulées, qui elles-mêmes dépendent de la contamination, de la matrice alimentaire ainsi que des conditions de production et d'emballage du produit (humidité, température, pH). Ainsi, l'irradiation dans le lait congelé serait le meilleur modèle pour l'élimination des spores pour réduire la dose nécessaire. Ces résultats démontrent que l'irradiation présente un avantage notable par rapport au traitement d'HoP sur l'élimination des bactéries sporulées. Ces données permettent de prédire grossièrement les doses qui seront à appliquer sur le lait maternel. En général, la contamination initiale dans du LM se situe entre 0 et 6 Log UFC/mL (Boix-Amorós, Collado, & Mira, 2016). En comparant le traitement de HoP à l'irradiation gamma sur du lait maternel qui contenant 2.5 Log UFC/mL de bactéries aérobie, il a été démontré qu'une dose de 5 kGy était suffisante pour procurer au LM une élimination de plus de 99,97% des bactéries totales aérobiques et une élimination totale des coliformes fécaux. Ainsi, la qualité microbiologique du lait irradié est équivalente au lait traité à la HoP sans chauffer le lait, ce qui évite de dénaturer les composantes.

Radiosensibilisation dans le lait maternisé

La combinaison de l'irradiation avec des additifs capables d'augmenter la radiosensibilité des pathogènes alimentaires a été testé dans l'optique de réduire les doses d'irradiation requises, particulièrement pour les bactéries sporulantes. Cela permet d'augmenter la sécurité du produit et d'éventuellement diminuer les coûts de traitement. En premier lieu, les additifs étaient choisis en fonction de leur capacité antioxydante à potentiel radiosensibilisant. Ainsi, l'acide ascorbique, le palmitate d'ascorbyl, la bêta-carotène et le phosphate de tocophérol ont été testés à des concentrations allant jusqu'à 2000 ppm. Aucun effet de radiosensibilisation n'a été trouvé pour les agents pathogènes sélectionnés dans le lait en formulation en poudre et congelé. Cependant, il est à prendre en considération que le lait n'a pas été préalablement stérilisé, ce qui peut avoir affecté les résultats. Par la suite, des additifs ont été sélectionnés dans la liste des additifs alimentaires permis par la Food and Agriculture Organization (FAO) dans les préparations pour nourrissons. Des pré-tests ont été effectués afin de déterminer la concentration de citrate de sodium, carbonate de sodium

ainsi que d'acide citrique afin de ne pas trop modifier le pH du produit. Ainsi, des concentrations de 2,5%, 0,5% et 0,75% (p/v) respectivement ont été sélectionnées pour le citrate de sodium, le carbonate de sodium ainsi que l'acide citrique. Ces produits sont sans danger pour les nourrissons, sont peu couteux et sont déjà amplement utilisés dans diverses industries depuis des décennies à des fins de régulateur d'acidité, émulsifiant, stabilisant et beaucoup plus. Il a été constaté que *B. cereus* était sensible à tous les additifs en combinaison avec l'irradiation. Seul *E. coli* était sensible au citrate de sodium, mais toutes les bactéries testées (*B. cereus, L. monocytogenes, S. aureus, E. coli et S.* Typhimurium) avaient une radiosensibilisation importante lorsqu'elles étaient exposées au carbonate de sodium, soit en poudre ou liquide congelé. Il est utilisé comme désinfectant pour les agrumes (Mahmoud *et al.*, 2011). Le mécanisme d'action repose sur l'ion CO^{2-} qui compromet les enzymes fonctionnelles et la membrane externe des bactéries à Gram négatif (Jarvis *et al.*, 2001). De plus, certaines études ont montré qu'il peut interférer dans la germination de bactéries sporulées à 0,1% p / v (Cheung, So, & Sun, 1998; Hachisuka, Kato, & Asano, 1956). Cela serait dû à l'alcalinisation du milieu ainsi l'altérations structurelles des protéines de type kératine. Globalement, le carbonate de sodium à 0,5% (p / v) est un excellent agent de radiosensibilisation à la fois sous forme congelée et en poudre pour toutes les souches testées.

Effet sur la qualité nutritionnelle et immunologique

L'effet de l'irradiation par rapport à la pasteurisation Holder sur la qualité nutritionnelle du lait maternel a été évalué dans le lait maternel et le lait en formulation. Dans un premier temps, l'irradiation a préservé la valeur nutritionnelle du lait maternel. Les protéines et le lactose n'ont démontré aucun changement après une dose d'irradiation de 10 kGy, ce qui est en accord avec Ham et al. (2009). Par contre, le contenu en vitamine C a été significativement diminuée ($p \leq 0.05$) lorsqu'irradié dans la préparation pour nourrissons. La sensibilité de l'acide ascorbique en présence d'irradiation avait également été remarquée par Wang and Chao (2003), mais la pasteurisation Holder est également connue pour diminuer cette vitamine très susceptible à la chaleur (Peila et al., 2016). L'irradiation a préservé les propriétés antioxydantes testées par DPPH, par contre, la formation de malondialdehyde est augmentée après irradiation dans le lait en formulation et dans le lait maternel de façon dose-dépendante entre 0 et 10 kGy. En effet, la peroxydation lipidique due aux rayons ionisant dans différentes matrices alimentaires a été relevée dans plusieurs études, ce qui est un désavantage par rapport à la pasteurisation Holder (El-Rawas et al., 2012; Sohn et al., 2009; Tesfai *et al.*, 2014).

Par la suite, le profil des protéines et peptides du lait maternel après irradiation et pasteurisation ont été comparés afin d'avoir une idée de l'impact des traitements sur les protéines et peptides. Dans tous les cas, le traitement d'irradiation a engendré une diminution des protéines de hauts poids moléculaires et des

peptides totaux, particulièrement pour le lait pasteurisé. Dans le lait en formulation, les protéines de poids moléculaire entre 300 et 15kDa ont été complètement éliminés et les peptides de moins de 15kDa diminués suite à l'irradiation. Par contre, ceci n'est pas observé dans le lait maternel, où aucun changement n'est observé dans ce groupes de protéines. Ainsi, la matrice alimentaire du lait maternel semble mieux protéger de l'agrégation et de la fragmentation des protéines que le lait en formulation ou que le traitement à la chaleur de pasteurisation (Afify & Shousha, 1988; Davies, 1987). Peu de différence ne fut observée dans tous les cas entre le lait en poudre ou congelé.

Finalement, l'activité enzymatique du lysozyme et de l'amylase dans le lait maternel a été évaluée après traitement d'irradiation et traitement à la chaleur. Aucun impact n'a été trouvé sur ces enzymes après irradiation. Cependant, l'activité du lysozyme dans le lait traité par Pasteurisation Holder (HoP) a été significativement diminuée ($p \leq 0.05$), comme il a été observé auparavant dans la littérature (Kim & Unger, 2010). D'autres études indiquent que l'amylase pourrait également être partiellement détruite par HoP (Peila et al., 2016). Toutefois, il a été observé que l'irradiation préserve l'intégrité de nombreuses enzymes telles que le lysozyme et l'amylase dans la salive humaine à 3,5 kGy (Ruhl et al., 2011).

6.1.2 Atomisation du lait maternel

La congélation du lait humain est la méthode courante de traitement des dons dans les banques de lait humain. Cependant, cette méthode comporte plusieurs désavantages car cela implique divers cycles de congélation/décongélation lors du traitement, transport et stockage. Cela requiert également du matériel de congélation et de l'électricité, ce qui peut constituer un défi pour le stockage et le transport dans certains pays. Comme alternative, la conversion du lait humain en poudre par atomisation est une option intéressante pour faciliter le stockage à long terme du lait maternel avec peu d'impact sur ses propriétés nutritionnelles et immunologiques (Lozano *et al.*, 2014). Un des paramètres critiques pour atteindre la stabilité du produit et l'inhibition de la croissance bactérienne est l'atteinte d'une très basse activité de l'eau (aw) du produit. En général, une valeur inférieure de aw à 0,4 est nécessaire pour limiter la prolifération microbienne et assurer la stabilité des aliments déshydratés (Feeney et al., 2014). La méthode de séchage par atomisation de la présente étude a permis d'obtenir une poudre ayant l'activité d'eau de 0,21, ce qui est comparable aux préparations pour nourrissons commerciales qui se situent normalement autour de 0,2.

Dans notre étude, la comparaison entre les nutriments du lait maternel (protéines, lipides et lactose) avant et après atomisation n'a montré aucun changement. Par contre, la vitamine C n'a pas pu être détectée (limite de détection de 0,0015mg/mL) sur le lait atomisé. L'acide ascorbique est une vitamine essentielle dans la nutrition de tous les groupes d'âges puisqu'elle n'est pas synthétisée ou stockée par le corps humain et

participe dans plusieurs voies métaboliques (Francis *et al.*, 2008). Il est possible que l'atomisation ait pu détruire cette vitamine puisqu'elle est sensible à la chaleur, à la lumière et à l'oxygène. En effet, Carlos, Antonio, and Misael (2018) mentionnent que généralement, une perte entre 30 et 90% de vitamine C peut être observée selon la matrice alimentaire atomisée. Or, il est également possible que la vitamine ait été détruite par le simple maniement du lait entre le prélèvement, la conservation et la manipulation des échantillons avant l'atomisation. En effet, il a été montré que le lait maternel peut perdre jusqu'à 90% de cette vitamine après seulement 20 minutes de manipulations. Cela est corroboré par le fait que le lait maternel dans les banques de lait maternel perd en moyenne 50% de sa valeur en vitamine C. Ainsi, plus de tests devront être effectués sur la qualité des micronutriments tels que les vitamines dans le lait humain atomisé. Il serait possible qu'une supplémentation du produit fini en vitamine C soit une étape nécessaire à la production d'un aliment fonctionnel à base de lait humain.

Rodríguez *et al.* (2018) ont précédemment évalué la conservation à long terme du lait maternel sous forme de poudre par séchage par pulvérisation (Rodríguez et al., 2018). Même si l'atomisation est une méthode efficace pour préserver les enzymes et les nutriments, une diminution de la teneur en immunoglobulines telles que les IgA d'environ 25% a été observée (Castro-Albarrán et al., 2016). Dans notre étude, la rétention des anticorps testés était de 82%, 78% et 70% pour IgA, IgG et IgM, respectivement. Les résultats peuvent varier selon les paramètres de température et de débit sélectionné. De façon générale, les données obtenues semblent être similaires à la lyophilisation et supérieures à la pasteurisation (Castro-Albarrán et al., 2016; Kim & Unger, 2010). Puisque très peu d'études ont été publiées sur le sujet, la méthodologie d'atomisation pourrait éventuellement être améliorée pour veiller à la conservation de ces protéines immunologiques importantes du lait humain.

Les analyses des propriétés antioxydantes et antibactériennes de la lactoferrine ont montré qu'il a été possible de déterminer après purification que même si la lactoferrine semble être présente en quantité normale, elle semble avoir perdu certaines des capacités antioxydantes normalement détectées par le test FRAP (Giansanti *et al.*, 2016; Safaeian *et al.*, 2015). Cependant, il convient de noter qu'une bonne partie des capacités protectrices de la lactoferrine sont dues aux peptides dérivés de la protéolyse lors de la digestion (Giansanti *et al.*, 2016). De façon générale, le lait atomisé semble également perdre des capacités antioxydantes, mais cela pourrait être relié à l'absence d'acide ascorbique dans nos échantillons. Finalement, la lactoferrine présentait des propriétés antimicrobiennes contre *E. coli* et *L. monocytogenes* après avoir été purifié du lait atomisé. De façon générale, l'atomisation est un procédé simple, rapide et peu coûteux qui permet d'obtenir un produit stable à température ambiante, avec un faible risque de contamination microbienne et une excellente préservation de la valeur nutritionnelle et des propriétés immunologiques

pour toutes les composantes testées. De plus amples études sont à mener afin de comprendre l'absence de vitamine C dans le lait maternel atomisé dans cette étude. Cette absence serait possiblement due à la manipulation du lait maternel avant et après traitement. En effet, cette vitamine est connue pour être très sensible à la chaleur, mais également à la congélation, à la réfrigération, à la manipulation et à plusieurs autres traitements (Schlotterer et Perrin, 2018). Bien que le séchage par atomisation utilise des températures élevées (160 à 230 ° C), le rapport surface / volume élevé des gouttelettes favorise un séchage rapide et le débit d'air fait en sorte que les particules atteignent une température de seulement 60 à 100° C, ce qui évite les dommages thermiques (Moreira *et al.*, 2010). Ainsi, il est possible d'avoir une rétention de plus de 97% de l'acide ascorbique après irradiation en fonction de la matrice alimentaire (Moreira *et al.*, 2010).

6.1.3 Tests *in vitro* du lait maternel enrichi en probiotiques

Les études sur les propriétés anticancers du lait humain ont montré une induction de la mort cellulaire de façon dose-dépendante entre 0 et 6 mg/mL de cellules cancéreuses et non-cancéreuses. Une double coloration de l'annexine V-FITC / PI a été réalisée afin de déterminer le mécanisme de mort cellulaire. Les résultats obtenus suggèrent que l'induction de l'apoptose dans les cellules cancéreuses colorectales pourrait être impliquée puisque le lait maternel a pu augmenter de manière significative le taux de cellules apoptotiques par rapport au témoin et n'a pas augmenté le taux de nécrose. En effet, il est souhaitable que le mécanisme de mort cellulaire impliqué soit associé au processus d'apoptose plutôt que de nécrose, qui elle est associée à de l'inflammation et à une réaction immunitaire (Haanen & Vermes, 1995). La combinaison de probiotiques et de lait maternel n'a produit aucun effet synergique. Les probiotiques testés n'ont présenté aucun effet anti-prolifératif ni cytotoxique à la concentration choisie de 9.5 Log UFC / mL. Les test concernant la NAD (P) H Quinone réductase (QR) ont permis de déterminer le potentiel détoxifiant du lait maternel et des probiotiques (Dennis & Witting, 2017). Il est suggéré que la consommation de 33 333 unités par jour de cette enzyme serait suffisante pour qu'un composé soit chimio préventif (Kirlin *et al.*, 1999). Dans notre étude, une unité (quantité nécessaire pour doubler la quantité de QR dans les cellules Hepa 1c1c7) correspond à environ 0,3 mg de lait maternel. Ainsi, il faudrait 100 mL (ou 10 g de lait maternel en poudre) par jour pour produire chez un adulte le caractère antiprolifératif et chimiopréventif au niveau des cellules cancéreuse.

6.1.4 Tests *in vivo* du lait maternel enrichi en probiotiques

La dernière partie de l'étude a été de démontrer *in vivo* qu'un aliment fonctionnel à base de lait maternel en poudre enrichi en probiotiques aurait la capacité de prévenir l'apparition de maladies chroniques liées à l'âge et à l'obésité. Ainsi, le lait humain en poudre a été administré à des rats âgés de 8 semaines en

combinaison avec des probiotiques de Bio-K Plus à une concentration de 9.5 Log UFC/mL. Les groupes ayant reçu du lait maternel ont eu une prise de poids significativement inférieure au groupe témoin. Par la suite, le poids des organes calculé en % du poids corporel montre que les rats ayant consommé le lait maternel enrichi avaient un poids hépatique significativement égal ou inférieur au groupe témoin, mais un poids des reins supérieurs lorsque le lait était combiné avec les probiotiques. Ainsi, le lait maternel semble prévenir l'accumulation de lipides dans le foie. Des tests ont été exécutés sur les reins afin de déterminer la présence de dommages oxydatifs qui sont les premiers signes de dommages aux reins (Dennis & Witting, 2017). Il a été montré que les reins sont mieux protégés contre les dommages liés au stress oxydatif lorsque les rats consomment le lait maternel enrichi en probiotiques. Dans notre étude, les taux de dommages oxydatifs aux lipides et aux protéines ayant consommé du lait maternel et des probiotiques étaient réduit de 97%. L'étude de l'activité de la catalase dans les reins n'a montré aucun changement entre les groupes.

Les analyses sur les niveaux sanguins de leptine, de glucose et d'insuline ont été évalués afin de contrôler l'impact de cet aliment fonctionnel sur le développement de l'obésité et du diabète. Ainsi, les rats gavés au lait maternel et aux probiotiques semblent être plus sensibles à la leptine comparativement au groupe contrôle. Il est admis que les individus obèses tendent à développer une résistance à la leptine. Dans notre étude, les rats gavés de lait maternel et probiotiques ont sécrété le même niveau de leptine mais semblent mieux y répondre puisqu'ils ont pris moins de poids. Le mécanisme d'action n'est pas connu, mais il est possible que des substances du lait agissent comme antagonistes à des récepteurs responsables de la résistance à la leptine (Higuchi *et al.,* 2016; Xue et al., 2016).

Le taux de glucose sanguin observé chez les rats nourris *ad libidum* a été mesuré chaque semaine. Les résultats ont montré que les rats qui consommaient le lait humain avaient un taux de glucose stable pendant la plus grande partie de l'étude alors que les rats gavés avec le lait enrichi en probiotiques ont vu leurs valeurs diminuer jusqu'à la fin de l'étude, tout comme le groupe témoin soumis à la diète haute en gras. Ce phénomène avait déjà été observé chez la souris et le rat soumis à une diète haute en gras dans des études antérieures et avait également été associé à une augmentation des niveaux d'insuline (Orhan *et al.*, 2017;Winzell & Ahrén, 2004), ce qui a été confirmé par notre étude. Ensuite, le rapport insuline / glucose a été calculé afin d'estimer indirectement la résistance à l'insuline des animaux (Orhan *et al.,* 2017; Winzell & Ahrén, 2004). Cela a démontré que les rats gavés de lait humain avaient un rapport plus bas, suggérant qu'il pourrait ralentir le processus de résistance à l'insuline comparativement aux rats nourris avec une diète haute en gras. Par contre, l'administration de probiotiques semble avoir exercé un effet peu important ou pouvait partiellement diminuer les effets bénéfiques du lait maternel.

Finalement, les marqueurs de l'inflammation et du syndrome métabolique ont été mesuré dans le sang des rats. Après 8 semaines, les marqueurs inflammatoires IL-1β et TNF-a sont à des niveaux normaux, puisque l'inflammation chronique sous-jacente de bas grade peut prendre plusieurs mois avant de s'installer. En effet, chez les rats obèses, l'inflammation de bas grade est souvent observée et est corrélée par des cytokines et des adipokines inflammatoires élevées dans les tissus et le sang, telles que PAI-1, IFN-g, TNF-a, IL-1β et IL- 6 (Francisqueti *et al.*, 2017; Tack *et al.,* 2012; Wu *et al.*, 2016). Dans notre étude, seul le facteur PAI-1 a significativement diminué ($p \leq 0.05$) pour le groupe ayant consommé le lait maternel enrichi en probiotiques, ce qui est un signe de santé cardiovasculaire et un indicateur de santé globale (Alessi & Juhan-Vague, 2006).

6.2 Conclusion générale

Ce projet de maîtrise s'est intéressé à la fabrication d'un aliment fonctionnel à base de lait maternel et de probiotiques ayant les capacités d'aider à prévenir les maladies liées à l'obésité et au vieillissement. Dans un premier temps, l'évaluation de l'irradiation gamma comme méthode de décontamination pour l'utilisation sécuritaire du lait maternel sous forme de poudre ou de liquide congelé a été testée sur cinq pathogènes alimentaires. Les tests visaient à démontrer l'efficacité et les avantages que représentent cette méthode de décontamination sans chaleur pour la préservation des nutriments et des propriétés du lait maternel, tout en assurant la sécurité alimentaire et la flexibilité de ce traitement sur les produits sous différentes formes. Ensuite, la possibilité d'utiliser des additifs alimentaires dans le lait maternel afin de sensibiliser les bactéries plus résistantes à l'irradiation a été évaluée. Par la suite, l'atomisation comme méthode de commercialisation et de préservation du lait maternel a été évaluée afin de s'assurer de la conservation et la préservation des nutriments et des propriétés du lait maternel. Finalement, la possibilité d'utiliser le lait maternel atomisé enrichi en probiotiques pour la prévention des maladies liées à l'obésité et la vieillesse ont été examiné *in vitro* et *in vivo*.

Ce projet de maîtrise a permis de mettre de l'avant l'irradiation comme méthode de décontamination du lait maternel liquide congelé ou en poudre comme méthode sûre, flexible qui pourrait éventuellement se substituer à la pasteurisation Holder qui est présentement la norme. Cette étude a démontré qu'une dose d'irradiation de 5kGy offre un degré de décontamination plus qu'acceptable pour les pathogènes alimentaires les plus communs tout en préservant les nutriments du lait à l'exception de la vitamine C. Les propriétés nutritionnelles et immunologiques du lait semblent mieux conservées sous la forme sèche, par contre, les spores et bactéries pathogènes sont plus facilement éliminées dans le lait congelé. Finalement, il est possible d'induire la radiosensibilisation des bactéries les plus résistantes telles que *Bacillus cereus* par

l’entremise d’additifs alimentaires sécuritaires dans le lait en formulation. Ces tests préliminaires laissent entendre que l’utilisation d’additifs dans le lait maternisé pourrait être une avenue afin de stériliser le lait humain dans le but d’augmente la sécurité et la qualité microbiologique du produit et d’une façon profitable.

Les résultats de cette étude ont permis de déterminer que l’atomisation du lait maternel permet une bonne rétention des propriétés nutritionnelles et immunologiques du lait maternel dans son ensemble. L’atomisation permet la production d’un produit stable pour plusieurs mois et facilement réhydratable.

Cette étude a aussi démontré par les expériences *in vitro* du lait maternel atomisé sur des cellules cultivées qu’il est possible que le produit présente des propriétés antiprolifératives et chimiopréventives. Par la suite, l’addition de probiotiques dans le lait maternel a augmenté son potentiel antioxydant de 25%. Finalement, la dernière partie de ce mémoire s’est intéressée à l’impact *in vivo* du lait enrichi en probiotiques sur des rats soumis à une diète riche en gras. L'étude montre que l'administration de lait humain et de probiotiques en tant qu'aliment fonctionnel pourrait ralentir l'accumulation de lipides, le stress oxydatif et le processus inflammatoire conduisant au syndrome métabolique.

De façon générale, ces résultats révèlent qu’il serait possible d’élaborer un produit sécuritaire, adapté à l’entreposage et au transport, qui pourrait être amener des bienfaits aux personnes à risque de souffrir de d’obésité et de maladies liées au syndrome métabolique. Également, l’expertise de l’irradiation du lait maternisé et du lait maternel répond à un réel besoin dans les banques de lait maternel et les industries de s’assurer de l’innocuité d’un produit destiné à une population immundéficiente. Finalement, un approfondissement des connaissances sera requis avant de pouvoir caractériser le lait maternel enrichi en probiotiques d’aliment fonctionnel. Cependant, les résultats de cette études *in vitro* et *in vivo* démontrent un fort potentiel. Ces résultats peuvent être d'un grand intérêt pour l'industrie alimentaire, pour la conservation des aliments sensibles aux traitements ainsi que pour le développement d’aliments qui pourront aider les populations à risques de développer des problèmes de santé.

Références

Afify, A. E. M. M. R., & Shousha, M. A. (1988). Effect of low-dose irradiation on soybean protein solubility, trypsin inhibitor activity, and protein patterns separated by polyacrylamide gel electrophoresis. *Journal of Agricultural and Food Chemistry*, *36*, 810–813.

Alessi, M.-C., & Juhan-Vague, I. (2006). PAI-1 and the metabolic syndrome: links, causes, and consequences. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *26*, 2200–2207.

Ben Miloud Yahia, N., Ghorbal, S. K. B., Maalej, L., Chatti, A., Elmay, A., Chihib, N.-E., & Landoulsi, A. (2018). Effect of temperature and gamma radiation on *salmonella* hadar biofilm production on different food contact surfaces. *Journal of food quality*, *2018*, 1–6.

Boix-Amorós, A., Collado, M. C., & Mira, A. (2016). Relationship between Milk Microbiota, Bacterial Load, Macronutrients, and Human Cells during Lactation. *Frontiers in microbiology*, *7*, 492.

Cacho, N. T., Harrison, N. A., Parker, L. A., Padgett, K. A., Lemas, D. J., Marcial, G. E., Lorca, G. L. (2017). Personalization of the Microbiota of Donor Human Milk with Mother's Own Milk. *Frontiers in microbiology*, *8*, 1470.

Carlos, L. A. J., Antonio, G. G. G., & Misael, C. R. (2018). Effect of the Spray Drying Process on the Quality of Coconut Powder Fortified with Calcium and Vitamins C, D_3 and E. *American Journal of Food Science and Technology*, *16*, 102–124.

Castro-Albarrán, J., Aguilar-Uscanga, B. R., Calon, F., St-Amour, I., Solís-Pacheco, J., Saucier, L., & Ratti, C. (2016). Spray and freeze drying of human milk on the retention of immunoglobulins (IgA, IgG, IgM). *Drying Technology*, *34*, 1801–1809.

Cheung, H. Y., So, C. W., & Sun, S. Q. (1998). Interfering mechanism of sodium bicarbonate on spore germination of Bacillus stearothermophilus. *Journal of Applied Microbiology*, *84*, 619–626.

Davies, K. J. (1987). Protein damage and degradation by oxygen radicals. I. general aspects. *The Journal of Biological Chemistry*, *262*, 9895–9901.

Dennis, J. M., & Witting, P. K. (2017). Protective role for antioxidants in acute kidney disease. *Nutrients*, *9*. doi:10.3390/nu9070718

El-Rawas, A., Hvizdzak, A., Davenport, M., Beamer, S., Jaczynski, J., & Matak, K. (2012). Effect of electron beam irradiation on quality indicators of peanut butter over a storage period. *Food chemistry*, *133*, 212–219.

Feeney, A., Johnston, C. D., Govender, R., O'Mahony, J., Coffey, A., & Sleator, R. D. (2014). Analysis of the role of the Cronobacter sakazakii ProP homologues in osmotolerance. *Gut Pathogens*, *6*, 15.

Francis, J., Rogers, K., Brewer, P., Dickton, D., & Pardini, R. (2008). Comparative analysis of ascorbic acid in human milk and infant formula using varied milk delivery systems. *International breastfeeding journal*, *3*, 19.

Francisqueti, F. V., Nascimento, A. F., Minatel, I. O., Dias, M. C., Luvizotto, R. de A. M., Berchieri-Ronchi, C., Corrêa, C. R. (2017). Metabolic syndrome and inflammation in adipose tissue occur at different times in animals submitted to a high-sugar/fat diet. *Journal of nutritional science*, *6*, e41.

Froh, E. B., Vanderpool, J., & Spatz, D. L. (2018). Best practices to limit contamination of donor milk in a milk bank. *Journal of Obstetric, Gynecologic, and Neonatal Nursing*, *47*, 547–555.

Giansanti, F., Panella, G., Leboffe, L., & Antonini, G. (2016). Lactoferrin from Milk: Nutraceutical and Pharmacological Properties. *Pharmaceuticals (Basel, Switzerland)*, *9*. doi:10.3390/ph9040061

Haanen, C., & Vermes, I. (1995). Apoptosis and inflammation. *Mediators of Inflammation*, *4*, 5–15.

Hachisuka, Y., Kato, N., & Asano, N. (1956). Inhibitory effect of sodium bicarbonate and sodium carbonate on spore germination of Bacillus subtilis. *Journal of Bacteriology*, *71*, 250–251.

Ham, J. S., Jeong, S. G., Lee, S. G., Han, G. S., Jang, A., Yoo, Y. M., Jo, C. (2009). Quality of Irradiated Plain Yogurt during Storage at Different Temperatures. *Asian-Australasian journal of animal sciences*, *22*, 289–295.

Higuchi, T., Mizuno, A., Narita, K., Ichimaru, T., & Murata, T. (2012). Leptin resistance does not induce hyperphagia in the rat. *The Journal of Physiological Sciences*, *62*, 45–51.

Jarvis, G. N., Fields, M. W., Adamovich, D. A., Arthurs, C. E., & Russell, J. B. (2001). The mechanism of carbonate killing of Escherichia coli. *Letters in Applied Microbiology*, *33*, 196–200.

Kim, J., & Unger, S. (2010). Human milk banking. *Paediatrics & child health*, *15*, 595–602.

Kirlin, W. G., Cai, J., DeLong, M. J., Patten, E. J., & Jones, D. P. (1999). Dietary compounds that induce cancer preventive phase 2 enzymes activate apoptosis at comparable doses in HT29 colon carcinoma cells. *The Journal of Nutrition*, *129*, 1827–1835.

Lozano, B., Castellote, A. I., Montes, R., & López-Sabater, M. C. (2014). Vitamins, fatty acids, and antioxidant capacity stability during storage of freeze-dried human milk. *International Journal of Food Sciences and Nutrition*, *65*, 703–707.

Mahmoud, G. A., Mohamed, G. A., Botros, H. W., & Sabri, M. A. (2011). Combined effect of gamma radiation, hot water and sodium bicarbonate on quality and chemical constituents of stored mandarin fruits. *Archives Of Phytopathology And Plant Protection*, *44*, 1115–1127.

Moreira, G. É. G., De Azeredo, H. M. C., De Medeiros, M. D. F. D., De Brito, E. S., & De Souza, A. C. R. (2010). Ascorbic acid and anthocyanin retention during spray drying of acerola pomace extract. *Journal of food processing and preservation*, *34*, 915–925.

Orhan, C., Şahin, N., Tuzcu, Z., Komorowski, J. R., & Şahin, K. (2017). Combined oral supplementation of chromium picolinate, docosahexaenoic acid, and boron enhances neuroprotection in rats fed a high-fat diet. *Turkish journal of medical sciences*, *47*, 1616–1625.

Peila, C., Moro, G. E., Bertino, E., Cavallarin, L., Giribaldi, M., Giuliani, F., Coscia, A. (2016). The Effect of Holder Pasteurization on Nutrients and Biologically-Active Components in Donor Human Milk: A Review. *Nutrients*, *8*. doi:10.3390/nu8080477

Rigourd, V., Barnier, J. P., Ferroni, A., Nicloux, M., Hachem, T., Magny, J. F., Bille, E. (2018). Recent actuality about Bacillus cereus and human milk bank: a new sensitive method for microbiological analysis of pasteurized milk. *European Journal of Clinical Microbiology & Infectious Diseases*, *37*, 1297–1303.

Rodríguez, A. A., Lacroix, M., Raymundo Solís Pacheco, J., García Morales, E., Alfonso Gutiérrez Padilla, J., Angulo Castellanos, E., Rosa Aguilar Uscanga, B. (2018). Assessment of the Biological Activity in Human Milk Power Treated with Different Processes for Their Conservation. *Journal of Food and Nutrition Research*, *6*, 329–334.

Ruhl, S., Berlenbach, P., Langenfelder, S., Hörl, D., Lehn, N., Hiller, K.-A., Durchschlag, H. (2011). Integrity of proteins in human saliva after sterilization by gamma irradiation. *Applied and Environmental Microbiology*, *77*, 749–755.

Safaeian, L., Javanmard, S. H., Mollanoori, Y., & Dana, N. (2015). Cytoprotective and antioxidant effects of human lactoferrin against H2O2-induced oxidative stress in human umbilical vein endothelial cells. *Advanced biomedical research*, *4*, 188.

Sohn, S. H., Jang, A., Kim, J. K., Song, H. P., Kim, J. H., Lee, M., & Jo, C. (2009). Reduction of irradiation off-odor and lipid oxidation in ground beef by α-tocopherol addition and the use of a charcoal pack. *Radiation Physics and Chemistry*, *78*, 141–146.

Tack, C. J., Stienstra, R., Joosten, L. A. B., & Netea, M. G. (2012). Inflammation links excess fat to insulin resistance: the role of the interleukin-1 family. *Immunological Reviews*, *249*, 239–252.

Tesfai, A., Beamer, S. K., Matak, K. E., & Jaczynski, J. (2014). Effect of electron beam on chemical changes of nutrients in infant formula. *Food chemistry*, *149*, 208–214.

Wang, J., & Chao, Y. (2003). Effect of 60Co irradiation on drying characteristics of apple. *Journal of food engineering*, *56*, 347–351.

Winzell, M. S., & Ahrén, B. (2004). The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. *Diabetes*, *53 Suppl 3*, S215–9.

Wu, P., Zhang, F., Dai, Y., Han, L., & Chen, S. (2016). Serum TNF-α, GTH and MDA of high-fat diet-induced obesity and obesity resistant rats. *Saudi pharmaceutical journal : SPJ : the official publication of the Saudi Pharmaceutical Society*, *24*, 333–336.

Xue, B., Yu, Y., Zhang, Z., Guo, F., Beltz, T. G., Thunhorst, R. L., Johnson, A. K. (2016). Leptin Mediates High-Fat Diet Sensitization of Angiotensin II-Elicited Hypertension by Upregulating the Brain Renin-Angiotensin System and Inflammation. *Hypertension*, *67*, 970–976.