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Effects of biopolymer-based microbeads using encapsulated essential oils combined with γ - irradiation on microbiological and physico-chemical properties of dry fermented sausages

Par

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Mémoire présenté pour l'obtention du grade de

Maître ès Sciences (M.Sc.) en sciences de Microbiologie Appliquée

Jury d'évaluation

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ACKNOWLEDGMENTS

I was so fortunate to have the support from a lot of people throughout my whole master program without whom this thesis would have been impossible. I am grateful to all of them who have contributed to this thesis directly or indirectly.

First and foremost, I would like to express my sincere gratitude to my director Prof. Monique

Lacroix for giving me an invaluable opportunity to gain knowledge and great experience in her laboratory. Her magnificent spirit, willpower and constancy of faith have impaired me a lot to aim higher and achieve my goals in my life.

I am highly thankful to Mr. Stéphane Salmieri for leading me into the field of microbiology and all the appreciative help during my master study and Dr. Shiv Shankar, Dr. Leila Bagheri for the great advice for my writing.

I am thankful to all my colleagues in the Labo-Resala for their assistance and friendship and my trainee Emma Juillet for her integrity and diligence. I would like to express my deepest sense of gratitude to my family in China for their blessings and inspiration. Finally, I would like to express my sincere thanks to Mitacs Glabalink and Fondation Armand-Frappier for their financial support.

ABSTRACT

Dry and fermented sausages (DFS), face high risks of contamination and outbreaks. Antimicrobial formulations based on essential oils (EOs) were developed. The EOs were selected by minimum inhibitory concentration (MIC) and synergy between them for formulas to be added in meat. A factorial design was conducted to develop the most efficient formula and fractional inhibitory concentration (FIC) was used to verify the synergistic effects. Alginate and cellulose nanocrystal (CNC) were used for the EOs encapsulation and X-/γ-irradiation was applied at end drying. Total aerobic mesophilic bacteria, *E. coli* O157:H7, *L. monocytogenes*, lactic acid bacteria, molds and yeasts were counted at several control points during manufacturing and storage. Color and texture were analyzed during storage. Results showed that EOs encapsulation and irradiation showed synergistic effects on reduction of *E. coli* O157:H7, total aerobic mesophilic bacteria and lactic acid bacteria. Encapsulation treatment of alginate-CNC showed the ability to improve the antimicrobial properties against lactic acid bacteria over time. All formulas showed high eliminating effects to *L. monocytogenes,* molds and yeasts. Irradiation and EOs extended the shelf life, assured the safety and protected the color and the texture deterioration without any negative effects on qualities.

Keywords:

Dry fermented sausage; Essential oils; Antimicrobial activity; Factorial design; Synergy; Foodborne pathogens; Microencapsulation; Gamma/X-ray irradiation; Microbiological quality; Physicochemical quality

RÉSUMÉ

Les saucisses fermentées et séchées (SFS) font face à des risques élevés de contamination et d'épidémies. Des formulations antimicrobiennes à base d'huiles essentielles (HEs) ont été développées. Les HEs ont été sélectionnés par concentration minimale inhibitrice (CMI) et de la synergie entre elles pour les formulations à ajouter dans la viande. Une conception factorielle a été menée pour développer la formulation la plus efficace et la concentration inhibitrice fractionnée (FIC) a été utilisée pour vérifier les effets synergiques. Des nanocristaux d'alginate et de cellulose (CNC) ont été utilisés pour l'encapsulation des HEs et rayons X ou γ a été appliquée à la fin du séchage. Les bactéries mésophiles aérobies totales, *E. coli* O157: H7, *L. monocytogenes*, les bactéries lactiques, les moisissures et les levures ont été comptées à plusieurs points de contrôle pendant la fabrication et l'entreposage. La couleur, la texture ont été analysées lors du stockage. Les résultats ont montré que l'encapsulation et l'irradiation des HEs ont des effets synergiques sur la réduction d'*E. coli* O157: H7, des bactéries mésophiles aérobies totales et des bactéries lactiques. Le traitement d'encapsulation de l'alginate-CNC a montré la capacité d'améliorer les propriétés antimicrobiennes contre les bactéries lactiques au fil du temps. Toutes les formulations ont montré des effets d'élimination élevés sur *L. monocytogenes*, les moisissures et les levures. De plus, les résultats ont montré que l'irradiation et les HE agissent en synergie pour assurer la salubrité, prolonger la durée de conservation, et protègent la couleur et la texture sans aucun effet négatif sur les qualités sensorielles.

Mots-clés :

Saucisse fermentée sèche; Huiles essentielles; Activité anti-microbienne; Conception factorielle; Synergie; Les agents pathogènes d'origine alimentaire; Microencapsulation; Irradiation gamma/rayons X; Qualité microbiologique; Qualité physicochimique

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LIST OF ABBREVIATIONS

Dry fermented sausages (DFS)

Ready-to-eat (RTE)

Essential oils (EOs)

Minimum inhibitory concentration (MIC)

Fractional inhibitory concentration (FIC)

Alginate and cellulose nanocrystal (CNC)

Generally Recognized as Safe (GRAS)

Food and Drug Administration (FDA)

lactic acid bacteria (LAB)

Total mesophilic bacteria (TMF)

Design of Experiments (DOE)

Inhibitory capacity (IC, %)

Control (CT)

EO-alginate (AE)

EO-alginate-CNC (ACE)

Gamma irradiation (GI)

Gram-positive catalase-positive cocci (GCC+)

Coagulase-negative *staphylococci* (CNS)

Verocytoxigenic Escherichia coli (VTEC)

Enterohaemorrhagic Escherichia coli (EHEC)

Shiga toxigenic E. coli (STEC)

Monoterpenes loaded with Chitosan to form nanoparticles (ChMNPs)

Chitosan (Ch)

Garlic essential oil GEO

Satureja essential oil (SKEO)

Rosmarinus officinalis essential oils (REO)

Cinnamon essential oil (CEO)

Thyme essential oil (TEO)

Thiobarbituric acid reactive substances (TBARS)

Oregano essential oil (OR)

Cinnamon essential oil (CN)

Radiosensitivity (RS)

Adenosine triphosphate (ATP)

Cumin essential oil nanoemulsion (CNE)

Metamodeling antimicrobial cocktail optimization (MACO)

Reactive oxygen species (ROS)

Lyophilized pomegranate peel (LPP)

1 INTRODUCTION

Meat market is rapidly increasing due to growing world population and economy (Lee *et al.*, 2020; Ponnampalam *et al.*, 2019). Meat and meat products contains various nutrient compositions including high-quality protein content, essential amino acids, and an excellent source of B-group vitamins, minerals, and other nutrients (Pateiro *et al.*, 2021) that is ideal for the growth and propagation of meat spoilage micro-organisms and common food-borne pathogens (Zhou *et al.*, 2010). Because of the relative high level of fat and distinctive processing features such as using diverse raw materials, absence of thermal treatment, fermented sausages are highly subjected to quality deterioration, including mainly lipid oxidation and bacterial growth (Tomović *et al.*, 2020). Many studies have reported that some pathogens (e.g., *Listeria monocytogenes, Salmonella* and *Escherichia coli* etc.) can survive in DFS (Lindqvist & Lindblad, 2009) and cause many cases of foodborne disease outbreaks in many countries (Heir *et al.*, 2013). Food poisoning from *S. aureus, Salmonella* spp. (Toldrá, 2010), *L. monocytogenes* (Christieans *et al.*, 2018; Meloni, 2015)*, Clostridium* (Barbuti & Parolari, 2002)*, Penicillia* (CHAVES-LÓPEZ1 *et al.*, 2012; Núñez *et al.*, 2015) and *Aspergillus* species (Iacumin *et al.*, 2017; Kocić-Tanackov *et al.*, 2020) has been widely implicated in DFS.

Synthetic additives have been accused for raising many carcinogenic and toxic problems (Jayasena & Jo, 2013). Due to the growing concerns regarding the food safety and harm of chemical and synthetic preservatives, natural antimicrobials have been the attractive alternative trend for the food market (Falleh *et al.*, 2020). Essential oils (EOs) are aromatic and volatile liquids extracted from plant materials, such as flowers, roots, bark, regarded as secondary metabolites (Hyldgaard *et al.*, 2012). EOs such as clove, oregano, thyme, nutmeg, basil, mustard, cinnamon oil are Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) (Kalogianni *et al.*, 2020). EOs and their components have shown great antibacterial, antiparasitic, insecticidal, antiviral, antifungal and antioxidant properties in previous research (Huang *et al.*, 2021).

The greater dose of EOs are required in applications to meat and meat products where fats, carbohydrates, proteins, and salts can interact with some phenolic compounds (Jayasena & Jo, 2013). Encapsulation is a promising technology to protect bioactive compounds from inactivation by reacting with the food ingredients discussed above (Castro-Rosas *et al.*, 2017). Encapsulation is tended to mask the unwanted smells or flavors of EOs by coating or entrapping EOs within another inert shell material, which isolates and protects the core materials (Gómez *et al.*, 2018;

Turasan *et al.*, 2015). Encapsulation offers a method in which core materials are protected from the environment, the wall materials carry, protect and can be used to deliver functional compounds to target specific sites or to improve their flow and organoleptic properties (Castro-Rosas *et al.*, 2017; Gómez *et al.*, 2018).

Food irradiation is a physical, safe, environmentally clean, and efficient technology that exposes foods to the direct action of electronic, electromagnetic rays to ensure the safety of foods and to prolong shelf life (Lacroix, 2014). The use of radiation may result adverse effects on the chemical and sensory quality of food products especially when applied at high doses (Criado *et al.*, 2019). It can be emphasized that gamma irradiation has proven a synergistic action when used in combination with essential oils (Caillet *et al.*, 2005).

This study therefore encapsulated EOs into biopolymers, combined treatment of irradiation and applied on DFS intended to improve microbial and physicochemical quality and extend shelf life.

2 LITERATURE REVIEW

2.1 Introduction to Dry Fermented Sausages (DFS)

2.1.1 Context Dry Fermented Sausage Manufacturing

Generally, dry fermented sausages are defined as sausages which have a final pH ranging between 5.2 and 5.8 after fermentation and drying in consistent with the lower lactic acid content (0.5% – 1.0%), the moisture lower than 30%, Aw ranging from 0.85 to 0.91, and moisture : protein ratio lower than 2.3 : 1 (Vignolo *et al.*, 2010). DFS hold a big portion in meat production in mediterranean countries and Germany. In Spain, one-fifth of the total meat products manufactured are dry-cured sausages (Fernández-López *et al.*, 2008). DFS are manufactured by mixing fresh or frozen meat (70–80%) and back fat (20–30%), food additives (nitrate, nitrite, antioxidants), salt, starter cultures and spices (Tomović *et al.*, 2020). The production of salchichón, a typical Spanish dry-fermented sausage, includes three well-defined steps: mixing of ingredients, fermentation and drying (Fernández-López *et al.*, 2008). During the three steps, the physical, chemical and microbiological diversifications occur on DFS are closely related to the raw material characteristics and the process conditions, and determine the shelf life and safety also the organoleptic properties of the final product (Fernández-López *et al.*, 2008; Houben & van 't Hooft, 2005).

From a technical standpoint, DFS are results of a series of consecutive events, and the proper occurrence of these events is the key to a successful outcome. According to Barbuti & Parolari (2002) and Franciosa *et al.* (2018), first phase is grinding of meat and fat chunks, usually in their original variable size and shape, into uniform condition of a certain particle size (typically 0.8–10 mm) followed by blending all the salts, spices, starter cultures and additives operated under vacuum or modified atmosphere. Then the mixture is stuffed into casings according to the size of products, the encased mass is thereafter tied with thread or fastened ropes or metal clips and transferred to a fermentation chamber with certain temperature and humidity. When the pH values of DFS have reached specific fermentation ending point, the DFS will then be transferred to a drying room operated under controlled temperature, humidity and air flows. Depending on the size of DFS, the drying period to achieve the expected Aw as the ending point could vary from several days to months (Houben & van 't Hooft, 2005).

2.1.2 Starter culture and mechanism of acidification

A quantity from 0.3–3% sugars are added in DFS to ensure the convert primarily to lactic acid by supernumerary lactic acid bacteria during fermentation, which reduces the pH of the sausage within a few days (Houben & van 't Hooft, 2005). Meat fermentation is a complex biological phenomenon in presence of a variety of synergistic or competing species (including bacteria, yeasts and molds), which can be accelerated by the ideal action of certain microorganisms (Barbuti & Parolari, 2002). Through fermentation and drying process, highly perishable raw materials are transformed into microbiologically stable and organoleptic defined products (Cocolin *et al.*, 2011). The aroma development of fermented sausages is tightly influenced by the process of fermentation and drying, therefore variations in the quality of final products are hard to minimize in traditional practices (Rantsiou & Cocolin, 2006). The metabolic activity of the added starter cultures standardizes product properties such as flavor and color and shorter ripening time to improve the production (Barbuti & Parolari, 2002; Hammes & Hertel, 1998).

A starter culture should dominate over other microorganisms, conduct the fermentation and colonize the sausages during the whole process (Rantsiou & Cocolin, 2006). The use of starter cultures that contain actively growing or resting forms of microorganisms is becoming more common in the modern sausage production. The common starter cultures used in meat industry are lactic acid bacteria (LAB), gram-positive catalase-positive cocci (GCC+) (mainly *staphylococci*), molds, and yeasts (Laranjo *et al.*, 2017). LAB produces large quantities of growth-inhibiting substances which contribute to the taste and texture of DFS (Cocconcelli & Fontana, 2008). Homofermentative and heterofermentative LAB are two types of LAB divided by the sugar fermentation metabolic patterns. The homofermentative LAB ferment hexoses through glycolysis by the Embden-Meyerhof-Parnas pathway producing basically only lactic acid, whereas the heterofermentative LAB ferment pentoses mainly through the phosphoketolase pathway producing lactic acid, CO_2 and ethanol or acetate (Von Wright & Axelsson, 2011). Laranjo *et al.* (2017) pointed out that in producing DFS, LAB acidify the batter, accelerate the coagulation of muscle protein, thereby improve the slice stability, hardness and cohesion of DFS. Moreover, LAB also form noticeable vinegary tastes contribute to the flavor of the final products (Molly *et al.*, 1997). Another meat starter cultures, coagulase-negative *staphylococci* (CNS), mainly attribute to the color development due to their nitrate reductase activity (Laranjo *et al.*, 2017).

Some yeasts have been shown to contribute to flavor and texture development throughout the curing process (Selgas & Garcıa, 2007). Flores *et al.* (2004) reported that *Debaryomyces* spp.

at optimized concentrations have a positive effect on the sausage aroma and sensory quality by inhibiting the production of rancidity and ethyl esters.

The use of molds can form appealing flavors to consumers (Berni, 2014; Laranjo *et al.*, 2017). Previous research of Sunesen & Stahnkehas (2003) reported that DFS produced with commercial molds showed more consistent taste, flavor, drying rate, and a more uniform appearance. The undesirable consequences caused by molds are health risks associated with the production of highly toxic secondary metabolites, mycotoxins by some unwanted growth of molds, such as penicillin produced by species of *Penicillium* (Papagianni *et al.*, 2007).

2.1.3 Dry Fermented Sausage associated risks

Because of the relative high level of fat and distinctive manufacturing processing features such as using diverse raw materials, absence of thermal treatment, fermented sausages are highly subjected to quality deterioration including lipid oxidation and bacterial growth (Tomović *et al.*, 2020). Food manufacturers need to demonstrate the safety of products by following the principles of HACCP and GMP, control or prevent growth of pathogens during the process and reduce contamination to the lowest possible level (Franciosa *et al.*, 2018). There exist several processing steps during the manufacturing of DFS that contribute to safety control: low water activity that could below the growth limit of most pathogens, and decreased pH resulting a better bacterial control (Barbuti & Parolari, 2002).

However, many studies have reported that some pathogens (e.g., *L. monocytogenes, Salmonella* and *E. coli* etc.) can survive in DFS (Lindqvist & Lindblad, 2009) and cause cases of foodborne disease outbreaks in many countries (Heir *et al.*, 2013). Food poisoning from *S. aureus, Salmonella* spp., and *Clostridium* has been widely implicated in DFS (Barbuti & Parolari, 2002; Toldrá, 2010). The major bacterial hazards of concern in DFS include *Verocytoxigenic Escherichia coli* (VTEC) from beef and *Salmonella* from pork (Ducic *et al.*, 2016). *Enterohaemorrhagic Escherichia coli* (EHEC), a subgroup of *Shiga toxigenic E. coli* (STEC), can cause severe human illness and is related to many of these outbreaks (Van Ba *et al.*, 2017). New guidelines governing manufacture of these products were introduced due to a series of outbreaks of *E. coli* O157:H7 related to consumption of DFS and product recalls, requiring a validation of the manufacturing process for eliminating *E. coli* O157:H7 (Holck *et al.*, 2011; Muthukumarasamy & Holley, 2007).

A high proportion of Salmonella-positive units especially *S.* typhimurium were reported for pork meat which is one of the optional meat types of some dry fermented sausages (Christieans

et al., 2018; Talon *et al.*, 2004; Van Ba *et al.*, 2017). *Salmonella,* one of the most frequently reported food-borne pathogens in meat-producing livestock, especially poultry and pigs, is therefore found in/on meat and meat products (Ducic *et al.*, 2016). In the production of DFS, raw meat and some possible non-meat ingredients may contain *Salmonella* that cause high initial, processing and post-processing contaminations, especially for sliced products (Gieraltowski *et al.*, 2013).

L. monocytogenes is a ubiquitous, psychrotrophic pathogen famous for its ability of forming biofilms and meat processing contamination (Gieraltowski *et al.*, 2013). Human cases of listeriosis are mostly related to *L. monocytogenes* (Meloni, 2015), which is also the most frequently-detected pathogens in DFS due to its high tolerance to low pH and high salty conditions (Christieans *et al.*, 2018; Meloni, 2015).

During the drying of DFS, ecological conditions are conducive to the development of uncontrolled mold populations, mainly composed by *Penicillia* (CHAVES-LÓPEZ1 *et al.*, 2012; Núñez *et al.*, 2015). López-Díaz *et al.* (2001) found that 38 strains out of 54 isolated from a natural white covering of Spanish fermented meat sausages characterized to be *Penicillium***.** Núñez *et al.* (2015) pointed out that several species of *Penicillium* are potentially able to produce mycotoxins such as ochratoxin A, patulin or cyclopiazonic acid on DFS that are hazardous for consuming. *Penicillium nordicum,* a moderate psychotropic organism and able to survive in environments rich in salts and proteinsis, is considered responsible for the production ochratoxin A (Kocić-Tanackov *et al.*, 2020). *Aspergillus ochraceus* and *A. westerdijkiae* which belong to *Aspergillus* species which present slightly less than *Penicillium* species (Kocić-Tanackov *et al.*, 2020)**,** are also capable to produce mycotoxin in fermented sausages (Lacumin *et al.*, 2017).

2.2 Essential oils

EOs are aromatic oily liquids that are extracted from parts of plants like flowers, buds, seeds, leaves, fruits, roots etc. (Burt, 2004). The extraction methods including conventional (steam distillation, hydrodistillation, solvent extraction) and innovative (supercritical fluid extraction, microwave-assisted extraction, ultrasound-assisted extraction) methods, should be appropriate selected for EOs without affecting their characteristics (Pateiro *et al.*, 2018). EOs are highly complex mixtures of hundreds of aroma compounds (Calo *et al.*, 2015) with diverse antimicrobial activities all characterized by low molecular weight (Jayasena & Jo, 2013). The active compounds can be divided into two groups of distinct biosynthetical origin (Bakkali *et al.*, 2008) including the

major one of terpenes and terpenoids and the other one of aromatic and aliphatic constituents (phenylpropanoids) (Jayasena & Jo, 2013). Many EOs and their active compounds have been proved with great antimicrobial activities in vitro individually and aggregately (Chouhan *et al.*, 2017; Van de Vel *et al.*, 2019).

The antimicrobial activity of EOs results from the complex interactions between their compounds such as phenols, alcohols, aldehydes, esters, ethers or methoxy derivatives (Burt, 2004; Jayasena & Jo, 2013). The bioactivities of EOs are closely related to the main components. Bassolé & Juliani (2012) have shown high antimicrobial properties of the EO components when tested separately. The interaction between EO compounds includes four possible types of effects: indifferent, additive, antagonistic, or synergistic effects (Burt, 2004).

The antimicrobial activity of EOs is not dependent on a single mechanism, the action varies when different components treat to different microorganisms (Pateiro *et al.*, 2021). Mechanisms have been proposed to be the actions of chemical compounds in EOs (Burt, 2004). The most common mechanism of antimicrobial effects is membrane disruption resulting in increasing fluidity and permeability, leakage of intracellular constituents, disturbing embedded proteins, inhibiting respiration etc. due to the accumulation of bioactive compounds in the phospholipid bilayer of the cytoplasmic membrane (Calo *et al.*, 2015; Huang *et al.*, 2014; Pateiro *et al.*, 2021).

2.3 Microencapsulation of bioactive compounds

When applying EOs in food, some bioactive compounds can interact with food ingredients or be degraded by proteolytic enzymes that may reduce their effectiveness against microorganisms (Burt, 2004; Castro-Rosas *et al.*, 2017; Jayasena & Jo, 2013). Therefore, higher concentrations of EOs are required when use in food models than *in vitro,* which could affect the organoleptic quality of food (Hyldgaard *et al.*, 2012). Encapsulation preserves shells to antimicrobials that protect the core compounds from inactivation by environmental factors and reactions with food components (Castro-Rosas *et al.*, 2017).

Microencapsulation is defined as a process in which tiny particles or droplets are surrounded by a coating or embedded in a homogeneous or heterogeneous matrix, usually polymers, resulting in small capsules (1–1000 μm) with many useful properties (Figure 2.1) (Gouin, 2004; Nazzaro *et al.*, 2012). Microencapsulation of EOs is generally achieved in two steps. First, an emulsion of volatile compounds is prepared in an aqueous dispersion using a wall material which also functioned as an emulsifier. The emulsion then is dried under controlled conditions to reduce

the loss of encapsulated material caused by volatilization (Desai & Jin Park, 2005; Nazzaro *et al.*, 2012).

EOs in microencapsulation is capable to prolong the shelf life of food without lessening their characteristics in terms of quality and hygiene. Using microencapsulation in food packaging is able to cover up unpleasant tastes and odors, or to provide a barrier between sensitive biologically active substances and the environment (represented by food or oxygen) (Maresca *et al.*, 2016).

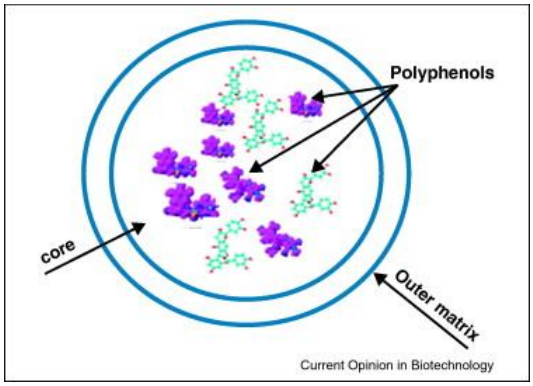

Figure 2.1 Example of a multilayer matrix used to entrap polyphenols (Adapted from Nazzaro *et al.*, 2012).

2.3.1 Alginate

Alginate, as a food grade, cheap, GRAS (generally recognised as safe), non-toxic and biocompatible polysaccharide is one of the most widely used materials for microencapsulation obtained mainly from marine algae and some bacteria (Maresca *et al.*, 2016). Alginate polymers have been applied to many different fields such as food, tissue engineering, drug delivery, and wound-dressing materials (Criado *et al.*, 2019). Alginate is composed of 1,4-linked α-L-guluronic acid (G) and β-D-mannuronic acid (M) residues therefore can form ionic hydrogels by cross-linking between the carboxylate anions of guluronic acid and divalent cations (Zarate *et al.*, 2011). When alginate gel contacts with calcium chloride, a so-called “egg box structure” (Figure 2.2) forms between the four G residues (Fernando *et al.*, 2020; Huq *et al.*, 2017). It’s hard to control permeability for alginate due to the wide pore-size distribution. The highly porous structure causes the leakage of macromolecules, which limits its use in whole-cell or organelle encapsulation (Zhang *et al.*, 2016a).

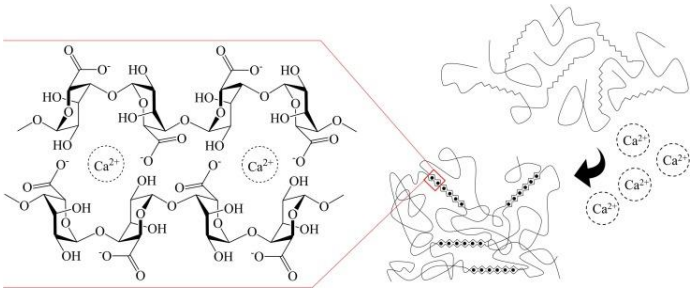

Figure 2.2 Gelation of alginate by calcium, the "egg-box" model (Adapted from Fernando *et al.*, 2020).

2.3.2 CNC

Health Canada defines cellulose nanocrystals (CNCs) as biodegradable and almost non-toxic nanomaterials, which exhibit excellent mechanical properties, emulsion stability and gel formation (Huq *et al.*, 2012; Zhu *et al.*, 2015). Due to its nanometer size and compositions of rod-like shaped nanoparticles, CNC holds the ability to create tortuous paths in a polymer matrix. Consequently, they can potentially delay the diffusion of compounds already dispersed in them and overcome the wide pore-size distribution of alginate (Criado *et al.*, 2019; Huq *et al.*, 2017).

The average diameter of these CNCs is 5-10 nm and the average length is about 100 nm (Khan *et al.*, 2014). The CNC used in this study has a negatively charged surface area at neutral pH, which may conduct an impact on alginate during probiotic encapsulation with $CaCl_2$ (Huq *et al.*, 2017; Huq *et al.*, 2012; Khan *et al.*, 2012). In previous study of Huq *et al.* (2017), it was found that CNC has an excellent reinforcing property with alginates which improves the poor mechanical properties of alginate matrix during the stabilization process (freeze-drying).

2.3.3 Alginate-CNC

Huq *et al.* (2017) developed modifications forming alginate with CNC. Probiotic (*Lactobacillus rhamnosus*) was encapsulated in alginate-CNC-lecithin microbeads to produce nutraceutical microcapsules by ionotropic gelation method. The research discovered that CNC increased the tensile strength of alginate microbead and improved the viability of probiotic during freeze drying and gastric passage. Nisin was microencapsulated into alginate-CNC beads by Huq *et al.* (2014) and applied on ready-to-eat ham. The encapsulation significantly lowered the counts of *L. monocytogenes* and retained the physicochemical properties (pH and color) of ham during storage. Criado *et al.* (2019) found that thyme EO loaded alginate contribute to *L. innocua* elimination and shelf-life extension applied on ground lean pork. The former work showed the

great potential of alginate-CNC microbeads applying in DFS to achieve good antimicrobial consequences without or with little organoleptic changes.

2.4 Food irradiation

Food irradiation has been historically in use and is increasingly being accepted and widely recognized as a part of overall good manufacturing practice (GMP) and hazard analysis critical control points (HACCP) systems (Diehl, 2002; Shah, Mir, & Pala, 2021). Food irradiation is a process of exposing food to the controlled amounts of ionizing radiations such as γ rays, X-rays and accelerated electrons, to extend shelf-life and maintain nutrition (Indiarto, Pratama, Sari, & Theodora, 2020; Singh & Singh, 2020). γ rays and X rays are short wavelength radiations with very high associated energy levels (Lacroix, 2014). Cobalt-60 is the most commonly used radionuclide for food in the form of γ rays. The emitted energy can be used as high as 95% (Indiarto & Qonit, 2020). The gamma irradiation is a promising technology to control pathogenic and spoilage bacteria and could be applied to fresh, frozen, or cooked products (Huq *et al.*, 2015). Radiation at 2 - 7 kGy can eliminate potentially pathogenic nonspore-forming bacteria such as *Salmonella, S. aureus, Campylobacter, L. monocytogenes*, or *E. coli* O157:H7 (Lacroix, 2014). X-rays that have high penetrating power and no left radiation hazards are raising interests as low risk-significant radioactive sources (Indiarto & Qonit, 2020). However, less studies have been done in use of X-ray for food pasteurization (Begum *et al.*, 2020).

The mechanism through which irradiation inactivates microbes is mainly the damage to nucleic acids, or direct or indirect damage caused by oxidative radicals originating from the radiolysis of water. The difference in radiation sensitivity between microorganisms is related to their chemical and physical structure and their ability to recover from radiation damage. Therefore, the amount of radiant energy required to control microorganisms in food varies according to the resistance of a specific species and the amount of microorganisms present (Lacroix, 2014).

Previous study reported that oregano EO and irradiation treatment showed synergistic effects increasing the bacterial radiosensitization of *L. monocytogenes* and *E. coli* O157:H7 (Lacroix *et al.*, 2009). The internal ATP concentration and the murein wall of both bacteria were affected by irradiation and EO treatments. Irradiation resulted in a significant decrease of the concentration of intracellular ATP. EO treatment resulted in a significant reduction of intracellular ATP and meawhile an increase of extracellular ATP. The synergistic overall effect was observed when radiation was used in combination with oregano EO. Furthermore, Lyu *et al.* (2018) has found that gamma radiation assisted cinnamon oil, which might primarily target on the cell structures

of *S. putrefaciens,* to increase the alteration of bacterial structures that induced to the changes of ATP and intracellular pH of *S. putrefaciens.*

2.5 Application of combined treatments on meat products

The use of radiation may result in adverse effects on the chemical and sensory properties of food products especially when applied at high doses (Criado *et al.*, 2019). It can be emphasized that gamma irradiation has proven a synergistic action when used in combination with EOs (Caillet *et al.*, 2005). Turgis *et al.* (2009b) reported that when applied irradiation in presence of EOs, irradiation would assist EOs to increase the radio-sensitization of food pathogens and spoilage bacteria. Criado *et al.* (2019) observed that thyme EO loaded alginate beads and gamma irradiation showed synergistic effect against *Listeria innocua* and mesophilic total flora in ground meat. Irradiation at 3 kGy with the active alginate beads had a complete inhibition of *Listeria*. Synergistic effect was also observed when the irradiation at 1 kGy combined with thyme EO loaded alginate beads, which extended the shelf-life of meat 12 days more than the control.

Research on microencapsulation by Huq *et al.* (2015) revealed synergistic antimicrobial effect on ready-to-eat meat products during storage by combing microencapsulated EOs-nisin and γ-irradiation. Microencapsulated cinnamon EO and nisin in alginate-CNC combined with γ-irradiation at 1.5 kGy significantly improved the radiosensitivity of *L.monocytogenes*. Microencapsulated oregano EO and cinnamon EO with nisin showed the highest bacterial radiosensitization of 2.89 and 5 times to the control separately. Abdeldaiem *et al.* (2018) also observed that the combined treatments of gamma irradiation at doses of 1, 3, and 5 kGy and edible rosemary EO coating improved the quality and safety of silver carp fish fillets by eliminating bacteria and extending the refrigerated shelf life up to 24 days compared to 6 days for uncoated control samples, without affecting chemical and sensory properties of fillets negatively. Dini *et al.* (2020) has reported the combination of chitosan, cumin EO nanoemulsion, gamma irradiation was the most effective treatment to control the population of microbial flora and inoculated pathogens, slow down some certain physicochemical changes, and therefore extend the storage period of beef loins.

A synergistic effect was also observed on the combined treatments of γ or X-ray irradiation with a mixture of oregano (*Origanum compactum*) and thyme (*Thymus vulgaris*) EO inhibiting *E. coli* O157: H7, *S.* typhimurium and *L. monocytogenes* applied in rice (Begum *et al.*, 2020). Addition of 1% ginger extract following with a treatment with γ-irradiation at 3 kGy or 5 kGy on processed frozen beef sausages were found sufficient to keep *E. coli* within safe levels for 3

months (Sediek *et al.*, 2012). The combination of EO or encapsulated EO treated with γ-irradiation also help to achieve a 5-log reduction for the procedures to assure the safety of fermented meats according to the USDA/FSIS (Porto-Fett *et al.*, 2008).

2.6 Hypothesis and objectives

2.6.1 Project goals

This project aims to extend the shelf-life of dry fermented sausages by treating with encapsulated natural antimicrobials combined with irradiation.

2.6.2 Hypothesis

1. The factorial design method can permit the identification of synergistic effects between EO combinations.

2. Microencapsulation can protect the active compounds against external stress and can improve the bioactivity and the stability of the antimicrobial properties over time.

3. The irradiation in presence of natural antimicrobial compounds can enhance the antimicrobial abilities of the irradiation treatment without quality alterations of the food.

4. The combined treatments of microencapsulation and γ-irradiation can act in synergy in inhibiting microbial flora and extending shelf life with no effects on physico-chemical and on organoleptic properties

2.6.3 Specific objectives

Objective 1: Develop essential oil contained formulation efficiently inhibit selected bacteria and fungi.

Objective 2: Encapsulate the formulations obtained in 1 and evaluate *in vitro* the antimicrobial effects of encapsulation.

Objective 3: Evaluate *in situ* the antimicrobial capacity of essential oil formulation, encapsulation, and irradiation.

Objective 4: Evaluate the physicochemical properties of sausages (color, texture) during storage.

2.6.4 Objective realizations

Objective 1:

The minimum inhibitory concentrations (MIC) according to Turgis *et al.* (2012a) was used for evaluating the antimicrobial activity of selected EOs against microorganisms *in vitro*. Then the selected EOs were used in a full factorial design targeted at bacteria and fungi separately. The significance of interactive effects between two-EO, three-EO until five-EO was analyzed and the combinations that were effective to more microorganisms were chosen as the most potential EO combinations of synergism. The combinations at last were verified synergism by Fractional inhibitory concentration (FIC) method (Turgis *et al.*, 2012a). The final compositions and concentrations of EO formulas were determined by FIC.

Objective 2:

The EO formula was encapsulated in alginate or alginate-CNC following the method of Huq *et al.* (2015) and added in fresh meat for sausage manufacturing. Total four groups of sausages were identified including control, EO formula, alginate encapsulation and alginate-CNC encapsulation. The BHI-agar well model and agar disk modified from the method of Bi *et al.* (2011), were used for evaluating the antimicrobial activities *in vitro*.

Objective 3:

Sausages were treated with X- or γ-irradiation at 1.5 kGy after drying. Then all sausages were stored at room temperature (20 - 21 °C). Microbial tests of analyzing counts of TMF, LAB, *E. coli* O157:H7 cocktail, *Listeria monocytogenes*, molds and yeasts were conducted during manufacturing and storage.

Objective 4:

Physico-chemical tests of interior and exterior color evaluations following the method of Lau (2019) and texture evaluation following the method of Houben & van ‘t Hooft (2005). Tests were conducted during storage.

3 PUBLICATION 1 – REVIEW ARTICLE

Essential oils as natural antimicrobials applied in meat and meat products – A review

Les huiles essentielles en tant qu'antimicrobiens naturels appliqués dans la viande et les produits carnés - Une revue

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Journal:

Critical Reviews in Food Science and Nutrition (2021): 1-17.

Submitted on 27-05-2021

Published online: 26 Jul 2021

DOI: https://doi.org/10.1080/10408398.2021.1957766

Contribution of authors:

JJ wrote the first draft of the manuscript. FR contributed to the collection of research papers. JJ, S Shankar, S Salmieri and ML contributed to manuscript revision, read, and approved the submitted version.

3.1 Abstract

Meat and meat products are highly susceptible to the growth of micro-organism and foodborne pathogens that leads to severe economic loss and health hazards. High consumption and a considerable waste of meat and meat products result in the demand for safe and efficient preservation methods. Instead of synthetic additives, the use of natural preservative materials represents an interest. Essential oils (EOs), as the all-natural and green-label trend attributing to remarkable biological potency, have been adopted for controlling the safety and quality of meat products. Some EOs, such as thyme, cinnamon, rosemary, and garlic, showed a strong antimicrobial activity individually and in combination. To eliminate or reduce the organoleptic defects of EOs in practical application, EOs encapsulation in wall materials can improve the stability and antimicrobial ability of EOs in meat products. In this review, meat deteriorations, antimicrobial capacity (components, effectiveness, and interactions), and mechanisms of EOs are reviewed, as well as the demonstration of using encapsulation for masking intense aroma and conducting control release is presented. The use of EOs individually or in combination and encapsulated applications of EOs in meat and meat products are also discussed.

Keywords: Essential oils; Antimicrobial activity; Foodborne pathogens; Encapsulation; Natural preservatives; Meat products

3.2 Résumé

La détérioration de la viande par les agents pathogènes d'origine alimentaire entraîne de graves pertes économiques et des risques pour la santé. Une consommation élevée et un gaspillage considérable de viande et de produits carnés entraînent une demande de méthodes de conservation sûres et efficaces. Au lieu d'additifs synthétiques, les huiles essentielles (HE), en tant que composés entièrement naturels et « clean label », ont été adoptées pour contrôler la sécurité et la qualité des produits carnés. Certaines HE, telles que le thym, la cannelle, le romarin et l'ail ont montré une forte activité antimicrobienne individuellement et en combinaison. Pour éliminer ou réduire les défauts organoleptiques des HE dans une application pratique et pour assurer une bioactivité durant toute la période d'entreposage, l'encapsulation des HE contribue à protéger la capacité antimicrobienne des HE dans les produits carnés. Dans cette revue, les détériorations de la viande, la capacité antimicrobienne (composants, efficacité et interactions) et les mécanismes des HE sont passés en revue, ainsi que la démonstration de l'utilisation de l'encapsulation pour masquer un arôme intense et effectuer une libération contrôlée est présenté. L'utilisation des HE individuellement ou en combinaison, et les applications des HE encapsulées dans la viande et les produits carnés sont également discutées.

Mots-clés: Huiles essentielles; Activité anti-microbienne; Pathogènes d'origine alimentaire; Encapsulation; Conservateurs naturels; Produits carnés

3.3 Introduction

Meat consumption is rapidly increasing due to the growing world population and world economy (Lee *et al.,* 2020; Ponnampalam *et al.,* 2019). Meat and meat products contain various nutrient compositions, including high-quality protein content, essential amino acids, B-group vitamins, minerals, and other nutrients (Pateiro *et al.,* 2021), ideal for the growth and propagation of meat spoilage micro-organisms and common foodborne pathogens (Zhou *et al.,* 2010). Atmospheric oxygen, temperature, moisture, light, endogenous enzyme activity, and growth of micro-organisms determine the quality and shelf life of meat (Chivandi *et al.,* 2016), of which the growth of micro-organisms is regarded so far the most significant factor in maintaining the safety and quality of meat although deteriorations can occur without micro-organisms (Zhou *et al.,* 2010). The major principle of meat quality control is to eliminate or reduce microbial deterioration (Niyonzima *et al.,* 2015) following Food safety objectives (FSO) and hazard analysis & critical control point (HACCP) systems (Liu *et al.,* 2021).

The spoilage of meat and meat products is associated with bacteria such as *Salmonella* spp., *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Clostridium* spp*, Pseudomonas*, *Acinetobacter, Brochothrix thermosphacta*, *Lactobacillus* spp., *Enterobacter* etc., as well as molds and yeasts, which can cause outbreaks which severely affect public health and the economy (Li *et al.,* 2020; Jayasena and Jo 2013). Current preservation methods include heating, chilling, high pressure, packaging, ionizing radiation, chemical preservative, bioactive compounds, and hurdle technologies (combining current and new food preservation techniques) (Jayasena and Jo 2013; Kalogianni *et al.,* 2020).

The high use of synthetic additives in food has raised many carcinogenic and toxic problems (Jayasena and Jo 2013; El-Wahab and Moram 2013). Colorants and flavor were found to cause cancer and lead to DNA damage (Kumar *et al.,* 2019). In addition, well-known food additives such as benzoates can initiate allergies such as erythrasma and asthma and are believed to result in brain damage (Pandey and Upadhyay 2012). Due to the growing concerns regarding the food safety and harm of chemical and synthetic preservatives, natural antimicrobials have been the attractive alternative trend for the food market (Falleh *et al.,* 2020). Plant extracts, essential oils, peptides, vitamin C (ascorbic acid), vitamin E (tocopherols), and protein hydrolysates have been proposed to prevent oxidation in processed meat products (Carocho *et al.,* 2014; Jiang and Xiong 2016).

Essential oils (EOs), a rich mixture of diverse bioactive chemical components, are aromatic and volatile liquids extracted from plant materials, such as flowers, roots, bark, regarded as

secondary metabolites (Hyldgaard *et al.,* 2012; Hassoun and Emir Çoban 2017). EOs are widely accepted by consumers, attributing their high volatility, ephemeral, and biodegradable nature (Falleh *et al.,* 2020). Some EOs are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) (Kalogianni *et al.,* 2020). EOs and their components have shown excellent antibacterial, antiparasitic, insecticidal, antiviral, antifungal, and antioxidant properties in previous research (Hyldgaard *et al.,* 2012). Considering the application in meat and meat products, EOs from oregano, rosemary, thyme, clove, cinnamon, mustard, and garlic have shown a greater potential to be used as an antimicrobial agent (Aziz and Karboune 2018; Chivandi *et al.,* 2016; Ghabraie *et al.,* 2016a).

Generally, higher doses of EOs are required for their application on meat and meat products (Jayasena and Jo 2013). Food pH, storing temperature, contamination levels, and the interactions of hydrophilic compounds of EOs with food matrix components such as fats, carbohydrates, proteins, and salts could affect the antimicrobial activity of EOs (Hyldgaard *et al.,* 2012). Encapsulation tends to mask the unwanted smells or flavors of EOs by coating or entrapping EOs within another inert shell material, isolating and protecting the core materials from inactivation by reacting with the food ingredients discussed above (Castro-Rosas *et al.,* 2017; Gómez *et al.,* 2018; Turasan *et al.,* 2015). The proper wall materials should have good mechanical strength that can offer firm protection to core materials, be compatible with food products, adapt to different environmental conditions, and conduct controlled release (de Souza *et al.,* 2018; Majeed *et al.,* 2015). There are several wall materials mostly used for encapsulation of EOs such as chitosan, gelatin, whey protein, gum arabic, maltodextrin, sodium caseinate, and modified starches (Gómez *et al.,* 2018; Majeed *et al.,* 2015). Generally, there are four main encapsulation types including (i) particles: matrix where EOs are dispersed; (ii) capsules: a membrane surrounds the core of EOs; (iii) complexes: EOs are stabilized in cavities by chemical interactions; and (iv) droplets: EOs dispersed in a solvent with surfactants (Maes *et al.,* 2019).

This review provides an overview of the published data on the antimicrobial activity of EOs and their components that could be potentially applied in meat and meat products. The current understanding of the possible mechanisms, synergies, limitations, and encapsulations of EOs was also presented.

3.4 Microbial deterioration of meats

Meat is a complex food ecological niche and rich in essential nutrients that strongly support the growth of a large number and variety of micro-organisms (Jayasena and Jo 2014; Russo *et*

al., 2006). The presence and growth of spoilage micro-organisms in meat and meat products can differ depending on the storage conditions such as temperature, water activity, and oxygen availability (Hernández-Macedo *et al.,* 2011; Labadie 1999). *Pseudomonas* spp. and lactic acid bacteria are always the dominant bacteria when meats are stored aerobically at chilled temperatures and refrigerated temperatures, respectively (Labadie 1999; Berruga *et al.,* 2005; Hernández-Macedo *et al.,* 2011; Russo *et al.,* 2006). Lactic acid bacteria can produce H_2S from cysteine, causing sour off-flavors, which thereafter oxidize myoglobin to metmyoglobin giving meat green colors (Hernández-Macedo *et al.,* 2011). Some LAB, like *Lactobacillus carnosum*, also produces CO_2 attributing to the "blowing" of vacuum packages (Doyle 2007; Hernández-Macedo *et al.,* 2011). *Brochothrix thermosphacta* has always been abundant in meats stored in aerobic or anaerobic conditions. It can metabolize glucose into lactic acid in anaerobic conditions, and subsequently, lactic acid into ethanol in aerobic conditions results in off-odors (Chaillou *et al.,* 2015; Pin *et al.,* 2002).

The great concern for causing outbreaks in the EU and USA includes *Salmonella* spp., *Escherichia coli* O157:H7, and other enterohemorrhagic *E. coli* (ETEC), *L. monocytogenes* and bacterial toxins produced by *Bacillus* spp., *Staphylococcus* spp. (*S. aureus*), and *Clostridium* spp. (Jayasena and Jo 2013; Kalogianni *et al.,* 2020). The growth of toxin-producing bacteria in meat is mainly responsible for the foodborne illness on consumption (Kalogianni *et al.,* 2020). *Escherichia coli* O157:H7 was reported in beef (Chaillou *et al.,* 2015; Gutema *et al.,* 2021), fermented and dried meats (Balamurugan *et al.,* 2020; Muthukumarasamy and Holley 2007) that can cause severe symptoms of hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Meng *et al.,* 2012). *L. monocytogenes* proved to be responsible for human listeriosis, which presents commonly in raw poultry, beef, and pork meat (Skowron *et al.,* 2020), ready-to-eat meats (Kurpas *et al.,* 2020), as well as in the meat processing plants which possibly transfered from the plant to meat and meat products during processing because of inefficient hygiene control (Duze *et al.,* 2021; Buchanan *et al.,* 2017). Fungi like *Penicillium* spp. and *Aspergillus* spp. were determined on dry-cured meats (Álvarez *et al.,* 2020; Zadravec *et al.,* 2019) or fermented sausages (López-Díaz *et al.,* 2001; Pleadin *et al.,* 2017), are responsible for the diseases (mycotoxicoses) caused by mycotoxins including majorly aflatoxin B_1(AFB_1) and ochratoxin A (OTA) (Zadravec *et al.,* 2020; Pleadin *et al.,* 2017).

3.5 Essential oils

3.5.1 Components of EOs

Essential oils are aromatic oily liquids extracted from parts of plants like flowers, buds, seeds, leaves, fruits, roots, etc., (Burt 2004). The extraction methods, including conventional (steam distillation, hydrodistillation, solvent extraction) and innovative (supercritical fluid extraction, microwave-assisted extraction, ultrasound-assisted extraction) methods, should be appropriately selected for EOs without affecting their characteristics (Pateiro *et al.,* 2018). Essential oils are highly complex mixtures of low molecular weight aromatic compounds (Calo *et al.,* 2015) with diverse antimicrobial activities (Jayasena and Jo 2013). The active compounds can be divided into two groups of distinct biosynthetic origin (Bakkali *et al.,* 2008), including the major one of terpenes and terpenoids and the other one of aromatic and aliphatic constituents (phenylpropanoids) (Jayasena and Jo 2013). Terpenes are the combination of isoprenes, a 5-carbon-base (C_5) unit, when contain oxygen terpenes are called terpenoids (Bakkali *et al.,* 2008). The most common terpenes are the monoterpenes (C_{10}) which make up 90% of the EOs, with various structures serving several functions (Bakkali *et al.,* 2008). Aromatic compounds derived from phenylpropane constitute less in EOs. The phenolic compounds with a polar functional group potentially determine the antimicrobial activity of the EOs (Pateiro *et al.,* 2021; Barbosa *et al.,* 2009). Therefore, generally, higher content of phenolic compounds present stronger antimicrobial abilities (Alirezalu *et al.,* 2020).

3.5.2 Mode of action of EOs

The antimicrobial activity of EOs is not dependent on a single mechanism, and the action is different for the different components of different micro-organisms (Pateiro *et al.,* 2021). Mechanisms have been proposed to be the actions of chemical compounds in EOs (Burt 2004). The most common mechanism of antimicrobial effects is membrane disruption (Pateiro *et al.,* 2021). The accumulation of bioactive compounds in the phospholipid bilayer of the cytoplasmic membrane results in damage of cytoplasmic membranes, increased fluidity and permeability, leakage of intracellular constituents, disruption of embedded proteins, and cell death (Calo *et al.,* 2015; Huang *et al.,* 2014; Pateiro *et al.,* 2021). Greater resistance of Gram-positive bacteria was reported probably due to the thick layer of peptidoglycan of the cell walls (Guimarães *et al.,* 2019). The obstructure of porin channels of the outer membrane of Gram-negative bacteria may have higher resistance to hydrophobic compounds (Bharti *et al.,* 2020). In the previous research, many EOs or their components, such as mustard, thyme, oregano, cinnamon, garlic EOs, and thymol,

carvacrol, cinnamaldehyde, eugenol have shown wide-spectrum antimicrobial activities against foodborne pathogens, including *E. coli* (Clemente *et al.,* 2016; Yuan *et al.,* 2019), *Listeria monocytogenes* (Dussault *et al.,* 2014; García-Díez *et al.,* 2017), *Salmonella typhimurium* (Ghabraie *et al.,* 2016a; Oussalah *et al.,* 2007), and food spoilage fungi such as *Aspergillus* spp. (Clemente *et al.,* 2019; Hossain *et al.,* 2016; Kocić-Tanackov and Dimić 2013), *Penicillium* spp. (Clemente *et al.,* 2019; Hossain *et al.,* 2016; Li *et al.,* 2014). Mustard EO has 10 times more bactericidal (EOs kill bacterial cells) or bacteriostatic (EOs inhibit the bacterial growth then the microbial cells may recuperate their reproductive ability) effect than cinnamon EO (Clemente *et al.,* 2016; Falleh *et al.,* 2020). This could be explained by the different actions of two EOs. Mustard EO could affect cell membrane, cause leakage of intracellular ATP (Turgis *et al.,* 2009), induce cell cycle arrest and filamentation (Clemente *et al.,* 2016). However, cinnamon EO could act on the membrane producing lumps, increase cell permeability, cause auto aggregation, leakage of electrolytes (Clemente *et al.,* 2016; Huang *et al.,* 2014). It was observed that Chinese cinnamon EO induced less depletion of the intracellular ATP concentration of bacteria than Spanish oregano and savory EOs but reduced more intracellular pH of *E. coli* O157:H7 that affected DNA transcription, protein synthesis, and enzyme activity of bacteria (Oussalah *et al.,* 2006). Garlic EO has great antifungal activities by acting on multiple sites of the hyphae of *P. funiculosum* (Li *et al.,* 2014). EOs act in several ways inhibiting fungal growth, including cell membrane disruption, alteration, inhibition of cell wall formation, dysfunction of the fungal mitochondria, inhibition of efflux pumps, produce reactive oxygen species (Nazzaro *et al.,* 2017).

3.5.3 Effects of individual EOs

Many EOs and their active compounds have been proved with great antimicrobial activities *in vitro,* individually and in combination (Chouhan *et al.,* 2017; Van de Vel *et al.,* 2019). Lists of the frequently used EOs or active compounds in antimicrobial activity testing used singly and in combination are presented in Table 3.1 and Table 3.2, respectively.

Several methods were used to test the antimicrobial capacities of EOs, including disk diffusion, agar wells, agar dilution method, broth dilution, time-kill analysis/survival curves, scanning electron microscopy (Burt 2004). The minimum inhibitory concentration (MIC) is cited by most researchers, defined as the lowest concentration of EOs to completely inhibit the growth (bacteriostatic) of micro-organism within a certain time and under specific conditions (Van de Vel *et al.,* 2019). Dussault *et al.* (2014) reported the broad-spectrum antibacterial activity of oregano (*Thymus capitatus* Hoff.) and thyme (Thymus vulgaris and *Thymus zygis* L.

var. *gacilis* Boissier) EOs against all groups of bacteria among the tested sixty-seven essential oils, oleoresins, and pure compounds. Chinese cinnamon (*Cinnamomum cassia*) was found to be the most effective EO from 32 EOs against five foodborne and spoilage bacteria with its lowest MIC values (Ghabraie *et al.,* 2016a). Mustard EO and its main component, allyl isothiocyanate, showed a strong antibacterial capacity to foodborne bacteria (Dussault *et al.,* 2014; Peng *et al.,* 2014; Turgis *et al.,* 2009). The effectiveness of EOs varies with the distilled parts of plants, plants' origins, and producing seasons (Burt 2004; Dussault *et al.,* 2014; Ghabraie *et al.,* 2016a).

Several studies on the effects of EOs on meat and meat products have been performed, showing great antimicrobial abilities for extending the shelf life of products (Calo *et al.,* 2015). Some studies about applications of single EOs in meat and meat products are mentioned in Table 3.3. Oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), orange (*Citrus sinensis* var. Valencia) EOs used in the vapor phase had been proved to have good antibacterial activities (Luna-Guevara *et al.,* 2021). The amount of 2000 mg/L of oregano EO reduced most *Salmonella* populations of 1.97 log CFU/g after 144 h storage and was organoleptically acceptable in the attributes of odor, texture, color, and general acceptance of sausages. Sage EO (*Salvia officinalis* L) at concentrations of 0.075 µL/g and 0.1 µL/g significantly reduced the total number of aerobic mesophilic bacteria and inhibited *Salmonella* spp., *Escherichia coli,* and *Listeria monocytogenes* and even resulted in better sensory properties of fresh pork sausages (Šojić *et al.,* 2018). According to Dussault *et al.* (2014), the growth rate of *L. monocytogenes* for hams containing EOs of garlic (*Allium sativum* L.) and red thyme (*Thymus vulgaris* and *Thymus zygis* L. var. *gacilis* Boissier) were not significantly different from the control. However, EOs of oregano (*Thymus capitatus* Hoff.) and Chinese cinnamon (*Cinnamon cassia*) contributed to 19% and 10% growth inhibition of *L. monocytogenes* in hams, respectively. Zhang *et al.* (2016) observed a reduction of lipids oxidation and a high inhibition of *Pseudomonas* spp. and *Enterobacteriaceae* at both concentrations of 0.1% and 0.5% of black pepper EOs (*Piper nigrum* L.) on fresh pork. Da Silveira *et al.* (2014) displayed that sensory characteristics of the bay leaf EO (*Laurus nobilis* L.) treated fresh Tuscan sausages were found acceptable for both tested concentrations (0.05% and 0.1%). They observed a reduction of the micro-organisms (total *coliforms)* by nearly 3 log CFU/g and an extension of the product shelf life by 2 days in the experiments. Kingchaiyaphum and Rachtanapun (2012) showed that kaffir lime peel EO (*Citrus hystrix* DC.) has a stronger antioxidative effect than fingerroot EO (*Boesenbergia pandurata* Roxb.). Then, 10 % kaffir lime peel and fingerroot can extend shelf life of Chinese sausages by 5 and 10 days, respectively.

3.5.4 Synergistic effects of EOs

The antimicrobial activity of EOs results from the complex interactions between their compounds such as phenols, alcohols, aldehydes, esters, ethers, or methoxy derivatives (Burt 2004; Jayasena and Jo 2013). The bioactivities of EOs are closely related to the main components; however, many researchers proved the high antimicrobial properties of the EO components when tested separately (Bassolé and Juliani 2012). The interaction between EO compounds includes four possible types of effects: synergistic, additive, no interactive, or antagonistic effects (Burt 2004). An additive effect is defined as the combined effect is equal to the sum of the individual effects. Antagonism is defined as the combined effect is less than the sum of individual effects. Synergism is when the combined substances are greater than the sum of the individual effects, while the no interactive is defined as indifference (Burt 2004). The assessment of the interaction between essential oil components is based on using macro- or micro-dilution techniques, among these techniques, the checkerboard is the most commonly used (Mackay *et al.,* 2000). The fractional inhibitory concentration index (FIC) is defined as the sum of FIC_A and FIC_B as it is shown in Equation (3), where FIC_A is the MIC of compound A in combination divided by the MIC of compound A alone (A pure), as shown in Equation (1), and FIC_B the MIC of compound B in combination, divided by the MIC of compound B alone (B pure), as shown in Equation (2):

$$FIC_A = MIC_A \text{ combined} / MIC_A \text{ alone} \quad (1)$$

$$FIC_B = MIC_B \text{ combined} / MIC_B \text{ alone} \quad (2)$$

$$FIC = FIC_A + FIC_B \quad (3)$$

Synergistic effect is defined for $FIC \leq 0.5$; additive effect for $0.5 \leq FIC \leq 1$; no interaction for $1 < FIC \leq 4$ and for $FIC > 4$, is defined as an antagonistic effect (Ayari *et al.,* 2020; Šimunović *et al.,* 2020).

Oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) EOs showed significant synergistic effects to several pathogenic micro-organisms like *A. flavus, A. parasiticus, P. chrysogenum* (Hossain *et al.,* 2016), and *S. aureus, Salmonella, E. coli, Bacillus cereus* (Gavaric *et al.,* 2015). To be noticed, phenolic monoterpenes and phenylpropanoids (typical strong antimicrobial activities), when combined with other components, were found to be able to increase the bioactivities of these mixtures. Phenolic monoterpenes and phenylpropanoids in combination with other components, were found to increase the

bioactivities (Bassolé and Juliani 2012). The combinations of phenolic compounds with monoterpenes alcohols were observed synergistic or additive; for example, the combination of phenolics (thymol, carvacrol, eugenol) was synergistically or additively active against *E. coli* strains (Ayari *et al.,* 2020; Ju *et al.,* 2020; Yuan *et al.,* 2019).

Combinations could also be used to decrease the quantities of EOs applied *in situ* and lower the organoleptic impacts of EOs, and then enable to use a broader range of them to treat meat and meat products (Hyldgaard *et al.,* 2012). Some studies on the effects of EOs in combinations on meat products are reported in Table 3. The combinations of EOs have been widely applied in fresh meat and processed meat products and showed great biopreservation potential to extend the shelf life. Thanissery and Smith (2014) applied thyme-orange combination at 0.5% to marinade broiler breast fillets and whole wings that significantly reduced the total aerobic and facultative mesophilic numbers on day 1, 7, and 10 compared with the controls. Ghabraie *et al.* (2016b) conducted experiments of 16 formulations consisting of nisin (12.5–25 ppm), nitrite (100–200 ppm), mixed essential oils (EOs) of Chinese cinnamon (*Cinnamomum cassia*) plus Cinnamon bark (*Cinnamomum verum*) (0.025–0.05%) and mixed of potassium lactate and sodium acetate (1.55–3.1%) with irradiation at 1.5 kGy against *Clostridium sporogenes* in a sausage model and revealed good antibacterial activities of formulations. EOs combination of shirazi-thyme (*Zataria multiflora*), cinnamon (*Cinnamomum zeylanicum*), and clove (*Syzygium aromaticum*) can efficiently act against *P. fluorescens* at low combination doses and decrease the adverse sensory concerns of EOs applied in chicken breast meat stored at 4 °C (Chaichi *et al.,* 2021). Anacardiaceae (*Pistacia lentiscus*) and Lamiaceae (*Satureja montana*) EOs showed synergistic effects to reduce *L. monocytogenes* growth and extend the shelf life of minced meat during refrigerated storage (Djenane *et al.,* 2011). Vasilijević *et al.* (2019) combined Juniper (*Juniperus communis* L.) and winter savory (*Satureja montana* L.) EOs applied on red wine marinades tested against *L. monocytogenes*, *Enterobacteriaceae*, lactic acid bacteria, and aerobic heterotrophic mesophyll bacteria. The EO mixtures decreased all the microbial counts during storage and were all sensory acceptable on beef. Menezes *et al.* (2018) observed that the addition of oregano (*Origanum vulgare*) essential oil enhanced the shelf-life of vacuum-packed cooked sliced ham based on LAB levels and more than 30 days were extended when cooked hams stored at 6 °C comparing to control. Reduced counts of Enterobacteriaceae, total coliform and *Staphylococcus aureus* during ripening were investigated with addition of oregano (*Coridothymus capitatus*) (0.25% v/v) or thyme (*Thymus vulgaris*) (0.25% v/v) EO in Tunisian dry fermented poultry meat sausages (El Adab and Hassouna 2016). Six EOs, basil (*Ocimum basilicum* L), garlic (*Allium sativum* L.), nutmeg (*Myristica fragans*), oregano

(*Origanum vulgare*), rosemary (*Rosmarinus officinalis* L) and thyme (*Thymus capitatus* Hoff. et Link), were used at 0.005% and 0.05% separately on dry cured sausages chouriço showing an inhibitory effect against *Salmonella* spp., *Listeria monocytogenes* and *Staphylococcus aureus* along processing (García-Díez *et al.,* 2016).

3.5.5 Limitations of EOs

The interaction of bioactive compounds with meat product compositions may decrease the effectiveness of the EOs. The fats, proteins, carbohydrates, water content, salts, and food additives as well as environmental determinants like temperature, packaging in vacuum/gas/air affect bacterial sensitivity (Calo *et al.,* 2015; Jayasena and Jo 2013). Meat products contain high fat and protein that could dramatically decrease the antimicrobial properties of EOs due to their high binding capacity to volatile compounds of EOs (Sultanbawa 2011). Thereafter, when EOs are applied in meats, it is always required in higher concentrations than *in vitro* to achieve sufficient antimicrobial activity, which raised the adverse organoleptic problems (Hyldgaard *et al.,* 2012; Yuan *et al.,* 2019). The possible solutions proposed by previous research to solve these challenges by combined synergistic effects of EOs or their bioactive compounds, incorporating volatile components of EOs in films or edible coatings, encapsulation of EOs in polymers of edible, and biodegradable coatings or sachets or into micro- and nanoemulsions (Hassoun and Emir Çoban 2017; Jayasena and Jo 2013; Singh *et al.,* 2019).

3.6 Encapsulation

Encapsulation is a technology that protects EOs by the action of one or more wall materials that could avoid direct interaction with food components and increase the effectiveness of EOs (Barbosa *et al.,* 2021; Gómez *et al.,* 2018), conduct a control release and mask unpleasant odors to decrease the sensory impact on foods (Gulin-Sarfraz *et al.,* 2021; Nazzaro *et al.,* 2012). Encapsulation can be performed either mechanically (spray-drying) or chemically; chemically by simple or complex coacervation (Castro-Rosas *et al.,* 2017). Essential oils can be encapsulated into biopolymers (Heckert Bastos *et al.,* 2020; Jain *et al.,* 2020), liposomes (Kamkar *et al.,* 2021; Wang *et al.,* 2021), micro- or nanoemulsions (Delshadi *et al.,* 2020; Rolim and Ramalho 2021; Yang *et al.,* 2021). Capsules of rosewood (*Aniba rosaeodora*) and cinnamon (*Cinnamon cassia*) EO encapsulated by Tween 80 and poly (butylene adipate-co-terephthalate) (PBAT) were found to have excellent antimicrobial activity against *E. coli, Salmonella, S. aureu*s and *Listeria* (Barbosa *et al.,* 2021). The synthesis of nanoemulsions, microencapsulation and packaging films

applied in food preservation are widely reviewed (Davarcı *et al.,* 2017; Kfoury *et al.,* 2019; Prakash *et al.,* 2018; Vishwakarma *et al.,* 2016). A summary of some experiments carried out on encapsulation of EOs is presented in Table 3.4.

3.6.1 Nanoparticles

Nanoencapsulation could be a way to develop closer interactions between antimicrobial components and micro-organisms (Hyldgaard *et al.,* 2012). Nanoparticles (NPs) are nano-vehicles with particle sizes below 100 nm (Rehman *et al.,* 2019). Several techniques have been used to achieve natural biopolymeric NPs, such as nanospray drying, self-assembly, electrospraying, and anti-solvent precipitation (Lammari *et al.,* 2020; Prakash *et al.,* 2018; Rehman *et al.,* 2020a; Rehman *et al.,* 2020b).

Hassan *et al.* (2021) have reported nanoencapsulation of oregano (*origanum syriacum*) EO by chitosan nanoparticles significantly suppress the growth of microbial species. Badawy *et al.* (2020) indicated that ChMNPs (Monoterpenes loaded with chitosan to form nanoparticles) could be used as a good preservation method for minced meat. The proposed mechanism is that monoterpenes are sensitive to the phospholipid bilayer of the cell membrane of the bacteria causing damages to the enzyme systems and growth inhibition. Furthermore, the positively charged amino groups of chitosan (Ch) would interact with the negatively charged macromolecules on the microbial cell surface to make the leakage of intracellular constituents of the microbial cell. The film-forming property of Ch plays an important role in the antimicrobial property due to Ch as the oxygen barrier. Morsy *et al.* (2018) carried out an experiment in which they reported that lyophilized nanoparticles of pomegranate (*Punica granatum* L.) peel (LPP-NPs) were effective in retarding lipid oxidation and improving the microbial quality and cooking characteristics of meatballs. Ghaderi-Ghahfarokhi *et al.* (2017) demonstrated that cinnamon (*Cinnamomum zeylanicum* L.) essential oil-incorporated chitosan nanoparticles (CEO-CSNPs) reduced the microbial population of beef patties, lipid oxidation, and improved consumer acceptance. Then, Ghaderi-Ghahfarokhi *et al.* (2016) investigated that thyme (*Thymus vulgaris* L.) essential oil (TEO) loaded chitosan nanoparticles (CS-NP-TEO) exhibited several distinct advantages of improving the microbial, chemical, and sensory quality during storage of beef burgers.

3.6.2 Microencapsulation

Microencapsulation could be a promising method to pack the active and/or sensitive components such as EOs as the core into a wall matrix that allows a controlled release and avoids contact with the environment (Castro-Rosas *et al.,* 2017; Hashim *et al.,* 2019; Yostawonkul *et al.,* 2021). Microencapsulation could be achieved using different methods, such as spray-drying, simple and complex coacervation, extrusion, and precipitation (Hashim *et al.,* 2019). The encapsulating materials, such as sodium alginate (sod-Alg), chitosan (Ch), and carboxymethyl cellulose (CMC), are essential for the formation of an effective system (Fadel *et al.,* 2020). Thyme (*Thymus zygis*) and rosemary (*Rosmarinus officinalis*) EO encapsulated in chitosan which was applied on dry fermented sausages as cotings, showed inhibition to moulds and yeasts during 3-month storage (Demirok Soncu *et al.,* 2020).

The encapsulation of bioactive compounds into calcium alginate microspheres or beads is arousing more attention presently, which is affected by ionic gelation of the calcium in the alginate droplets and their conversion into hydrogel beads (Davarcı *et al.,* 2017). Fadel *et al.* (2020) conducted a comparative study on the microencapsulation of 10 commercial EOs into alginate beads. They found that the microencapsulation in the sodium alginate and chitosan improved the antioxidant activity and phenolic content of the encapsulated clove (*Syzygium aromaticum*) EO compared with carboxymethyl cellulose. Huq *et al.* (2015) presented a study in which microencapsulation of antimicrobials (EOs and nisin) combined with γ-irradiation treatments showed synergistic antimicrobial effect during storage on ready to eat (RTE) meat products. The micro-encapsulation had increased the bacterial radiosensitivity (RS) of oregano (*Origanum compactum*) and cinnamon (*Cinnamomum cassia*) both with nisin by 39 and 113% compared to free ones. Criado *et al.* (2019) has found that introduction of cellulose nanocrystals (CNCs) from 0 to 30% in alginate beads exhibited an increase of thyme (*Thymus vulgaris*) EO loading capacity and a longer continuous release period was noticed when thyme EO was 3% in beads. The microbeads contributed to a 2-log reduction of *L. innocua* during more than 10 days storage as compared to the control and a synergy between microbeads and irradiation was observed.

3.6.3 Active packaging

Essential oil incorporation in polymers can lead to physical changes such as the film structure, water barrier properties, and transparency, whereas it may provide edible films with antioxidant and/or antimicrobial properties (Atarés and Chiralt 2016). There are uses of packaging films and coatings in the active packaging technology (Ribeiro-Santos *et al.,* 2017). Using the technology

of incorporating EOs in functional packaging films can reduce the diffusion rate of EOs into food products, conduct a controlled release of active compounds to product surface that extend the shelf life of products without affecting the organoleptic properties (Hyldgaard *et al.,* 2012; Pateiro *et al.,* 2021) and help to maintain temperature, moisture, and quality control of the food (Sharma *et al.,* 2021). Biopolymers like proteins and polysaccharides, due to their nature of biodegradability, are drawing great interests in using for antimicrobial packaging films (Cha and Chinnan 2004; Vieira *et al.,* 2011). The mobility of volatile compounds of EOs introduced in the polymer matrix is a key point for understanding release mechanisms (Wicochea-Rodríguez *et al.,* 2019). The mechanism of the action of active packaging could be direct contact with food or through mass transfer to the headspace inside the package (Ribeiro-Santos *et al.,* 2017; Wicochea-Rodríguez *et al.,* 2019). The antimicrobial effectiveness could depend on the diffusion of active agents onto the food surface through the headspace from the packaging, sachet, coating, or pad (Marturano *et al.,* 2019). The non-contact approach allows a slower release of aromatic compounds, prolongs the efficiency period, and decreases the toxic level (Ribeiro-Santos *et al.,* 2017; Varghese *et al.,* 2020; Wicochea-Rodríguez *et al.,* 2019).

Clove and oregano EOs incorporated with palm oil in fish gelatin formed biodegradable packaging film showing antimicrobial and antioxidant activities (da Silva e Silva *et al.,* 2021). Esmaeili *et al.* (2020) showed that the chitosan film containing nano encapsulated garlic EO exhibited the best microbiological and chemical results. Pabast *et al.* (2018) highlighted that nano-encapsulation of Satureja EO coated in chitosan contributed to the sensory and microbial qualities and extension of shelf-life of lamb meat during chilled storage. Zhang *et al.* (2020) found that nano-encapsulation of tarragon EO (TEO) enabled the controlled release of the active compounds on the surface of pork samples and a chitosan-gelatin coating containing encapsulated TEO inhibited lipid oxidation, microbial growth, and improved sensory attributes that extended the shelf life of fresh pork slice by 8 days more than the control.

3.7 Conclusions

Meat and meat products are sufficient in nutrients that are highly conducive for the growth of spoilage and pathogenic micro-organisms. Esssential oils, as clean-label alternatives, can avoid the carcinogenic and toxic problems caused by synthetic food additives. The biological activity of EOs is intently related to the bioactive compounds of EOs, especially phenolic compounds, which can interact with cell membranes, affect permeability, and leak cell contents. Several EOs are observed to have synergistic effects eliminating or delaying the growth of micro-organisms. The

most common contradictory and tricky problem of applying EOs in food products is the maintenance of organoleptic properties of food products with relatively low doses of EOs at which EOs still show high antimicrobial abilities against micro-organisms. Generally, a higher concentration of EOs is required for food models, which usually leads to other unpleasant odors and tastes. Encapsulating EOs into one or more wall materials that carry, delivers, and release EOs controllably is one of the novel technologies to solve this problem. The use of combination of EOs, encapsulation, nisin, irradiation, high hydrostatic pressure, modified atmosphere packaging, etc., are novel technologies to be applied for safety and quality of meat and meat products. Moreover, with the trending use of EOs, it is necessary to develop the regulations including the maximum permissible limits, toxicity studies for food preservation.

3.8 Acknowledgments

The authors are grateful for the financial support from Natural Sciences and Engineering Research Council of Canada (NSERC, project no CRDPJ 531475 – 18), from Montpak Int. (Laval, Québec, Canada) under a research contract agreement, from the Ministry of Economiy and Innovation of the province of Quebec (PSO-1-2-44530) and from a chair granted by Quebec Ministry of Agriculture, Fishery and Food (MAPAQ) (PPIA12).

Table 3.1 Minimum inhibitory concentrations (MIC) of selected commonly used EOs against food borne pathogens.

Common name	Species	Main components	Pathogens, MIC ppm	References
Mustard	*Sinapis alba*	Allyl isothiocyanate 71%	*Staphyloccocus aureus*, 128 ppm *Micrococcus luteus*, 128 ppm *Staphyloccocus epidermidis*, 256 ppm *Escherichia coli*, 512 ppm *Bacillus subtilis*, 512 ppm *Shigella sonnei*, 512 ppm *Salmonella lignieres,* 256 ppm *Pseudomonas aeruginosa,* 256 ppm *Pseudomonas fluorescens,* 512 ppm	(Peng *et al.*, 2014).
Oregano	*Origanum vulgare*	Carvacrol, thymol	*Aspergillus niger,* 625 ppm *Aspergillus flavus,* 2500 ppm *Aspergillus parasiticus,* 2500 ppm *Penicillium chrysogenum,* 625 ppm	(Hossain, Follett, Dang Vu, *et al.*, 2016).

	Thymus capitatus Hoff.	Cavacrol (81.2), p-Cymene (5)	*L. monocytogenes,* 521 ppm *Staphyloccocus aureus,* 417 ppm *B. cereus,* 261 ppm *S. enterica serovar typhimurium,* 625 ppm *E. coli O157:H7,* 625 ppm *Pseudomonas aeruginosa,* 2083 ppm	(Dussault *et al.*, 2014), (Casiglia, Bruno, Scandolera, Senatore, & Senatore, 2019).
	Origanum compactum	Carvacrol (22), γ-terpinene (23), thymol (19)	*E. coli O157:H7,* 250 ppm *Salmonella typhimurium,* 500 ppm *Staphylococcus aureus,* 130 ppm *L. monocytogenes,* 1000 ppm	(Oussalah *et al.*, 2007).
Cinnamon Chinese cassia	*Cinnamomum cassia*	*Trans*-cinnamaldehyde (87.58), cinnamyl acetate (7.53)	*L. monocytogenes,* 625 ppm *S. aureus,* 625 ppm or 470 ppm or 1042 ppm *B. cereus,* 208 ppm or 261 ppm *S. enterica serovar typhimurium,* 417 ppm or 625 ppm *E. coli O157:H7,* 417 ppm or 470 ppm or 625 ppm *Pseudomonas aeruginosa,* 1250 ppm	(Ghabraie, Vu, *et al.*, 2016a),(Dussault *et al.*, 2014).
Cinnamon bark	*Cinnamomum verum*	*Trans*-cinnamaldehyde (40.71-68.52), cinnamyl acetate (2.15-14.25), β-phellandrene (9.02), β-caryophyllene	*L. monocytogenes,* 780 ppm or 0.0313% *S. aureus,* 1250 ppm or 2.5	(Ghabraie, Vu, *et al.*, 2016a),(Kang & Song, 2018),(D. F. Huang *et al.*, 2014).

		(7.41)	mg/mL *B. subtilis,* 5 mg/mL *E. coli,* 780 ppm or 0.0313% or 10 mg/mL *S. typhimurium,* 1250 ppm or 0.0625% or 10 mg/mL *P. aeruginosa,* 2500 ppm	
Red bergamot	*Monarda didyma* L.	Carvacrol (48.21), *p*-cymene (13.98), ɣ-terpinene (12.69)	*L. monocytogenes,* 1250 ppm *S. aureus,* 2500 ppm *E. coli* ,1250 ppm *S. typhimurium,* 5000 ppm *P. aeruginosa,* >10000 ppm	(Ghabraie, Vu, *et al.*, 2016a).
Lemongrass	*Cymbopogon citratus*	*Citral (63)*	*L. monocytogenes,* 1250 ppm *S. aureus,* 625 ppm *B. cereus,* 156 ppm *S. enterica serovar Typhimurium,* >5000 ppm *E. coli O157:H7,* 5000 ppm *P. aeruginosa,* >5000 ppm	(Dussault *et al.*, 2014).
Red Thyme	*Thymus vulgaris*	Thymol, carvacrol, γ-terpinene	*A. niger,* 1250 ppm *A. flavus,* 1250 ppm *A. parasiticus,* 1250 ppm *P. chrysogenum,* 312.5 ppm *L. monocytogenes,* 833 ppm *S. aureus,* 313 ppm *B. cereus,* 417 ppm *S. enterica serovar*	(Ghabraie, Vu, *et al.*, 2016a),(Hossain, Follett, Dang Vu, *et al.*, 2016).

			typhimurium, 2083 ppm *E. coli O157:H7,* 1250 ppm *P. aeruginosa,* 3333 ppm	
Winter savory	*Satureja montana* L.	Cavacrol 43.84, γ-Terpinene 12.66, Thymol 6.71	*L. monocytogenes,* 625 ppm *S. aureus,* 625 ppm *B. cereus,* 313 ppm *S. enterica serovar typhimurium,* 1250 ppm *E. coli O157:H7,* 1250 ppm *P. aeruginosa,* >5000 ppm	(Dussault *et al.*, 2014),(Ben Lagha, Vaillancourt, Maquera Huacho, & Grenier, 2020).
Garlic	*Allium sativum* L.	Diallyl sulphides 42%-53%	*S. aureus,* 24 ppm MRSA, 32 ppm *Candida albicans,* 16 ppm *Candida krusei,* 24 ppm *Candida glabrata,* 32 ppm *Aspergillus niger,* 20 ppm *Aspergillus flavus,* 40 ppm *Aspergillus fumigatus,* 32 ppm	(TSAO & YIN, 2001).
Clove	*Eugenia caryophyllus*	Eugenol (83–95), eugenyl acetate (9.96), β-caryophyllene (4.01)	*L. monocytogenes,* 3750 ppm *S. aureus,* 1875 ppm *E. coli,* 1875 ppm *S. typhimurium,* 3750 ppm *P. aeruginosa,* >10000 ppm	(Ghabraie, Vu, *et al.*, 2016a).

Table 3.2 **Combination of essential oils or their components and antimicrobial interactions against several micro-organisms by checkerboard method.**

EO combination	Microorganisms	Interaction	Reference
Oregano + thyme	*Paenibacillus amylolyticus, Bacillus cereus*	Synergism	(Ayari *et al.*, 2020)
	A. flavus, A. parasiticus, P. chrysogenum	Synergism	(Hossain, Follett, Vu, *et al.*, 2016)
	E. Cloacae, P. fluorescens, L. innocua	Addition	(Gutierrez, Barry-Ryan, & Bourke, 2009)
	L. monocytogenes, S. aureus, Salmonella enteritidis	Addition	(REYES-JURADO, LÓPEZ-MALO, & PALOU, 2016)
	S. Aureus, Salmonella, E. coli, Bacillus cereus	Synergism	(Gavaric *et al.*, 2015)
Cinnamon + mandarin	*A. niger, A. flavus, A. parasiticus, P. chrysogenum,*	no interaction	(Hossain, Follett, Vu, *et al.*, 2016)
Mandarin + oregano	*Paenibacillus amylolyticus, Bacillus cereus*	no interaction	(Ayari *et al.*, 2020)
Eucalyptus + thyme			
Mandarin + tea tree			
Cinnamon + tea tree	*A. niger, A. flavus, A. parasiticus, P. chrysogenum,*	Synergism,	(Hossain, Follett, Vu,

		Addition	*et al.*, 2016)
Peppermint + thyme	*Paenibacillus amylolyticus, Bacillus cereus*		(Ayari *et al.*, 2020)
Oregano + peppermint			
Tea tree + thyme			
Cinnamon + thyme			
Cinnamon + thyme	*L. monocytogenes, E. coli*	Synergism	(García-Díez *et al.*, 2017)
	Botrytis cinerea, Penicillium expansum	Synergism	(Nikkhah, Hashemi, Habibi Najafi, & Farhoosh, 2017)
Cumin + cinnamon	*E. coli*		(García-Díez *et al.*, 2017)
Thyme + cumin	*L. monocytogenes, Salmonella spp.*		
Cinnamon + parsley	*L. monocytogenes*		
Garlic + bay	*Salmonella spp.*		
Thyme + rosemary	*Salmonella spp.*	Synergism	(García-Díez *et al.*, 2017)
	Botrytis cinerea.	Synergism	(Nikkhah *et al.*, 2017)

	Penicillium expansum	No interaction	(Nikkhah *et al.*, 2017)
Cinnamon + rosemary	*Botrytis cinerea, Penicillium expansum*	No interaction	(Nikkhah *et al.*, 2017)
Callistemon lanceolatus+citronella java	*S. aureus, M. luteus, B. subtilis, E. coli*	Synergism	(K. Sharma, Guleria, Razdan, & Babu, 2020)
Callistemon lanceolatus+cymbopogon flexuosus			
Ocimum gratissimum+cymbopogon flexuosus	*S. aureus, M. luteus, B. subtilis, K. pneumoniae*	Synergism	
Carvacrol + cinnamaldehyde;	*F. coli, L. innocua*	Synergism	(Requena, Vargas, & Chiralt, 2019)
	P. roqueforti	No interaction	(Ju *et al.*, 2020)
	A. niger	Antagonism	(Ju *et al.*, 2020)
Eugenol + carvacrol;	*A. niger*	No interaction	(Ju *et al.*, 2020)
	P. roqueforti,	Synergism	(Ju *et al.*, 2020)
	Escherichia coli O157: H7	Addition	(Yuan *et al.*, 2019)
Oregano + mustard	*L. monocytogenes, S. aureus, Salmonella enteritidis*	Addition	(REYES-JURADO *et*

			al., 2016)
Thyme + mustard			
Eugenol + cinnamaldehyde	*E. coli, L. innocua*	Synergism	(Requena *et al.*, 2019)
Cinnamon + mustard	*A. ochraceus*	Synergism	(Clemente *et al.*, 2016)
	Penicillium verrucosum, Fusarium oxysporum, Penicillium expansum, Aspergillus niger, Botryotinia fuckeliana, Aspergillus flavus, Geotrichum spp., Rhizopus stolonifer	Addition	
Chinese cinnamon + cinnamon bark	*L. monocytogenes, S. aureus, E. coli, S. Typhimurium*	Addition	(Ghabraie, Vu, Tata, Salmieri, & Lacroix, 2016b)
Cinnamon + tea tree	*A. Niger, A. flavus, A. parasiticus, P. chrysogenum*	Addition	(Hossain, Follett, Vu, *et al.*, 2016)
Eucalyptus + tea tree			
Cinnamon + eucalyptus			
Basil + peppermint			
Thymol + trans-cinnamaldehyde	*Escherichia coli* O157: H7	Addition	(Yuan *et al.*, 2019)

Thymol + eugenol			
Thymol + vanillin			
Vanillin + eugenol			
Vanillin + carvacrol			
Eugenol + trans-cinnamaldehyde			
trans-cinnamaldehyde + carvacrol			
Carvacrol + thymol	*Campylobacter jejuni*	Synergism	(Šimunović *et al.*, 2020)
	S. aureus, Salmonella, E. coli, Bacillus cereus	Synergism	(Gavaric *et al.*, 2015)
	P. roqueforti, A. niger	Synergism	(Ju *et al.*, 2020)
	Escherichia coli O157 : H7	Addition	(Yuan *et al.*, 2019)
Citral + eugenol	*P. roqueforti, A. niger*	Synergism	(Ju *et al.*, 2020)
Citral + thyme	*P. roqueforti, A. niger*		
Thyme + cinnamaldehyde Citral + carvacrol	*A. niger*		
Cinnamon bark + citronella	*P. corylophilum*	Synergism	(Ji, Kim, Beuchat, &

			Ryu, 2019)
Pelargonium asperum + ormenis mixta	*Staphylococcus aureus*	Synergism	(Ouedrhiri *et al.*, 2018)
Eucalyptus caesia Benth + dracocephalum multicaule Montbr & Auch	*Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes*	Synergism	(Hashemi & Jafarpour, 2020)
	Escherichia coli, Shigella flexneri, Pseudomonas aeruginosa, Salmonella typhi	Addition	
Rosmarinus officinalis + carvacrol	*Bacillus subtilis*	Synergism	(Fadil *et al.*, 2018)

Table 3.3 Applications of EOs in meat preservation.

EOs	Concentrations applied	Tested micro-organisms	Major effects	Types of meat	Storage conditions	References
Oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), orange (*Citrus sinensis* var. Valencia)	700-2000 mg/L^{-1} of air	*Salmonella enterica*	Reduced the *Salmonella* population in sausages stored until 144 hrs, alter sensory properties	Meat sausage	4 °C	(Luna-Guevara *et al.,* 2021)
Sage (*Salvia officinalis* L.)	0.05 µL/g, 0.75 µL/g, 0.1 µL/g,	Total number of aerobic mesophilic bacteria, *Salmonella* spp., *Escherichia coli* and *Listeria monocytogenes*	Reduced total number of aerobic mesophilic bacteriaand inhibited *Salmonella* spp., *Escherichia coli* and *Listeria monocytogenes*, better sensory properties	Fresh pork sausage	3 ± 1 °C, under dark conditions, for 8 days.	(Šojić *et al.,* 2018)
Bay leaf (*Laurus nobilis* L.)	0.05 g/100 g or 0.1 g/100 g	*Psychrotrophs*, *Mesophiles*, Lactic acid bacteria and Total *coliforms*	Reduced the population of total coliforms (2.8 log CFU/g) and	Tuscan sausage	7°C for 14 days	(da Silveira *et al.,* 2014)

			to extend the product shelf life for two days			
Garlic (*Allium sativum* L.), oregano (*Thymus capitatus* Hoff.), thym (red) (*Thymus vulgaris* and *Thymus zygis* L. var. *gacilis* Boissier), Chinese cinnamon (*cinnamon cassia*)	500 ppm (0.05% v/w)	*L. monocytogenes*	A reduction of the growth rate by 19 and 10% was observed when oregano and cinnamon cassia EOs were respectively added in ham at a concentration of 500 ppm.	Ham	4°C for 35 days	(Dussault *et al.,* 2014)
Black pepper essential oil (*Piper nigrum* L.)	0, 0.1 and 0.5%, v/v	*Pseudomonas* spp., *Enterobacteriaceae*	Inhibition of *Pseudomonas* sp p., *Enterobacteriacea e*	Fresh pork	4 °C for 9 days	(Zhang *et al.,* 2016)
Thyme and orange	0.5%	Total aerobic and facultative mesophiles	Extended shelf life	Broiler breast fillets and whole wings	Vacuum tumbling, 4°C	(Thanissery and Smith 2014)

Chinese cinnamon (*Cinnamomum cassia*), Cinnamon bark (*Cinnamomum verum*)	0.025–0.05%	*Clostridium sporogenes*	Reduced *Clostridium sporogenes*	Pork sausage	4 °C	(Ghabraie *et al.,* 2016)
Shirazi-thyme (*Zataria multiflora*), cinnamon (*Cinnamomum zeylanicum*), and clove (*Syzygium aromaticum*)	20 mg kg^{-1}	*P. fluorescens*	Reduced *P. fluorescens*, extend shelf life	Chicken breast meat	4 °C for 12 days	(Chaichi *et al.,* 2021)
Anacardiaceae (*Pistacia lentiscus*), Lamiaceae (*Satureja montana*)	*S. montana* 0.06%, *P. lentiscus* 0.20%	*Listeria monocytogenes*	Synergy, reduction of *Listeria monocytogenes*, extend shelf life	Minced beef	5 ± 1 °C	(Djenane *et al.,* 2011)
Juniper (*Juniperus communis* L.) and winter savory (*Satureja montana* L.)	0.25% *J. communis* EO; 0.125% *S. montana* EO	*Listeria monocytogenes*, *Enterobacteriaceae*, aerobic heterotrophic mesophilic bacteria, lactic acid bacteria	Reduction of tested strains, extend shelf life	Red wine-marinated beef	4 °C for 15 days	(Vasilijević *et al.,* 2019)

Oregano (*Origanum vulgare*)	0.4% (v/w)	LAB natural microbiota	Decreased growth rates of LAB, extend shelf-life	vacuum-packed cooked sliced ham	6, 12, 15, 20 and 25 °C for 45 days	(Menezes *et al.,* 2018)
Oregano (*Coridothymus capitatus*), thyme (*Thymus vulgaris*)	0.25% (v/v) each	Enterobacteriaceae, total coliform, *Staphylococcus aureus*	Reduced the Enterobacteriaceae counts, total coliform counts and *Staphylococcus aureus* counts	Tunisian dry fermented poultry meat sausage	0, 7, 14, 21, 28 days during ripeing	(El Adab and Hassouna 2016)
Basil (*Ocimum basilicum* L), garlic (*Allium sativum* L.), nutmeg (*Myristica fragans*), oregano (*Origanum vulgare*), rosemary (*Rosmarinus officinalis* L) and thyme (*Thymus capitatus* Hoff. et Link)	0.005% and 0.05%	*Salmonella* spp., *Listeria monocytogenes, Staphylococcus aureus*	Inhibition of *Salmonella* spp., *Listeria monocytogenes, Staphylococcus aureus,* shorten the drying period	Dry cured sausage chouriço	0, 3, 8, 15, 21 days during ripeing	(García-Díez *et al.,* 2016)

Table 3.4 Applications of EOs encapsulated in meat and meat products.

EOs	Concentrations applied	Encapsulation and types	Tested micro-organisms	Majoer effects	Types of meat	Storage conditions	References
Anise (*Pimpinella anisum L.*), caraway (*Carum carvi L.*), Nutmeg (*Myristica fragrans*)	Anise 0.5%, caraway 1%, nutmeg 1%	Manihot esculenta and Carrageenan functionized wiith anise, caraway, fim	Total plate count, *psychrophilic* count, *Coliform* and, yeast and mold	Total plate count, psychrophilic count and yeast and mold count were also significantly ($P < 0.01$) lower in treatment groups	Chicken nuggets	4 ± 1 °C,15days	(Bharti *et al.,* 2020)
Thyme (*Thymus vulgaris*)	1 – 3%	Alginate, cellulose nanocrystals (CNCs), beads	*L. innocua* and mesophilic total flora (MTF)	Eliminated *L. innocua* and reduce the mesophilic total flora (MTF)	Ground lean pork	4 °C for 14 days	(Criado *et al.,* 2019)
Thyme	Silk fibroin nanofibers: plasma-thyme EO	Silk fibroin nanofibers	*Salmonella typhimurium*	Aftercombined treatment, the number of Salmonella Typhimurium in chicken meat and duck meat decreased by 6.1 and 6.06 Log CFU/g compared with control group at 25 °C, respectively.	Poultry meat (chicken and duck)	4 or 25 °C for 7 days	(Lin *et al.,* 2019)

Garlic (*Allium sativum)*	Garlic essential oil or nanoencapsulated garlic EO (2% v/v)	Chitosan, whey protein, film	Aerobic plate count, lactic acid bacteria, psychrotrophic bacteria, *Staphylococcus aureus*, coliforms	Retarded the growth of main spoilage bacterial groupsl (aerobic plate count 3.69 log CFU/g) compared to the control	Vacuum-packed sausages	4 °C, 50days	(Esmaeili *et al.,* 2020)
Thyme (*Thymus zygis*), rosemary (*Rosmarinus officinalis*)	1%	Chitosan, coating	aerobic total viable count (TVC), lactic acid bacteria (LAB), Gram (+) catalase (+) cocci, *Enterobacteriaceae* and mold/yeast	Retarded fungal mycelium development on the casing.	Dry-fermented sausages	4 °C, 3 months	(Demirok Soncu, Özdemir, Arslan, Küçükkaya, & Soyer, 2020)
Tarragon (Artemisia dracunculus L.)	Mass ratios of chitosan to Tarragon EO (1:0, 1:0.2, 1:0.4, 1:0.6, 1:0.8 and 1:1)	Chitosan, coating	Total viable count	Inhibited the quality deterioration	24 h post-mortem fresh pork slices	4°C for 16 days	(Zhang *et al.,* 2020)
Satureja (*Satureja khuzestanica*)	1% v/v, proper amounts of free EO and SKEO-loaded	Chitosan, coating	Total Viable Count (TVC), Lactic Acid Bacteria (LAB) and *Pseudomonads* (PBC)	Slowed down the microbial growth significantly	Lamb meat	°C for 20 days	(Pabast *et al.,* 2018)

	nanoliposomes						
Rosemary (*Rosmarinus officinalis*)	5000 mg/L	Chitosan-benzoic acid (CS-BA), nanogel	*Salmonella typhimurium*	Nanogel-encapsulation led to higher antibacterial activity against *Salmonella typhimurium* on beef.	Beef cutlet	4°C for 1, 4, 8, and 12 days	(Hadian *et al.,* 2017)
Pomegranate (*Punica granatum* L.) peel extracts	1 and 1.5%	Lyophilized nanoparticles	Total viable bacterial count, psychrophilic bacteria, and lipolytic bacteria	Effective antioxidant and antimicrobial properties, increased sensory acceptabilities.	Meatballs of minced beef meat	4°C for 15 days	(Morsy *et al.,* 2018)
Clove (*Syzygium aromaticum*)	2 mg/g beef	Chitosan (CS)-Myristic acid (MA) nanogel	*S. entericaser. Enteritidis*	Nanogel had more efficiency in controlling the investigated pathogen than the free CEOs	Beef cutlets	4°C for 12 days	(Rajaei *et al.,* 2017)
Cinnamon (*Cinnamomum zeylanicum* L.)	(0.1% of encapsulated CEO	Chitosan, nanoparticles	*S. aureus*, total mesophilic aerobic viable count (TMVC), *Enterobacteriaceae*, yeasts and molds	The encapsulation increased the antimicrobial abilities	Beef patties	4°C for 8 days	(Ghaderi-Ghahfarokhi *et al.,* 2017)

			(Y&M) and lactic bacteria (LAB)				
Thyme (*Thymus vulgaris* L.)	Chitosan	0.05 or 0.1% of encapsulated thyme EO, nanoparticles	*S. aureus* *Enterobacteriaceae*	Encapsulation process improved the shelf life, maintained antimicrobial activities during storage.	Beef burgers	4°C for 8 days	(Maryam Ghaderi-Ghahfarokhi *et al.,* 2016)
Jabuticaba (*Myrciaria cauliflora*) extract (JE)	2 and 4% of MJE	Maltodextrin, microencapsulation	Aerobic mesophiles, Aerobic psychrotrophics, Lactic acid bacteria, thermotolerants coliforms and *S. aureus*	The extract had no positive effect on microbial stability during storage.	Fresh sausages	1 ± 1 °C for 15 days	(Baldin *et al.,* 2016)
Oregano (*Origanum compactum*) or Cinnamon (*Cinnamomum cassia*)	EOs in alginate-CNC microbeads was 250 µg/ml	Alginate-CNC, microencapsulation	*L. monocytogenes*	Microencapsulation significantly ($P \leq 0.05$) improved the radiosensitivity of *L. monocytogenes*. Microencapsulated oregano and cinnamon essential oil in combination with nisin showed the highest bacterial radiosensitization 2.89 and 5, respectively, compared to the control.	Ready-to-eat cooked ham.	4°C for 35 days	(Huq *et al.,* 2015)

4 PUBLICATION 2 – RESEARCH *IN VITRO* TESTS

A rapid way of formulation development revealing potential synergic effects on numerous antimicrobial combinations against foodborne pathogens

Un moyen rapide de développement de formulation révélant des effets synergiques potentiels sur de nombreuses combinaisons antimicrobiennes contre les agents pathogènes d'origine alimentaire

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Journal:

Microbial Pathogenesis, 105047.

Received 8 March 2021, revised 7 May 2021, accepted 7 June 2021, available online 12 June 2021.

DOI : https://doi.org/10.1016/j.micpath.2021.105047

Contribution of authors:

ML is the research responsible and coordinator of the project. She has planned the research experiment. JJ and JF conceived and the design of the experiments. JJ and EJ performed the experiments. JJ organized the database, performed the statistical analysis and wrote the first draft of the manuscript. JJ, S Shankar, S Salmieri and ML contributed to manuscript revision, read, and approved the submitted version.

4.1 Abstract

The interactions between various essential oils (EOs) were evaluated for the development of antimicrobial formulations. A full factorial design was applied for testing eight EOs (Mustard, Thyme, Garlic, Oregano, Chinese cinnamon, Cinnamon bark, Red bergamot, Winter savory) against nine bacteria (*E. coli* O157:H7 RM1239, *E. coli* O157:H7 RM 1931, *E. coli* O157:H7 RM 1933, *E. coli* O157:H7 RM 1934, *E. coli* O157:H7 380-94, *Listeria monocytogenes* LM 1045, *Listeria innocua* ATCC 51742, *Salmonella* Typhimurium SL 1344, *Salmonella enterica Newport* ATCC 6962) and two molds (*Penicillium chrysogenum* ATCC 10106, *Aspergillus niger* ATCC 1015). Results showed that combinations of Thyme + Oregano, Oregano + Cinnamon bark, Chinese cinnamon + Cinnamon bark have shown high interactions in Factorial design and validated to be mostly additive effects against tested bacteria. The combination of Mustard + Thyme, Mustard + Garlic, and Thyme + Garlic EOs showed high interactions and also all additive effects against tested molds. The corresponding results of factorial design and checkerboard render the designation to demonstrate the highly efficient formulations and interactions rapidly among abundant mixtures.

Keywords: Factorial design; Synergy; Essential oil formulation; Antimicrobial activity; Foodborne pathogens

4.2 Résumé

Les interactions entre différentes huiles essentielles (HE) ont été évaluées pour le développement de formulations antimicrobiennes. Un plan factoriel complet a été appliqué pour tester huit HE (moutarde, thym, ail, origan, cannelle de Chine, écorce de cannelle, bergamote rouge, sarriette) contre neuf bactéries (*E. coli* O157:H7 RM1239, *E. coli* O157:H7 RM 1931, *E. coli* O157:H7 RM 1933, *E. coli* O157:H7 RM 1934*, E. coli* O157:H7 380-94, *Listeria monocytogenes* LM 1045, *Listeria innocua* ATCC 51742, *Salmonella Typhimurium* SL 1344, *Salmonella enterica Newport* ATCC 6962) et deux moisissures (*Penicillium chrysogenum* ATCC 10106, *Aspergillus niger* ATCC 1015). Les résultats ont montré que les combinaisons de thym + origan, origan + écorce de cannelle, cannelle chinoise + écorce de cannelle ont montré des interactions élevées dans la conception factorielle et validées comme étant principalement des effets additifs contre les bactéries testées. La combinaison des HE moutarde + thym, moutarde + ail et thym + ail a montré des interactions élevées ainsi que tous les effets additifs contre les moisissures testées. Les résultats obtenus en utilisant un design factoriel et en utilisant le test de «checkerboard» a permis de mettre au point des formulations antimicrobiennes hautement efficaces agissant en synergie et dans les concentratins optimales pour chaque HE.

Mots-clés: Conception factorielle; Synergie; Formulation d'huiles essentielles; Activité anti-microbienne; Pathogènes d'origine alimentaire

4.3 Introduction

Foodborne infections have always been a serious threat to public health and the industrial economy (Yeni *et al.*, 2016). A broad spectrum of foodborne pathogens could cause illness and death both in advanced and developing countries (Tauxe, 2002). *E. coli* O157:H7 is found majorly in beef, pork, poultry, and lamb has become one of the most concerned foodborne pathogens (Doyle, 1991; Lim *et al.*, 2010). Also, *Listeria monocytogenes,* mostly found in meat, poultry, and dairy products (Todd & Notermans, 2011), can survive under diverse conditions such as low humidity, high salt concentrations, and broad-gauge temperatures from −1 to 45 °C (Farber & Peterkin, 1991). Unlike *L. monocytogenes*, *L. innocua* is not pathogenic but frequently shows possibly genetic information transfer (Gómez *et al.*, 2014). Together with other pathogens*, Salmonella* caused infections to around 15% population of the USA in 2012 (Crim *et al.*, 2014). Fungi like *Aspergillus*, *Fusarium,* and *Penicillium* species cause spoilage and toxicity also distort the organoleptic properties (Aoudou *et al.*, 2010).

There is a growing demand for safe, healthy, and natural foods increase since people are concerned about chemical residue existing in the foods they consume (Mishra & Datta-Gupta, 2017). Consumers' high demand for foods free from synthetic chemical compounds calls preservation methods by adding natural antimicrobial compounds. Antimicrobial compounds are used to kill undesired microorganisms in foods or to prevent and inhibit their growth.

Essential oils (EOs) are aromatic, volatile, and complex oily liquids extracted from plants (Hyldgaard *et al.*, 2012). Major compounds present in essential oils (EOs) can be identified into two groups: (1) terpene and terpenoid as the main group and (2) aromatic and aliphatic components (Faleiro, 2011). Numerous studies indicated that essential oils and their compounds possess excellent antibacterial, antiparasitic, antifungal, insecticidal, and antioxidant properties (Burt, 2004). Cinnamon, oregano, and thyme (red) EOs were found to possess great antimicrobial activities against *L. monocytogenes*, *Salmonella* Typhimurium, *P. aeruginosa*, *S. aureus*, *B. cereus,* and *E. coli* (Dussault *et al.*, 2014; Scotti *et al.*, 2021). Clove EO was observed effective against *P. solitum, P. roqueforti, P. chryogenum, A. versicolor, A. niger* (Bagheri *et al.*, 2020) and *S.* Typhimurium, *B. cereus* and *S. aureus* (Budiati *et al.*, 2018). Garlic EO was found efficiently inhibiting *P. roqueforti, A. niger, Listeria* spp., *C. divergens, C. tyrobutiricum,* and *B. thermosphacta* (Bagheri *et al.*, 2020). Due to their excellent properties and natural flavors, EOs have been used for a long time as natural additives in food preservation under proper doses and are generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA) (Dima & Dima, 2015; Tisserand & Young, 2013). However, in food systems, the lipophilic compounds

could interact with food matrix components such as fat, starch, and proteins (Hyldgaard *et al.*, 2012), resulting in a demand for higher concentrations of EOs to achieve the same antimicrobial effects as *in vitro* (Burt, 2004). Thus EOs may affect organoleptic properties like taste, odor negatively (Ghabraie *et al.*, 2016). The application of EOs in combination could be the solution to increase the efficacy of EOs capitalized on their synergistic and additive effects (Bassolé & Juliani, 2012). Former studies have observed synergies of cinnamon and mustard EOs on *P. putida* and *E. coli* OI57:H7 (Clemente *et al.*, 2016), combinations of thyme and cinnamon EOs, garlic and bay EOs, thyme and rosemary EOs, thyme and cumin EOs on *E. coli*, *L. monocytogenes*, *S. aureus* and *Salmonella* spp. (García-Díez *et al.*, 2017), oregano and thyme EOs on *A. flavus, A. parasiticus,* and *P. chrysogenum* (Hossain *et al.*, 2016).

Checkerboard and time-kill methods are the most commonly used techniques to study the synergistic effect between compounds (Bassolé & Juliani, 2012; White *et al.*, 1996). The FIC (checkerboard) index is the sum of individual FIC values that defines the essence of interactions between binary combinations (Turgis *et al.*, 2012b). The time-kill method assesses interactions by measuring the time- and concentration-dependent sub-inhibitory abilities of two compounds (Singh *et al.*, 2000). However, the time-kill method cannot distinguish additive or no interactive effects (Singh *et al.*, 2000), while FIC is time-consuming to perform (Mackay *et al.*, 2000). Hence, to explore synergy effects among more EOs, an exhaustive search of possible combinations is not practicable in the lab using FIC or time-kill method as the number of tests increases rapidly with the number of concluded antimicrobials (Antony, 2014; Feala *et al.*, 2010). Statistical Design of Experiments (DOE) analyzes the experiment's output according to the input factors efficiently (Mukerjee & Wu, 2007). DOE-based software testing approaches in engineering and computer sciences are well-established in the pharmaceutical process in recent years and have been advocated as a tactic to investigate highly complex systems at relatively low costs (Feala *et al.*, 2010; Kuhn & Reilly, 2002; Pressman & Ince, 2000). Thus DOE could be considered as a feasible method when facing many factors (Mishra & Datta-Gupta, 2017). Among multiple types of DOE, the factorial design is a statistical information collection strategy that allows studying the joint effects on a response, commonly used in engineering research and industries (Antony, 2003). The factorial design, especially fractional factorial design, allows a realization of interaction screening with large quantities of EOs or other antimicrobial compounds and can substantially attenuate the number of experiments by defining trials that capture the individual and interactive effects on the response of a complex system (Chen *et al.*, 2010; Mukerjee & Wu, 2007).

In this test, the full factorial design was used with modifications on the time-kill method and verified with the FIC method to reveal the synergic effects among different five essential oils targeted to nine bacteria and two molds, in order to generate more detailed information of interactions and develop effective antimicrobial formulations with relatively less work and time.

4.4 Materials and methods

4.4.1 Preparation of essential oil emulsion

The EOs used in this test and their main constituents are presented in Table 4.1. EOs were stored at 4 ℃ before tests. The EOs emulsions were prepared as an oil-in-water emulsion containing essential oil(s) (2 %, v/v, ratios were calculated by design method), Tween® 80 (2 %, v/v) (Sigma–Aldrich Ltd) and sterile water (96 %, v/v). Mixtures were homogenized using an Ultra-Turrax (T25 digital) for 1 min at 15,000 rpm, filtered using a 0.2 µm sterile filter, and stored at 4 ºC (Ghabraie *et al.*, 2016).

Table 4.1 List of essential oils (EOs) and their origin and main compounds[a].

Latin name	Common name	Origin	Distilled part	Main compounds
Brassica Juncea	Mustard oil	Canada	Seeds	Allyl isothiocyanate, Diallyl trisulfide, Diallyl sulfide
Thymus thymoliferum	Thymus vulgaris (thyme) oil	Spain	Flowers	Carvacrol, thymol, b-caryophyllene
Allium sativum	Garlic	China	Crushed bulb	Dimethyl trisulfide, Diallyl disulfide, Diallyl sulfide, Diallyl tetrasulfide
Origanum compactum	Oregano Compact	Morocco	Flowers	Carvacrol, P-cymene, thymol, Y-terpinene
Cinnamomum cassia	Chinese cinnamon	China	Bark	Trans cinnamaldehyde, Trans o méthoxycinnamaldéhyde, Coumarine
Cinnamomum verum	Cinnamon, Ceylon bark	Madagascar	Bark	Trans-cinnamaldehyde, cinnamyl acetate, b-phellandrene, b-caryophyllene
Monarda didyma	*Red Bergamot (Beebalm)*	*Canada*	*Flowers*	*Carvacrol, p-cymene, ɣ-terpinene*

Satureja montana	*Savory*	*Spain*	*Flowers*	*Thymol, p-cymene*

a Mustard EO was provided by Hilltech (Vankleek Hill, ON, Canada), Garlic EO was provided by Novotast (Montreal, Quebec, Canada), the rest were purchased from Zayat Aroma (Montreal, Quebec, Canada).

4.4.2 Preparation of bacterial cultures

Nine bacterial strains, including *Listeria monocytogenes* LM 1045, *Listeria innocua* ATCC 51742, *Salmonella* Typhimurium SL 1344, *Salmonella enterica Newport* ATCC 6962, and five *Escherichia coli* O157:H7 strains (RM1239, RM 1931, RM 1933, RM 1934, 380-94) were used as target bacteria. All bacterial strains were maintained at -80 ºC in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD, USA) containing glycerol 10 % (v/v). Before each experiment, a working culture was prepared by sub-culturing 1 ml of stock culture in TSB (9 ml), then incubated at 37 ºC for 24 h for two consecutive growth cycles to obtain approximately 10^9 CFU/m*L.* Cultures were then centrifuged at 1300 ×g for 15 min and re-suspended in saline solution 0.85% (w/v) before use (Dussault *et al.*, 2014).

4.4.3 Preparation of fungal cultures

Aspergillus niger ATCC 1015 and *Penicillium chrysogenum* ATCC 10106 were used for fungal tests. Two strains were maintained at -80 ºC in potato dextrose broth (PDB, Difco, Becton Dickinson) containing glycerol (10% v/v). Prior to each experiment, stock cultures were subcultured through two 48 h growth cycles in the PDB medium at 28 ºC. The cultures were pre-cultured in PDA for 3 days at 28 ºC. Conidia were collected from the agar media using sterile saline containing 0.05 % Tween 80, and the filtrate was adjusted to 1× 10^5 conidia/mL for tests using a microscope (Hossain *et al.*, 2016).

4.4.4 Determination of the minimum inhibitory concentrations (MIC)

A modified broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of the EOs as described by Turgis *et al.* (2012a). A 100 µL aliquot of 2 - fold serial dilution (from 10,000 to 10 µg/mL) of EOs suspension was prepared and deposited in each well of a 96-well microplate (SARSTEDT, St. Leonard, QC, Canada) using Mueller-Hinton Infusion (MHI) broth for bacteria or PDB for fungi. Each well was then inoculated with 100 µL of a pathogen at a concentration of 10^5 CFU/mL (bacteria) or 10^5 conidia/mL (fungus), making a final volume of 200 µL, a final concentration of 0.5 x 10^5 CFU/mL (bacteria) or 10^5 conidia/mL (fungus) each well.

The microplates were incubated under the aerobic condition and agitated at 100 rpm in a shaker for 24 h at 37 °C (bacteria) and 48 h at 28 °C (fungi). The absorbance was measured at 595 nm in a BioTek ELx800® absorbance microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The MIC is the lowest concentration of the antimicrobial agent, demonstrating the complete growth inhibition of microorganisms showing equal absorbance as blank.

4.4.5 Experimental designs for determining effective combinations of antimicrobials

To determine the effective combinations of antimicrobials, one design was used for each strain to study the effects of selected EOs on each strain of bacteria and fungus; therefore, 9 and 2 factorial designs were used for bacteria (9 strains) and fungi (2 strains) separately.

A full factorial design 2^5 (5 EOs at 2 levels) was performed in triplicate to evaluate the antimicrobial capacities. Two hundred eighty-eight and 64 mixtures were prepared for bacteria and fungi, respectively, with the selected EOs (5 EOs each) either at their high level or low level according to good antimicrobial abilities to most bacteria or fungi from MIC results. The evaluated concentrations were tested based on MIC results by dividing these concentrations by 5 (high level) and 10 times (low level). This factorial design allows the estimation of the main effect (for each compound) and the two-, three- four- and five-factor (five EOs) interactions.

4.4.6 Pathogenic growth conditions and analysis of factorial design experiments

The growth analysis of microorganisms in the presence of EOs was carried out according to Nazer *et al.* (2005) with modifications. A sterile 96-well microplate (Sarstedt, Montreal, QC, Canada) were filled with 100 µL of the corresponding mixture of essentials and 100 µL of a fresh 24-h culture of bacteria/ 48-h culture of fungi, adjusted at a concentration of 10^5 CFU/mL using Mueller–Hinton (MH) broth (bacteria)/ Potato Dextrose Broth (PDB) medium (fungi). The microplate was incubated at orbital shaking at 100 rpm for 24 h at 37 ºC for bacteria and 48 h at 28 ºC for fungi. Controls of the process culture broth + EOs, culture broth only, culture broth + bacteria/fungi were included and incubated under the same conditions as the treatment. The optical density (OD) at 595 nm was measured at the beginning of the experiment and after 24 h for bacteria and 48 h for fungi.

The percentage of growth inhibition was estimated using the following equations:

$$\boldsymbol{Growth\ inhibition}\% = \frac{\left(OD_T - OD_{T_0}\right)_{test}}{\left(OD_T - OD_{T_0}\right)_{control}} \times \mathbf{100} \qquad (4)$$

Where OD is the optical density at 595 nm, T is the time after 24 h for bacteria and 48 h for fungi, T0 is the initial time 0. The test refers to the culture grown with antimicrobials, and the control refers to the culture grown without antimicrobials. This variable indicates how much the growth is reduced in the presence of antimicrobials.

4.4.7 Determination of synergic effects of selected EOs

The checkerboard method was used to determine the efficacy of possible interaction between the antibacterial and the antifungal, which could be synergistic, additive, or antagonist against the pathogens. This test was elucidated that the interaction of two antibacterial and antifungal in the liquid phase for evaluating the most efficient combinations which can inhibit the tested microorganisms. Fractional inhibitory concentration (FIC) index of EOs and natural extracts in combinations were performed against each bacteria and fungi (Turgis *et al.*, 2012a). The FIC values of the antibacterial and the antifungal were calculated by the following equation:

$$\boldsymbol{FIC_a = \frac{Combined\ MIC_a}{MIC_a\ alone}} \quad (5)$$

$$\boldsymbol{FIC_b = \frac{Combined\ MIC_b}{MIC_b\ alone}} \quad (6)$$

$$\boldsymbol{FIC = FIC_a + FIC_b} \quad (7)$$

MICa alone is the MIC value of EOa, MICb alone is the MIC value of EOb, Combined MICa is the concentration of EOa in the selected well, combined MICb is the concentration of EOa in the selected well.

Results are considered as synergy if FIC< 0.5, additive if 0.5 ≤ FIC ≤ 1, no interaction if 1 < FIC ≤ 4, antagonist effect if FIC > 4 (Ayari *et al.*, 2020).

4.4.8 Statistical Analysis

All tests were carried out in triplicate. Data from three independent replicate trials were subjected to statistical analysis using Minitab 19 (Minitab, LLC). Differences between means were tested using Duncan Multiple Range Test and P values $\leqslant$ 0.05 were considered significantly different. The influence of the factors on the experimental response was estimated while using the multiple linear regression method.

4.5 Results

4.5.1 Evaluation of minimum inhibitory concentrations (MIC) of EOs

The MIC values of EOs against 9 bacteria and 2 fungi are presented in Table 4.2. The EOs were classified into three distinct groups corresponding to their MIC values (i) high efficiency (MIC $\leqslant$ 625 µg/mL), (ii) medium efficiency (625 < MIC $\leqslant$ 2500 µg/mL), (iii) low efficiency (MIC > 2500 µg/mL). Based on MIC values against pathogens, mustard EO showed high efficiency against all microorganisms tested with very low MIC values ranging from 156 to 625 µg/mL. Thyme EOs have a high to medium inhibition efficiency to all the bacteria and molds with MIC values ranging from 313 to 1250 µg/mL. Chinese cinnamon has high efficiency against all *E. coli* and molds tested, *S.* Typhimurium and *L. monocytogenes*, medium efficiency against *S. enterica* and *L. innocua*. Cinnamon bark exhibited high efficiency against *E. coli* O157:H7 RM 1239, -RM 1931, and two molds; however, medium efficiency against all other microorganisms tested. Oregano has high efficiency against *E. coli* O157:H7 RM 1933, medium efficiency against all other *E. coli*, *Salmonella,* and *Listeria* strains tested. Red bergamot and Winter Savory have a medium efficiency against all microorganisms tested. To be noticed that Garlic EO has shown very low inhibition against all bacteria but a high efficiency against molds.

Among the tested EOs, mustard, thyme, oregano, Chinese cinnamon, and cinnamon bark were the most effective in inhibiting bacteria, and for fungi, mustard, thyme, garlic, Chinese cinnamon, and cinnamon bark EOs were most effective. These EOs were selected for factorial design to further check the interactions in combinations.

Table 4.2 Minimum Inhibitory Concentrations (MIC) of selected essential oils against different micro-organisms[a].

Essential oils	*E. coli O157:H7*					*Salmonella*		*Listeria*		*P.chryso genum*	*A.niger*
	380-94	1239	1931	1933	1934	*Typhim urium*	*enterica*	*mono cytoge nes*	*innocua*		
Mustard	625	625	625	313	625	625	313	625	313	156	156
Thyme	625	1250	625	313	1250	1250	625	1250	1250	625	1250

Garlic	>10000	>10000	>10000	10000	10000	>10000	>10000	10000	>10000	625	625
Oregano	1250	1250	1250	625	2500	1250	1250	1250	2500	1250	1250
Chinese cinnamon	625	625	625	625	625	625	1250	625	1250	156	313
Cinnamon bark	1250	625	625	1250	1250	1250	1250	1250	2500	313	313
Red bergamot	2500	1250	2500	1250	2500	2500	2500	2500	2500	1250	2500
Winter Savory	2500	2500	2500	1250	2500	2500	2500	2500	2500	1250	1250

[a]MIC values are presented in µg/mL.

4.5.2 Experiment design for bacteria

The factorial effects for 9 strains of bacteria are presented in Table 4.3. The factorial effects including the “main effects of each EOs”, “two-factor interaction”, “three-factor interaction” of two or three EOs, respectively, and this full design provides interaction factors until five EOs. The factorial effects indicate the inhibition percentage combining to the constant. The factor is added to the constant when the compound is at a high level and subtracted at a low level. This means that when a factor is negative, the inhibition is higher with a low concentration of compounds but lower with high-level concentrations. The factorial effects indicate the contributions to the inhibition from different interactions between compounds but are not equal to the synergy of interactions, which refers to a higher antimicrobial activity than the sum of individual compounds (Bassolé & Juliani, 2012).

Results showed that the main effects of each compound are mostly larger than interactions (Table 4.3). Oregano, Chinese cinnamon, and cinnamon bark EOs have shown a significant contribution to the inhibition of all bacteria. For *E. coli*, oregano against the strain 1934 has shown a significantly much higher effect than other EOs at 21.66 followed by cinnamon bark EO to strain 1933 at effect value 9.64, Thyme EO to strain 1934 at value 7.92 and cinnamon bark EO to strain 1934 and oregano to strain 1933 at 7.43 and 7.22 respectively. For *Salmonella*, cinnamon bark has a higher effect to strain SL1344 at 6.50. Cinnamon bark and Chinese cinnamon reached effect at 10.58 and 9.6 to *L. innocua*, respectively. Oregano, Chinese cinnamon and cinnamon bark showed significant contributions to all the bacteria but no interactions at two or more that

were found to have significance to all strains. However, the significance still exists among all the interactions until five-factors where the inhibition is significant to *L. innocua* at value 0.92. Four-factor interaction like mustard*thyme*Chinese cinnamon*cinnamon bark contributed significantly to strain 1931 at effect 0.54 and thyme*oregano*Chinese cinnamon*cinnamon bark to *L. innocua* at effect 1.40. Three-factor interaction like oregano*Chinese cinnamon*cinnamon bark contributed significantly to strain 1934 at effect 2.49 and mustard*oregano*cinnamon bark to strain SL1344 at effect 2.35. In order to have a final formula that could be effective for all the pathogens and with lower concentrations of EOs, the two-level interaction in this test could be considered for further study. Thyme*oregano, oregano*Chinese cinnamon and Chinese cinnamon*cinnamon bark that has significant contributions to more bacteria were selected for synergy check.

Table 4.3 Factorial effects of the experimental design for bacteria.

	E. coli O157:H7					*Salmonella*		*Listeria*	
Term[a]	380-94	1239	1931	1933	1934	*enterica*	*Typhimurium*	*innocua*	*monocytogenes*
Constant	35.10(***)	18.93(***)	10.54(***)	-17.70(***)	37.25(***)	39.59(***)	28.86(***)	37.04(***)	9.77(***)
M	-0.08	-0.06	-0.31	0.41	-0.68	-1.20(***)	-0.45	-0.94(*)	0.47
T	0.05	0.97(***)	0.21	2.65	7.92(***)	-0.35	1.69(*)	1.40(**)	0.59
O	1.24(**)	1.00(***)	0.88(***)	7.22(***)	21.66(***)	-0.79(*)	4.21(***)	2.69(***)	1.93(***)
C	1.06(**)	2.82(***)	3.01(***)	4.46(*)	4.44(***)	4.68(***)	3.54(***)	9.60(***)	2.72(***)
B	2.92(***)	2.05(***)	3.28(***)	9.64(***)	7.43(***)	4.53(***)	6.50(***)	10.58(***)	4.29(***)
M*T	-0.13	0.02	0.29	2.89	1.32	-0.14	0.50	-0.35	1.31(***)
M*O	-0.20	-0.19	0.23	-1.23	0.61	0.24	2.15(**)	-1.47(**)	1.33(***)
M*C	0.36	0.34	-0.01	-0.03	-1.26	0.36	0.71	-0.90(*)	0.02
M*B	-0.92(*)	0.22	0.62(*)	-2.89	0.20	-0.13	0.75	-1.27(**)	-0.23
T*O	-0.93(*)	-0.37	0.26	-0.13	2.40(**)	-0.24	2.66(***)	-2.05(***)	1.50(***)
T*C	0.28	0.46(*)	0.29	2.10	0.83	-0.25	-0.32	0.84	0.11
T*B	-0.53	0.89(***)	0.70(**)	0.74	2.43(**)	0.22	-0.63	0.55	-0.11
O*C	1.68(***)	0.48(*)	0.34	-0.45	0.70	-0.40	0.59	0.72	-0.06
O*B	-1.77(***)	1.47(***)	1.53(***)	-0.95	5.24(***)	0.05	1.19	1.17(*)	-0.36
C*B	-0.49	-0.70(**)	0.49(*)	0.30	1.77(*)	-0.09	1.95(**)	6.15(***)	1.38(***)
M*T*O	0.03	-0.25	-0.13	-2.29	-0.48	-0.54	0.37	-0.33	0.42
M*T*C	-0.18	-0.05	0.11	-0.87	-0.43	-0.13	-0.39	-0.15	-0.37
M*T*B	-0.25	-0.16	-0.01	-1.76	-0.16	-0.15	0.29	0.39	-0.06
M*O*C	0.47	-0.38	0.35	1.05	-0.89	0.01	-0.71	-0.78	-0.26
M*O*B	-0.50	0.14	0.12	-0.82	1.55(*)	-0.05	-2.35(**)	-0.70	-0.16
M*C*B	0.76	-0.25	-0.15	2.84	0.33	0.20	-0.33	-0.50	0.46

T*O*C	0.30	0.02	0.33	0.66	1.06	0.12	-0.25	0.96(*)	-0.27
T*O*B	-0.37	0.27	-0.07	-2.74	2.39(**)	-0.01	-1.75(*)	1.03(*)	-0.47
T*C*B	0.95(*)	-0.43(*)	-0.14	-2.49	1.72(*)	-0.23	0.94	0.98(*)	0.10
O*C*B	-0.11	-0.05	-0.30	-1.88	2.49(**)	-0.05	-1.04	0.58	-0.18
M*T*O*C	0.13	0.08	-0.42	-1.08	-1.05	-0.39	-0.84	-0.80	-0.30
M*T*O*B	0.63	0.18	-0.25	-2.20	-0.13	-0.51	0.36	-1.16(*)	-0.06
M*T*C*B	0.20	-0.04	0.54(*)	0.31	0.82	0.03	1.05	0.13	-0.46
M*O*C*B	-0.52	0.24	0.24	-1.86	0.37	-0.27	0.75	-0.12	-0.63(*)
T*O*C*B	0.09	0.32	0.22	1.47	1.22	-0.47	-0.92	1.40(**)	-0.36
M*T*O*C*B	-0.07	0.27	-0.23	3.29	0.79	0.44	-0.06	-0.92(*)	-0.04
95% confidence interval(*)	0.78	0.42	0.47	3.40	1.49	0.63	1.43	0.88	0.61
99% confidence interval (**)	1.03	0.56	0.63	4.51	1.98	0.84	1.90	1.16	0.82
99.9% confidence interval (***)	1.34	0.72	0.82	5.86	2.57	1.09	2.47	1.51	1.06

[a] Factor M: Mustard, T: Thyme, O: Oregano, C: Chinese cinnamon, B: Cinnamon bark.

4.5.3 Factorial effects to fungi

Table 4.4 shows the effects of 5 EOs (mustard, thyme, Chinese cinnamon, cinnamon bark, and garlic) combinations on fungus. The combinations of EOs exhibited higher inhibition in fungi than bacteria which gave the general mean of 80.52 % and 43.44 % against *A. niger* and *P. chrysogenum*, respectively. The effects of one factor (one EO) presented significantly higher inhibition against two strains of fungi except mustard EO against *A. niger*. Garlic EO showed the biggest effects of 8.43 and 8.30 against *A. niger* and *P. chrysogenum,* respectively. Four- and five- factor interactions didn't show any significant contributions to inhibition, and for three-factor interaction, only garlic*Chinese cinnamon*cinnamon bark exhibited significant inhibition against *A. niger* at value 1.48. More significant inhibition was observed among two-factor interactions such as mustard*thyme, mustard*garlic, and thyme*garlic against both fungi that were selected for synergy check.

Table 4.4 Factorial effects of the experimental design for fungi[a].

Term	*A. niger*	*P. chrysogenum*
Constant	80.52(***)	43.44(***)
M	0.15	0.98(*)
T	2.44(***)	2.86(***)
G	8.43(***)	8.30(***)
C	6.90(***)	2.43(***)
B	5.48(***)	3.19(***)
M*T	-1.13(*)	-1.40(**)
M*G	-2.06(***)	-2.28(***)
M*C	-0.31	-0.15
M*B	-0.47	-0.85
T*G	-2.58(***)	-3.33(***)
T*C	-0.14	0.08
T*B	-0.76	-0.37
G*C	-0.62	-0.27
G*B	-1.75(**)	-0.83
C*B	-0.54	-4.32(***)
M*T*G	0.00	-0.09
M*T*C	0.69	-0.25
M*T*B	-0.53	-0.25
M*G*C	-0.24	-0.09
M*G*B	-0.07	0.24
M*C*B	-0.31	0.67
T*G*C	-0.14	-0.25
T*G*B	-0.54	0.11
T*C*B	0.15	0.41
G*C*B	-1.48(**)	0.21
M*T*G*C	0.00	0.21
M*T*G*B	-0.22	0.24
M*T*C*B	-0.23	0.11
M*G*C*B	0.69	-0.09
T*G*C*B	0.21	0.55
M*T*G*C*B	0.37	-0.29
95% confidence interval (*)	1.09	0.91
99% confidence interval (**)	1.44	1.21
99.9% confidence interval (***)	1.87	1.57

[a]Factor M: Mustard, T: Thyme, C: Chinese cinnamon, B: Cinnamon bark, G: Garlic

4.5.4 Synergy check of selected combinations by FIC method

The FIC index of selected combinations that have significant effects on most bacteria is presented in Table 4.5. Results showed that thyme*oregano and Chinese cinnamon*cinnamon bark showed additive effects against all the bacteria tested. Oregano*cinnamon bark also showed additive against all bacteria except *E. coli* O157:H7 strain 380-94, RM 1239, and RM 1931, where no interactive effect was obtained. The FIC index of selected combinations to inhibit fungi is presented in Table 4.6. Mustard*thyme, mustard*garlic, and thyme*garlic all showed additive effects against both fungi.

Table 4.5 Fractional inhibitory concentration (FIC) of combined EOs against target bacteria.

Bacteria	T+O[a]		O+B		C+B	
	FIC	Effect	FIC	Effect	FIC	Effect
E. coli O157:H7 380-94	0.75	AD	1.06	NI	0.75	AD
E. coli O157:H7 RM1239	0.63	AD	1.06	NI	0.63	AD
E. coli O157:H7 RM1931	0.56	AD	1.06	NI	0.63	AD
E. coli O157:H7 RM1933	0.75	AD	0.75	AD	0.75	AD
E. coli O157:H7 RM1934	0.56	AD	0.75	AD	0.75	AD
S. enterica	0.75	AD	1.00	AD	0.63	AD
S. Typhimurium	0.75	AD	0.75	AD	0.63	AD
L. innocua	0.75	AD	1.00	AD	0.63	AD
L. monocytogenes	1.00	AD	0.75	AD	0.75	AD

FIC≤0.5: synergic effect (S); 0.5<FIC≤1: additive effect (AD); 1<FIC≤4: no interactive effect (NI); FIC>4: antagonistic effect (A). [a] Factor T: Thyme, O: Oregano, C: Chinese cinnamon, B: Cinnamon bark.

Table 4.6 Fractional inhibitory concentration (FIC) of combined EOs against target fungi.

Fungi	M+T[a]		M+G		T+G	
	FIC	Effect	FIC	Effect	FIC	Effect
A. niger	0.56	AD	0.56	AD	0.56	AD
P. chrysogenum	0.63	AD	0.63	AD	1.00	AD

FIC≤0.5: synergic effect (S); 0.5<FIC≤1: additive effect (AD); 1<FIC≤4: no interactive effect (NI); FIC>4: antagonistic effect (A). [a] Factor M: mustard, T: Thyme, G: garlic.

4.6 Discussion

EOs have been widely applied in food due to their great antibacterial and antifungal properties contributed by small constituent molecules (Bassolé & Juliani, 2012). The inhibitory capabilities may vary with types of EOs and target pathogens (Raut & Karuppayil, 2014). Most of the selected EOs tested in MIC have shown good antimicrobial abilities except garlic EO, which is not effective against target bacteria but showed good inhibition against the targeted fungi. Mustard, thyme, oregano, Chinese cinnamon, and cinnamon bark EOs have shown good antimicrobial activities, especially mustard EO, which exhibited a very low MIC value of 156 µg/mL against *A. niger* and *P. chrysogenum*, 313 µg/mL against *E. coli* O157:H7 1933, *Salmonella enterica* and *L. innocua*. Turgis *et al.* (2009) have reported that mustard EO is an effective antimicrobial agent against *Escherichia coli* O157:H7 and *Salmonella typhi.* Allyl isothiocyanate, the major component of mustard EO, has been reported efficiently inhibiting *L. monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *S.* typhimurium, *E. coli O157:H7* and *Pseudomonas aeruginosa* at MIC values 625, 625, 625, 625, 625 and 78 µg/mL, respectively (Dussault *et al.*, 2014). Carvacrol and thymol are the major components of Oregano and thyme EOs, which are recognized as strong antimicrobial agents (Memar *et al.*, 2017). According to Lambert *et al.* (2001), the antibacterial properties of carvacrol and thymol could depend upon their permeability, depolarization, and membrane fracture resulting in intracellular contents leak. Ahmad *et al.* (2011) discovered that thymol and carvacrol, two major compounds in thyme EO, present as lipophilic compounds that could impact the biosynthesis of ergosterol and destroy membrane with reactive hydroxyl groups to all the clinical and laboratory *candida* isolates. Cinnamaldehyde, as the main compounds of Chinese cinnamon and cinnamon bark, is also found widely inhibiting the growth of Gram-positive and Gram-negative bacteria, as well as yeasts and molds (Ooi *et al.*, 2006).

Chinese cinnamon oil induced less depletion of intracellular ATP than Spanish oregano and savory EO but more effective in reducing significantly the intracellular pH of *L. monocytogenes* and *E. coli O157:H7* that leads to cell damage (Oussalah *et al.*, 2006). Unlike thymol and carvacrol, cinnamaldehyde does not collapse the membrane causing large cell constituents release like ATP leaking but small ions; however, it restrains ATP generation and membrane-bound adenosine triphosphatase (ATPase) activities, therefore, induce a quick cease of energy metabolism (Gill & Holley, 2004; Helander *et al.*, 1998). Di Pasqua *et al.* (2007) reported that cinnamaldehyde showed rapid antimicrobial abilities by tremendously impacting the fatty acids in the membrane that brings to structural alterations of cell membrane. Research also showed that garlic EO highly inhibits *P. funiculosum* by destroying the membranes of cells and organelles as well as affecting the expression of some key proteins in physiological metabolism (Li *et al.*, 2014).

The test done in this research is a design method in order to identify interactions among five essential oils. The combinations that showed strong interactions were also evaluated the potential synergy between them to inhibit foodborne bacteria and molds. The design experiments revealed the potential synergy of interactions of different numbers of factors by factorial effects to the inhibition, which also offered the accessibility of interactions of more than two compounds at one time. To analyze the synergy effects from the design, the factorial effect is important as it presents the influence of all interactions and also can be used for calculating the final mean value of inhibition. From Table 4.3 and 4.4 we can calculate the inhibition of bacteria and fungi using different combinations of EOs. For example, for strain *E. coli* O157:H7 RM1934, when oregano was at a high level (MIC/5), the growth inhibition was 37.25% + 21.66% = 58.91% (constant adds the main effect of oregano), while the inhibition was 37.25% - 21.66% = 15.59% (constant substracts the main effect of oregano) when oregano was at its low level (MIC/10), together with other compounds. This means adding high-level oregano in occurrence with other compounds is more efficient in inhibition. Consistently, the inhibition efficiency of two, three, and four EOs against different bacteria or fungi could also be calculated (Nazer *et al.*, 2005). When oregano and cinnamon bark were both at their high values, the growth inhibition was 37.25% + 21.66% (main effect of oregano) + 7.43% (main effect of cinnamon bark) + 5.24% (two-factor effect of oregano*cinnamon bark) = 71.58%. Thus, the factorial effects provided the inhibition rate of different interactions. However, it should be noticed that the interaction could possibly not be accurately represented independently by the values calculated this way due to the collective effects of other inhibitors. In this consideration, we didn't use the calculated values for synergy analysis but focused on the significance of interactions. When an interaction gives the significance of factorial effects, that means the interaction dedicates significantly affect to the inhibition; thus,

it would be the interest to check further the precise synergy effects which could still possibly be synergy, addition, no interaction or antagonism depended on the FIC index (Ghabraie *et al.*, 2016). Consequently, the combinations with stronger interactions against more pathogenic species were further checked synergy by checkerboard method.

From the FIC results (Table 4.5 and 4.6), thyme*oregano, Chinese cinnamon*cinnamon bark showed additive effects against all bacteria strains, and mustard*garlic, mustard*thyme and thyme*garlic also showed additive effects against both fungi. Ghabraie (2016) found additive effects of Chinese cinnamon*cinnamon bark against *E. coli*, *L. monocytogenes*, *S. aureus*, *S.* typhimurium, and *P. aeruginosa*. Thyme*oregano and thymo*L**carvacrol were also found to have additive effects against targeted bacteria (Gavaric *et al.*, 2015). Carvacrol or thymol combined with eugenol were proved to have synergistic effects against *E. coli* which may be due to the disintegration of the outer membrane of *E. coli* by carvacrol and thymol that allows eugenol easier to enter the cytoplasm (Pei *et al.*, 2009). The structure, antibacterial capacity, and mechanism of thymol and carvacrol are similar (Lambert *et al.*, 2001); however, the mechanism of synergy is not clear yet. Zhou *et al.* (2007) proposed three hypotheses for a synergistic effect of thymol and carvacrol against *S.* typhimurium: (a) the antibacterial mechanism of thymol and carvacrol might be different for acting targets of *S.* typhimurium; (b) the synergistic effect exists because of the similarity of their mechanism; and (c) may only specific to *S.* Typhimurium (Bassolé & Juliani, 2012). Ji *et al.* (2019) discovered partial synergistic effect of garlic and thyme against *Penicillium corylophilum* strains. Mustard EO was found to have synergy with clove EO (Aguilar-González *et al.*, 2015) and cinnamon EO (Clemente *et al.*, 2019) against fungi.

Comparing both the factorial effects and FIC index of selected interactions, some interactions were not significantly efficient in factorial effects but still showed an addition in the FIC test. Also, the significant factorial effects could show no interactive effects in the checkerboard. For example, oregano*cinnamon bark presented no significance against *L. monocytogenes* but showed additive effect while no interaction to *E. coli* O157:H7 RM 1931, which showed 99.9% confidence interval significance. This actually offers proof that there are no strict corresponding relationships between factorial effects and synergy effects. However, all the selected interactions showed additive effects to all the targeted pathogens except oregano*cinnamon bark which presented no interactive effects against three *E. coli* strains, but these FIC values are still as low as close to 1. This fact gives the factorial design a great potential to largely reveal the synergy effects of two- and more than two-factor interactions as a synergistic screening method.

In previous research, several designs have been applied in the synergy study of EOs (Fadil *et al.*, 2018; Ouedrhiri *et al.*, 2016). Mixture designs were carried out against *Salmonella* typhimurium with *Thymus vulgaris*, *Rosmarinus officinalis,* and *Myrtus communis* EOs showing potential synergy, which was afterward verified to be synergistic effect by FIC, between *Thymus vulgaris* and *Myrtus communis* EOs and offering optimal portions of the combinations (Fadil *et al.*, 2018). Ouedrhiri *et al.* (2016) also applied mixture designs of *Origanum compactum*, *Origanum majorana,* and *Thymus serpyllum* essential oils against *S. aureus*, *E. coli*, *B. subtilis,* and *P. aeruginosa* to obtain combinations of EOs with optimized concentrations based on the statistical synergy effects. A 2^5 complete factorial design and a 2^{7-1} regular fractional factorial design were conducted with 5 aromatic compounds at 2 levels and a half-complete factorial design of 7 compounds (aromatic and acidic compounds) at 2 levels, respectively, to substantiate their ultimate synergistic potentials (Nazer *et al.*, 2005). A metamodeling antimicrobial cocktail optimization (MACO) scheme was demonstrated for rapid screening of potent antimicrobial cocktails, using fractional factorial design at only 18 parallel trials for 6 drugs with 3 concentration levels out of 729 (3^6) total combinations (Chen *et al.*, 2010). A validation study also confirmed the synergy effects under the concentrations selected in the prior sensitivity tests when the combination of trimethoprim and gentamicin at the same concentrations inhibited the bacteria more than the sum of the two drugs used individually.

Since the number of potential EO combinations is practically numerous, this design offers a systematic statistical approach, instead of the time-consuming conventional methods, for rapidly identifying the most promising mixtures and hierarchizing their effects among a large number of EOs based on the factorial factors. Furthermore, there are many research showing synergy between EOs but it is not fully explored about which interactions of molecules that lead to synergistic, additive, or antagonistic effects (Hyldgaard *et al.*, 2012). The results in this study could provide a starting point for investigating the molecular mechanisms responsible for this synergy and contribute to the development of new and more component combinations (Chen *et al.*, 2010).

4.7 Conclusions

In the present study, MIC results of eight EOs (mustard, thyme thymol, garlic, oregano compact, Chinese cinnamon, cinnamon bark, red bergamot, winter savory) against nine bacteria and two molds showed high antimicrobial abilities, except garlic EO that exhibited high MIC value against bacteria but low against fungi. Especially mustard EO is extremely efficient in inhibiting tested *E. coli*, *Salmonella,* and *Listeria* strains as well as molds (*A. niger* and *P. chrysogenum*) at

MIC value of 156 µg/mL. Besides the good inhibitory activity against bacteria, Chinese cinnamon and Cinnamon bark also showed good antifungal activities. A full 2^5 factorial design with five EOs at 2 concentration levels was conducted for each targeted microorganism. The factorial effects indicate the significantly strong interactions being highly potentially synergistic or additive effects, including thyme*oregano, oregano*cinnamon bark, Chinese cinnamon*cinnamon bark to bacteria and mustard*thyme, mustard*garlic, and thyme*garlic to fungi. These combinations obtained from factorial design were verified by checkerboard method. In checkerboard assay, the combination of thyme*oregano and Chinese cinnamon*cinnamon bark showed additive effects against all the tested bacteria. Combination of oregano*cinnamon bark showed no interactive effects against three *E. coli* O157:H7 strains but additive effects against the rest of bacteria. Combination of mustard*thyme, mustard*garlic, and thyme* garlic showed additive effects against the tested molds. Thus, the three combinations tested against bacteria and three combinations tested against fungi in checkerboard could be regarded as powerful antimicrobial formulations at concentrations obtained in FIC.

In summary, the potential synergistic or additive combinations from factorial design showed mostly additive effects in the checkerboard method, which allocates the feasibility of using factorial design for rapidly synergistic screening on various antimicrobial mixtures for formula development.

4.8 Acknowledgments

The authors are grateful for the financial support from Natural Sciences and Engineering Research Council of Canada (NSERC) and by Montpak (project number: RDCPJ 531475-18) and by a chair granted by Quebec Ministry of Agriculture, Fishery and Food (MAPAQ).

4.9 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5 ARTICLE 3 – *IN SITU* TESTS WITH GAMMA IRRADIATION

Combined effects of microencapsulated essential oils and γ-irradiation on microbiological and physicochemical properties of dry fermented sausages during ripening and storage

Effets combinés des huiles essentielles microencapsulées et de l'irradiation γ sur les propriétés microbiologiques et physico-chimiques des saucisses sèches fermentées pendant la maturation et le stockage

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Title of the journal:

Food Control

Submitted on 24-06-202

Revised on 08-09-2021

Manuscript Number: FOODCONT-D-21-01757

Contribution of authors:

ML is the research responsible and coordinator of the project. She has planned the research experiment. JJ conceived the design of the experiments, performed the experiments, organized the database, performed the statistical analysis, and wrote the first draft of the manuscript. S. Shankar edited and revised the manuscript. S. Salmieri and ML contributed to manuscript revision, read, and approved the submitted version.

5.1 Abstract

The present study illustrates the effect of combined treatments of encapsulated essential oils (encapsulated in alginate or alginate-CNC) and γ-irradiation (at the dose of 1.5 kGy) on *Escherichia coli* O157:H7, *Listeria monocytogenes*, molds and yeasts, lactic acid bacteria (LAB), and total mesophilic bacteria (TMF), as well as pH, water activity, color, and texture in dry fermented sausages during ripening and vacuum-packed during storage at room temperature (20 - 21 °C) for 20 weeks. Combined treatments showed synergetic effects on inhibiting resistant *E. coli* O157:H7 cocktail isolated from dry fermented sausage responsible of a *E. coli* outbreak and LAB. Results also showed a strong inhibition of *L. monocytogenes*, molds and yeasts, and TMF. Also, the combined treatments did not affect the texture of the sausages and encapsulation of the essential oils contributed to the color protection of the sausages.

Keywords: Essential oils; Microencapsulation; Gamma irradiation; Dry fermented sausage; Microbiological and physicochemical properties

5.2 Résumé

La présente étude illustre l'effet de traitements combinés d'huiles essentielles encapsulées soit dans de l'alginate ou dans de l'alginate en présence de nanocrystal cellulose (CNC) (alginate-CNC) en combinaison avec un traitement d'irradiation à une dose de 1.5 kGy. L'effet de ces combinaisons de traitements a été vérifié sur l'élimination d'*Escherichia coli* O157:H7, *Listeria monocytogenes*, moisissures et levures, acide lactique bactéries (LAB) et bactéries mésophiles totales (TMF), ainsi que sur le pH, l'activité de l'eau, la couleur et la texture des saucisses fermentées et séchées et emballées sous vide pendant la maturation et pendant le stockage à température ambiante (20 - 21 °C) soit 20 semaines. Les traitements combinés ont montré des effets synergiques sur l'inhibition du cocktail résistant d'*E. coli* O157:H7 isolé à partir de saucisse fermentée et séchée responsable d'une épidémie d'*E. coli*. Les résultats ont également montré une forte inhibition de *L. monocytogenes*, des moisissures et des levures, et une réduction du TMF. De plus, les traitements combinés n'ont pas affecté la texture des saucisses et l'encapsulation des huiles essentielles a contribué à la protection de la couleur des saucisses.

Mots-clés : Huiles essentielles; Microencapsulation; Irradiation gamma; Saucisson sec fermenté; Propriétés microbiologiques et physico-chimiques

5.3 Introduction

Dry fermented sausages (DFS) are manufactured by mixing ground lean meat, animal fat, salt, starter cultures, spices, and food additives (Lau, 2019; Van Ba *et al.*, 2017). One popular type of dry fermented sausages is salchichón (Rubio *et al.*, 2008). The production has three well-defined phases including mixing, fermentation, and drying (Fernández-López *et al.*, 2008). Dry fermented sausages (DFS) are uncooked meat products that are risky to be contaminated by spoilage strains like *Lactobacillus spp.*, *Pseudomonas*, yeasts, and molds or pathogenic microorganisms like *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* O157:H7 (Van Ba *et al.*, 2017) causing quality problems and numerous foodborne infections (Tomović *et al.*, 2020). Following the 1994 outbreak of *E. coli* O157:H7 of salami, FSIS required a validation of manufacturing process of a reduction of 5 log CFU / g of *E. coli* O157: H7 for all fermented sausages (GETTY *et al.*, 2000).

Essential oils (EOs) are aromatic and volatile liquids extracted from plants, considered secondary metabolites (Hyldgaard *et al.*, 2012). Essential oils are widely applied as flavorings and natural antimicrobial agents in food factories owing to multiple properties of EOs. Essential oils posses strong antibacterial properties (Gavaric *et al.*, 2015; Ghabraie, Vu, Tnani, *et al.*, 2016; Lionis *et al.*, 2021), antiviral (Asif *et al.*, 2020; Lionis *et al.*, 2021; Panikar *et al.*, 2021), antifungal (Clemente *et al.*, 2019; Hossain *et al.*, 2016) and antioxidant (Jayasena & Jo, 2014) properties. Essential oils are generally recognized as safe (GRAS) as food additives (Ayari *et al.*, 2020). Carvacrol, the main component of oregano EO, affects the outer membrane of bacteria resulting in ions leakage in bacteria that led to cell inhibition (Lambert *et al.*, 2001). Eugenol is an active constituent in clove EO that can permeabilize the bacterial cell membrane or suppresses their virulence factors, including the production of intracellular reactive oxygen species (ROS), inhibition of some bacterial enzymes, and inhibition of bacterial and fungal biofilm formation (Marchese *et al.*, 2017). However, EOs are often required higher concentrations when applied on food to achieve as good antimicrobial effects as *in vitro;* therefore, the high volatility of EOs always leads to a decrease in acceptance (Bozkurt, 2006; Busatta *et al.*, 2008; Van Ba *et al.*, 2017). In addition, the high content of fat in meat products can protect the microorganisms from the action of EOs (Burt, 2004). Encapsulation of EOs is one of the most efficient solutions to increase the solubility and stability of EOs when applied in food models, masking the unacceptable odor and taste besides control release (Ghaderi-Ghahfarokhi *et al.*, 2017). Alginate is a polysaccharide extracted from brown algae. Due to the biodegradability and easy gelation with alkaline metals, it is used in many fields (Criado *et al.*, 2020). Cellulose nanocrystal (CNC) is a biodegradable

nanomaterial; when mixed with alginate, CNC creates pathways in alginate emulsion that delays the release of EOs and enhances the stability of alginate and EO solutions (Criado *et al.*, 2019; Huq *et al.*, 2012; Kaboorani *et al.*, 2016; Moon *et al.*, 2011).

Gamma irradiation is an efficient food decontamination method. A dose of 3 kGy γ-irradiation can be applied to meat without alterations of sensory properties (Lacroix, 2014). Combining irradiation with other food preservation methods can lower the dose of irradiation, keep the stability of organoleptic and nutritional properties of food, increase radiosensitization of microorganisms and extend the shelf life of food (Ben Fadhel *et al.*, 2016; Lacroix, 2014). Previous research has discovered a synergy between marination of vegetable extracts and 1.5 kGy of γ-irradiation to inactivate pathogenic bacteria and increase the shelf-life of ready-to-cook pork loins without influencing its sensorial and nutritional qualities (Ben Fadhel *et al.*, 2016). The synergetic effect was also observed when thyme loaded alginate-CNC microbeads combined with γ-irradiated at 1 kGy to eliminate *L. innocua* in ground meat (Criado *et al.*, 2019) and cinnamon/oregano and nisin loaded Alginate-CNC beads with 1.5 kGy γ-irradiation to inhibit *L. monocytogenes* applied on ready-to-eat ham (Huq *et al.*, 2015).

The main objective of this study was to evaluate the effects of combined treatments of microencapsulated EOs and γ-irradiation on inhibiting resistant *E. coli* cocktail isolated from fry fermented meats which is responsible for the health outbreak, *L. monocytogenes*, molds and yeasts, LAB, TMF and on physicochemical properties during the ripening and storage of DFS.

5.4 Materials and methods

5.4.1 Preparation of bacterial cultures

The mixture of five *E. coli* O157:H7 strains (RM1239, RM1931, RM1933, RM1934, 380-94) prealably isolated from fermented dry sausage responsible of outbreaks and *L. monocytogenes* (LM 1045) was kept at − 80 °C in Tryptic Soy Broth (TSB; Becton-Dickinson, Sparks, MD, USA) containing glycerol (10% v/v). Before each experiment, stock cultures were propagated through three successive growth cycles at 37 °C for 24 h in TSB for *E. coli* cocktail and *L. monocytogenes* to obtain stationary phase cells approximately 10^{12} CFU/mL and 10^{9} CFU/mL, respectively.

5.4.2 Antimicrobial formulation preparation

Formulations were prepared according to the method of Huq *et al.* (2015) with some modifications. An aqueous suspension of the encapsulation polymer was prepared by dissolving 2% (w/v) alginate (Sigma-Aldrich, Ontario, Canada) in deionized water for 24 h under magnetic stirring. Spray-dried CNC powder (Pointe-Claire, QC, Canada) was dispersed in deionized water under magnetic stirring to form 1% (w/v) CNC suspension. Then, ultra-sonication (QSonica Q-500, Misonix, Qsonica, LLC, Newtown, CT, USA) at 1000 J/g was applied on CNC suspension. A 5% (w/w) CNC from 1% CNC suspension (according to wt% of alginate) was homogenized using an Ultra-Turrax TP18/1059 homogenizer (Janke and Kunkel, Staufen, Germany) at 20 ºC and 20,000 rpm for 2 min. Alginate-CNC suspension was thereafter emulsified with the formulation based on 3 % cinnamon EOs according to Huq *et al.* (2015) using Tween 80 (5% w/v, Sigma–Aldrich Ltd). The coarse emulsion was homogenized using an Ultra-Turrax TP18/1059 homogenizer at 15,000 rpm for 2 min. A quantity of 0.01 M $CaCl_2$ (Sigma-Aldrich, Ontario, Canada) solution was dropped into emulsified suspension according to portion alginate-CNC-EOs: $CaCl_2$: 75:25. The final concentration of EOs in alginate-CNC microbeads was 2.25%. The free EOs and EOs encapsulated in alginate were also verified to evaluate the effectiveness of microencapsulation of alginate-CNC and the effectiveness of CNC in microencapsulation. In the end, four formulations were prepared, including $CaCl_2$ solution, non-encapsulated EOs, EOs encapsulated in alginate, and EOs encapsulated in alginate-CNC.

5.4.3 BHI-agar deep-well model to evaluate the depletion of formulations

In order to evaluate the depletion of the antimicrobial formulations over time, BHI-agar deep well model was used according to a modified methods of Bi *et al.* (2011). To prepare the BHI-agar deep-well model for depletion test, 225 mL of sterilized BHI-agar solution was poured into a 600-mL beaker to a height of 40 mm. After gel solidification, a 7.0-mm pipet tip was used to make four wells (from gel surface to bottom) in each beaker. Subsequently, 1.0 mL of each formulation was added to each well and beaker was stored at 4 ℃. A 100-µL aliquot of each formulation in the well was transferred to a bioassay plate to determine the antimicrobial activity at day 0 and after 1, 3, 7, 14 and 21 days of storage.

5.4.4 Antimicrobial activities evaluation of formulations

Antimicrobial activities of available free or microencapsulated antimicrobial contents in formulations was evaluated by bioassay method as described by Huq *et al.* (2015) with some

modifications. The sterile BHI-agar were inoculated with *L. monocytogenes* or *E. coli* cocktail (10^6 CFU/mL). A 25 mL of inoculated broth was added to each Petri dish plate (95 mm ×15 mm) and allowed to solidify. Thereafter, holes of 7.0 mm in diameter were made and 100-µL of the antimicrobial formulation(s) from BHI-agar deep-well model were added to each agar well. The plates were incubated for 24 h at 37 ℃ temperature and the inhibition zone (mm) was measured to determine the antimicrobial activity against bacteria. The zone of inhibition around the cellulose disc (transparent zone presenting the absence of colonies) was measured using traceable carbon fiber digital caliper (resolution: 0.1 mm accuracy: 0.2 mm; Fisher Scientific). The inhibitory capacity (IC, %) is calculated as follow by Equation:

Inhibitory capacity (%) = (diameter of the inhibition zone / diameter of Petri dish) x 100 (8)

5.4.5 Sausage manufacture

Sausages were manufactured in a biosafety level 2 laboratory at INRS according to a developed manufacturer procedure. Inoculum of 4% *E. coli* cocktail (10^{12} CFU/mL) and 0.1% *L. monocytogenes* (10^8 CFU/mL) were inoculated to meat. Then, the meat was mixed with meat spices (3.03%) and starter culture (0.05%, *Staphylococcus camosus*, *Kocuria salsicia*, *Lactobacillus sakei*) for fermentation. Finally, the EO concentration of 0.45% (10 mL formulations were added to each 50-g sausage) was added to the formulation. The concentration of EO was selected based on preliminary studies whose data are not shown herein. The meat mixture was embossed in the casings using a Tre Spade sausage filler (Mod. 10 Deluxe; P/N 21100/L; FACEM SpA, Turin, Italia) to obtain diameter approximately 18 mm and weight around 50 g per sausage. Afterwards, sausages were fermented for 48 h (25±0.5 °C, 90±2% RH) to reach pH approximate 5.20 followed by 5 days of drying (14± 1°C, 70 ± 5% RH) to reach Aw approximately 0.85. Sausages without inoculum were manufactured follow the same way as the control.

5.4.6 Sample irradiation

The irradiation procedure was done in a cobalt-60 Underwater Calibrator UC-15A (Nordion, Ottawa, ON, Canada) having an energy level of 1.25 MeV and dose rate of 6.37 kGy/h. Dry fermented sausages (DFS) samples of each formulation group were sealed under 96% vacuum in eight transparent bags (Winpak Ltd., Vaudreuil-Dorion, QC, Canada) separately using a packaging machine (model 250 Single Chamber, Sipromac Inc., St-Germain-de-Grantham, QC, Canada) and kept at 4 °C. Then, sausages were irradiated at 1.5 kGy. Finally, eight groups of different treatments on sausages were tested including control (CT), EOs treated (EO), EO-

alginate treated (AE), EO-alginate-CNC treated (ACE), γ-irradiation treated (CT+GI), EOs + γ-irradiation treated (EO+GI), EO-alginate + γ-irradiation treated (AE+GI), and EO-alginate-CNC + γ-irradiation treated (ACE+GI).

5.4.7 Microbiological analysis

Each sausage sample (10 g) was mixed in 90 mL of peptone water (0.1%) in sterile Whirl-Pak sampling bags (Fisher Scientific, Ontario, Canada). Then, samples were homogenized in a Seward 400 Circulator Stomacher® (Fisher Scientific) at 260 rpm for 1 min. Then the homogenates were serially diluted (1/10) in peptone water (Alpha Biosciences Inc., Baltimore, MD, USA). Subsequently, dilutions (100 µL) were spread-plated following official methods. In order to improve the limit of detection (LOD) from 2 log to 1 log, when applicable, dilutions were also pour-plated in molten cooled agar. Aerobic mesophilic bacteria were plated and counted on tryptic soy agar (Alpha Biosciences Inc., Baltimore, MD, USA) incubated at 37 ℃ for 48 h; Lactic acid bacteria were counted on Man Rogosa Sharpe agar (Alpha Biosciences Inc., Baltimore, MD, USA) incubated at 30 ℃ for 72 h; molds and yeasts were counted on potato dextrose agar (Alpha Biosciences Inc., Baltimore, MD, USA) after incubation at 25 ºC for 72 h; *E. coli* O157:H7 were counted on MacConkey sorbitol agar (Oxoid Unipath Ltd. Nepean, Ottawa, Canada) incubated at 37 ºC for 24 h; *L. monocytogenes* were counted on Palcam agar (Oxoid Unipath Ltd. Nepean, Ottawa, Canada) with addition of antibiotics acriflavine (5 mg/mL), polymyxin B (10 mg/mL) and ceftazidime (8 mg/mL), incubated at 37 °C for 48 h. Samples were taken for microbial counts before fermentation (Time 0), after fermentation (Time F), after drying and applied irradiation (End dry) and during storage weeks (1 - 20 weeks).The detection limit of the above mentioned techniques is 10 CFU/g.

5.4.8 pH, temperature and Aw measurement

The pH, temperature and Aw were tested during fermentation and drying to monitor the right condition and ending point for each stage. Color and texture were tested during storage to verify the effects of antimicrobial formulations, irradiation, and storage time.

Monitoring of the temperature and the pH of sausages during fermentation was conducted using Omega OM-CP-pHTEMP2000 data loggers (Omega Canada, Laval, QC, Canada) equipped with glass pH electrodes AlphaSeries PHE-2385 and temperature probes RTD PRTF-11-3-100-(3/16)-16-E. Data were collected by using OM-CP data logger software (Omega Engineering, Norwalk, CT, USA). Aw measurements were performed at regular intervals during drying (weekly during

the first 2 weeks and then daily), using a Rotronic AwQuick A2101 system equipped with an AWC probe (Géneq Inc., Montreal, QC, Canada). The Aw-meter was calibrated using 0.5, 10, 35, 80 and 95% humidity standards. Aw measurements were applied only on non-inoculated sausages for biosafety purposes.

5.4.9 Color measurement

Both the exterior and the interior color of DFS were evaluated using a Minolta Colorimeter Color reader CR10 (Konica Minolta Sensing, Inc., Mahwah, NJ, USA). The surface color of DFS was measured immediately after removing the sausage casing, whereas the interior color was measurements after slicing the sausages into slices of 2 cm thick with diameter of around 18 mm (Lau, 2019). Three samples were taken on each treatment. Color coordinates were determined by the CIE-LAB system and the results were expressed as lightness (*L**), redness (*a**) and yellowness (*b**). In addition, hue angle of the polar representation, was calculated according to Equation (9) and total change of color ΔE* calculated according to Equation (10) were quantified on each sample.

$$h = \tan^{-1}(b^*/a^*) \tag{9}$$

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \tag{10}$$

5.4.10 Texture analysis

During storage, three sampling sausages were selected randomly from the same treatment. The casing was removed and sausages were cut into 2 cm thick slices with flat contact surfaces before tests (Houben & van 't Hooft, 2005). Texture was measured at ambient temperatures with a Universal Tensile Machine (Model H5KT, Tinius-Olsen Inc., Horsham, PA, USA,) equipped with a 100 N-load cell (type FBB). A compression method was selected, and samples were compressed 20% of original height. The max stress and Young's modulus were obtained by using the corresponding UTM software.

5.4.11 Statistical analysis

Each experiment was done in triplicate (n = 3). For each replicate, 2 samples from each treatment group were analyzed for microbial tests and 3 samples from each treatment group were analyzed for physicochemical analysis. A one-way analysis of variance (ANOVA) for equal variances, Tamhane's test for unequal variances and Duncan's multiple-range test were

performed. Differences between means were considered significant when the confidence interval was smaller than 5% ($P \leq 0.05$). The analysis was performed by PASW Statistics 18 software (IBM Corporation, Somers, NY, USA).

5.5 Results and discussion

5.5.1 *In vitro* evaluation of the antimicrobial activities of formulations

Fig. 5.1 represents the effects of available free or microencapsulated antimicrobials against *L. monocytogenes* and *E. coli* cocktail, described by inhibitory capacity (IC, %). Formulation with only EO showed higher inhibition against both *L. monocytogenes* and *E. coli* at Day 0, but loss antibacterial ability fast at Day1 which had smaller inhibition zone comparing to 2 encapsulated formulations that shows the microencapsulation has protected the EOs from fast volatizing. The rapid decrease of antimicrobial abilities of free EOs is similar to the results obtained by Huq *et al.* (2015), in which cinnamon EO and oregano EO encapsulated in alginate-CNC microbeads presented control release of EOs. At Day 3, the 3 formulations showed not much difference inhibiting *L. monocytogenes*, however encapsulated EOs in alginate and CNC showed higher inhibition to *E. coli* than EOs only encapsulated in alginate. At Day 7, EOs encapsulated in alginate and CNC showed higher inhibition against *L. monocytogenes* than other 2 formulations. However, EOs encapsulated in alginate-CNC showed similar effects with EOs non-encapsulated and EOs encapsulated in alginate against *E. coli*. Release of free antimicrobials was tested for 21 days. At the end of storage, there was no inhibition of the three solutions against the pathogens tested. The mechanism of EOs antimicrobial action was studied by Oussalah *et al.* (2006) and they demonstrated that oregano and savory EOs can affect the membrane integrity of *L. monocytogenes* and *E. coli* O157:H7 and induced depletion of the intracellular ATP. In another study also it was observed that mustard EO could affect the membrane integrity of *E. coli* O157:H7 and *Salmonella typhi* and caused leakage of the intracellular ATP (Turgis *et al.*, 2009). Cinnamaldehyde as the major active component of cinnamon EO, inhibits transmembrane ATPase (Hyldgaard *et al.*, 2012) and decreases intracellular ATP (OUSSALAH *et al.*, 2006) that inhibit the growth of bacteria.

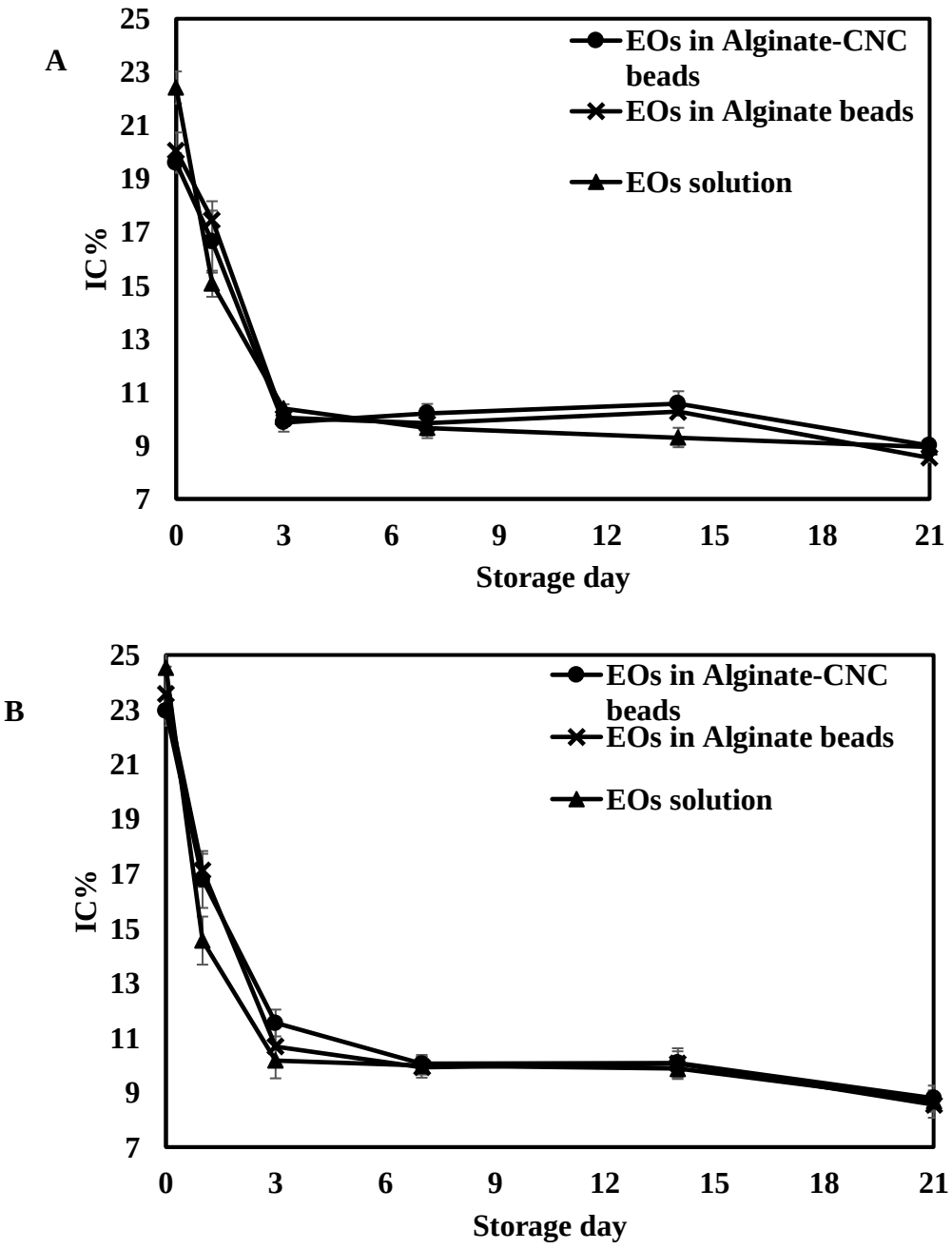

Figure 5.1 **Antimicrobials against (A) *L. monocytogenes* and (B) *E. coli* cocktail during storage at 4 ℃.**

5.5.2 *E. coli* elimination by the antimicrobial formulations: *in situ* test without and with irradiation

The reduction of *E. coli* during whole process is shown in Table 5.1. For control group, drying reduced by 2.9-log *E. coli*; no *E. coli* was detected after 8 weeks of storage. EO reduced 3.2-log *E. coli* after drying and no *E. coli* was detected at 2nd week storage. AE reduced 3.2-log *E. coli* after drying and no *E. coli* was detected at 4th week of storage. ACE reduced 3.4-log *E. coli* after

drying and no *E. coli* was detected after 2 weeks storage. Irradiation was applied after drying. The results suggested that irradiation and EO, AE, ACE have the same effect, irradiation eliminated *E. coli* only at 4th week of storage. In the presence of EOs combined with irradiation, no matter if they were encapsulated or not, no detection of *E. coli* was observed. In presence of EOs encapsulated or not, around 3 log reduction of *E. coli* after drying and no reduction after fermentation were observed. EO+GI reduced the period of *E. coli* elimination from 4 weeks to 2 weeks and encapsulation reduced the elimination period of 4 weeks compared to only irradiated group. Irradiation did a complete elimination of *E. coli* at the end of drying in presence of EOs and EOs in encapsulation.

Samples treated with EO, EO-alginate and EO-alginate-CNC combined with 1.5 kGy γ-irradiation had inhibited *E. coli* to a non-detective level at the end of drying, which indicated that all three antimicrobial treatments (EO, EO-alginate and EO-alginate-CNC) combined with 1.5 kGy γ-irradiation had showed a synergetic effect to inhibit *E. coli* O157:H7 cocktail. Take EO-GI for example, CT samples reduced 2.9-log (7.7 - 4.8 = 2.9 log CFU/g) after drying. Samples treated with only EO reduced *E. coli* from 7.3 to 4.1 log CFU/g which caused a reduction of 3.2-log, excluding the 2.9-log reduction for fermentation and drying process, the real reduction contributed by EOs is 0.3-log. The samples treated with only γ-irradiation reduced *E. coli* from 7.7 to 4.3 log CFU/g which contributed to a real reduction of 0.5-log (7.7 - 4.3 -2.9 =0.5 log CFU/g). When samples treated with EO combined to γ-irradiation, the reduction was 3.4-log (7.3 -1 - 2.9 = 3.4 log CFU/g) after drying which is more than the total sum of the reduction of EO and CT+GI groups (0.3 + 0.5 =0.8 log CFU/g).

Previous research has observed good inhibition to *E. coli* by using EOs. Carvacrol or thyme EOs showed significant reduction to *E. coli* applied on beef (Stratakos & Grant, 2018). Carvacrol nanoemulsion disposed in modified chitosan coating has significantly increased the radiosensitization of *E. coli* O157:H7 by 1.32-fold (Severino *et al.*, 2015). Addition of 1% ginger extract following with a treatment with γ-irradiation at 3 kGy or 5 kGy on processed frozen beef sausages were found sufficient to keep *E. coli* within safe levels for 3 months (Sediek *et al.*, 2012). The combination of EO or encapsulated EO treated with γ-irradiation also help to achieve a 5-log reduction for the procedures to assure the safety of fermented meats according to the USDA/FSIS (Porto-Fett *et al.*, 2008). Gamma irradiation is a type of ionizing radiation that inactivates the microorganisms by direct breakdown the chemical bonds within DNA or by the indirect damage of oxidative radicals originating from the radiolysis of water on cell membranes and chromosomes (Lacroix, 2014). This may facilitate the contact between antimicrobial molecules and cell

membranes and thus increase the inhibitory effects (Lacroix, 2014). Therefore, irradiation could enhance the antimicrobial capabilities of essential oils meanwhile the addition of essential oils could increase the radiosensitivity of microorganisms (Turgis *et al.*, 2009; Turgis *et al.*, 2012).

Table 5.1 Growth of *E. coli* cocktail during sausage processing and storage at room temperature[1].

	Concentration of *E. coli* cocktail (log CFU/g)										
Samples	Time 0	Time F	End dry	1week	2weeks	4weeks	6weeks	8weeks	12weeks	16weeks	20weeks
CT	7.7 ± 0.2^{dB}	7.4 ± 0.1^{dB}	4.8 ± 0.0^{cB}	4.6 ± 0.7^{cC}	3.3 ± 0.0^{bB}	2.2 ± 0.3^{aA}	1.8 ± 1.1^{aA}	<1	<1	<1	<1
EO	7.3 ± 0.3^{cA}	7.1 ± 0.2^{cA}	4.1 ± 0.9^{bA}	2.4 ± 0.5^{aA}	<1	<1	<1	<1	<1	<1	<1
AE	7.4 ± 0.2^{dA}	7.2 ± 0.1^{dAB}	4.2 ± 0.4^{cA}	2.4 ± 0.4^{bA}	1.5 ± 0.1^{aA}	<1	<1	<1	<1	<1	<1
ACE	7.4 ± 0.1^{dAB}	7.2 ± 0.1^{dA}	4.0 ± 0.2^{cA}	2.4 ± 0.4^{bA}	<1	<1	<1	<1	<1	<1	<1
CT+GI	7.7 ± 0.2^{dB}	7.4 ± 0.1^{dB}	4.3 ± 0.3^{cA}	3.0 ± 0.5^{bB}	1.5 ± 0.3^{aA}	<1	<1	<1	<1	<1	<1
EO+GI	7.3 ± 0.3^{aA}	7.1 ± 0.2^{aA}	<1	<1	<1	<1	<1	<1	<1	<1	<1
AE+GI	7.4 ± 0.2^{bA}	7.2 ± 0.1^{aAB}	<1	<1	<1	<1	<1	<1	<1	<1	<1
ACE+GI	7.4 ± 0.1^{bAB}	7.2 ± 0.1^{aA}	<1	<1	<1	<1	<1	<1	<1	<1	<1

[1] Numbers are expressed as means ± standard deviations. Within each row, means with the same lowercase letter are not significantly different (P > 0.05). Within each column, means with the same uppercase letter are not significantly different (P > 0.05).

5.5.3 *L. monocytogenes* elimination by the antimicrobial formulations: *in situ* test without and with irradiation

The reduction of *L. monocytogenes* during whole process is shown in Table 5.2. Fermentation did not have any effect on *Listeria* whatever the treatment was. All treatments with γ-irradiation of 1.5 kGy eliminated *Listeria* after drying. For control group, 1.5-log reduction at the end of drying and no reduction by fermentation was observed. Complete elimination was observed after 4 weeks storage. For EO, no significant reduction by fermentation but a complete elimination at the end of drying compared to control was observed. Encapsulation of alginate showed a delay in the elimination of *Listeria*. At the end of drying a 3.4-log reduction was observed

and a complete elimination occurred after 1 week storage. Alginate-CNC encapsulated EO exhibited similar antimicrobial effect like EO, and a complete elimination was observed at the end of drying.

L. monocytogenes is observed to be more resistant to γ-irradiation than *E. coli* O157:H7 (Tawema *et al.*, 2016). However, results in the present study showed that *L. monocytogenes* is more sensitive to the antimicrobials and to γ-irradiation than *E. coli*, which allows a strong inhibition during drying. This could be possibly due to the manufacturing procedures of DFS, the conditions of anaerobiosis and salinity occurred in drying process of manufacturing that also contribute to the decrease in the number of bacteria (Fernández-López *et al.*, 2008). This result is persistent with previous research (Lindqvist & Lindblad, 2009) that inactivation rates of *E. coli* is higher than *L. monocytogenes* at 20 ℃ for *E. coli* and 22 ℃ for *L. monocytogenes* for fermented sausages. Chinese cinnamon and cinnamon bark combined with nitrite, nisin, and organic acid salts encapsulated in Alginate-CNC microbeads showed efficient reduction of *L. monocytogenes* in fresh pork sausages (Ghabraie, Vu, Tata, *et al.*, 2016). The combination of γ-irradiation and natural antimicrobials was discovered good to inhibit *L. monocytogenes* (Sediek *et al.*, 2012; Tawema *et al.*, 2016). Samples treated with EO-Alginate and control group showed non *L. monocytogenes* at 1st week and 4th week of storage, respectively. Oregano and cinnamon EO with nisin encapsulated in Alginate-CNC microbeads in combination with 1.5 kGy γ-irradiation also has shown synergistic effect during storage and microencapsulation significantly improved the radiosensitivity of *L. monocytogenes* on ready-to-eat meat products (Huq *et al.*, 2015).

Table 5.2 **Reduction of *L. monocytogenes* during sausage processing and storage at room temperature[1].**

	Concentration of *L. monocytogenes* (log CFU/g)									
Samples	Time 0	Time F	End dry	1week	2weeks	4weeks	6weeks	12weeks	16weeks	20weeks
CT	5.2 ± 0.5^{bA}	5.1 ± 0.7^{bA}	3.7 ± 0.9^{aB}	3.7 ± 0.4^{aA}	3.3 ± 0.3^{aB}	<1	<1	<1	<1	<1
EO	4.7 ± 0.7^{bA}	4.0 ± 0.2^{aA}	<1	<1	<1	<1	<1	<1	<1	<1
AE	4.8 ± 0.6^{bA}	4.3 ± 0.6^{bA}	1.4 ± 0.6^{aA}	<1	<1	<1	<1	<1	<1	<1
ACE	4.7 ± 0.3^{bA}	4.3 ± 0.3^{aA}	<1	<1	<1	<1	<1	<1	<1	<1
CT+GI	5.2 ± 0.5^{aA}	5.1 ± 0.7^{aA}	<1	<1	<1	<1	<1	<1	<1	<1
EO+GI	4.7 ± 0.7^{bA}	4.0 ± 0.2^{aA}	<1	<1	<1	<1	<1	<1	<1	<1
AE+GI	4.8 ± 0.6^{bA}	4.3 ± 0.6^{aA}	<1	<1	<1	<1	<1	<1	<1	<1
ACE+GI	4.7 ± 0.3^{bA}	4.3 ± 0.3^{aA}	<1	<1	<1	<1	<1	<1	<1	<1

[1]Numbers are expressed as means ± standard deviations. Within each row, means with the same lowercase letter are not significantly different (P > 0.05). Within each column, means with the same uppercase letter are not significantly different (P > 0.05).

5.5.4 Molds and yeasts elimination by the antimicrobial formulations: *in situ* test without and with irradiation

The reduction of molds and yeasts during whole process is shown in Table 5.3. In summary, fermentation and drying didn't affect the growth of molds and yeasts. Molds and yeasts were eliminated only after 8 weeks of storage. Only 0.7-log reduction was found after drying. In presence of EOs whatever encapsulated or not, around 2-log reduction of molds and yeasts was observed during fermentation and were completely eliminated at the end of drying. In one study, cinnamon EO encapsulated in chitosan nanoparticles was found to reduce molds and yeasts during first 4 days of storage on beef patties (Ghaderi-Ghahfarokhi *et al.*, 2017). Cinnamaldehyde as the main active component of cinnamon EO, showed reduction in the growth of fungi by inhibiting the cell wall synthesizing enzymes that discourages the cell division (Bang *et al.*, 2000). The irradiation group showed a complete elimination of mold and yeasts only at 6th week of storage. This shows molds and yeasts could be more resistant to irradiation than *L. monocytogenes* and *E. coli* O157:H7. This corresponds to the fact that molds and yeasts are generally more resistant to irradiation than vegetative bacterial cells (Monk *et al.*, 1995). Until 8th week of storage, the control group inhibited molds and yeasts to a non-detective level.

In summary, results showed that EOs, EOs encapsulated in alginate and EOs encapsulated in alginate-CNC have similar inhibition effects to the resistant *E. coli* under dry and acidity condition. EOs and EOs encapsulated in alginate-CNC showed stronger inhibition effects to *L. monocytogenes* than EOs encapsulated in alginate without CNC. Antimicrobials combined with 1.5 kGy γ-irradiation have inhibited all three microorganisms below the detection limit at the end of drying. All three antimicrobials also showed synergetic effects when combined with 1.5 kGy γ-irradiation against *E. coli* O157:H7 cocktail right after irradiation and until 1 week storage. EOs combined with 1.5 kGy group has also showed synergistic effects until 2 weeks storage.

Table 5.3 Reduction of molds and yeasts during sausage processing and storage at room temperature[2].

	Concentration of molds and yeasts (log CFU/g)										
Samples	Time 0	Time F	End dry	1week	2weeks	4weeks	6weeks	8weeks	12weeks	16weeks	20weeks
CT	7.4 ± 0.2^{fB}	7.7 ± 0.5^{fB}	6.7 ± 0.4^{eB}	6.0 ± 0.3^{dB}	5.4 ± 0.4^{cB}	4 ± 0.5^{bBC}	2.1 ± 0.9^{aA}	<1	<1	<1	<1
EO	6.8 ± 0.2^{bA}	4.6 ± 0.4^{aA}	<1	<1	<1	<1	<1	<1	<1	<1	<1
AE	6.8 ± 0.5^{bA}	4.9 ± 0.7^{aA}	<1	<1	<1	<1	<1	<1	<1	<1	<1
ACE	6.7 ± 0.3^{bA}	4.8 ± 0.4^{aA}	<1	<1	<1	<1	<1	<1	<1	<1	<1
CT+GI	7.4 ± 0.2^{eB}	7.7 ± 0.5^{eB}	5.7 ± 0.3^{dA}	4.0 ± 0.4^{cA}	3.1 ± 0.2^{bA}	1.7 ± 0.9^{aAB}	<1	<1	<1	<1	<1
EO+GI	6.8 ± 0.2^{bA}	4.6 ± 0.4^{aA}	<1	<1	<1	<1	<1	<1	<1	<1	<1
AE+GI	6.8 ± 0.5^{bA}	4.9 ± 0.7^{aA}	<1	<1	<1	<1	<1	<1	<1	<1	<1
ACE+GI	6.7 ± 0.3^{bA}	4.8 ± 0.4^{aA}	<1	<1	<1	<1	<1	<1	<1	<1	<1

[1]Numbers are expressed as means ± standard deviations. Within each row, means with the same lowercase letter are not significantly different (P > 0.05). Within each column, means with the same uppercase letter are not significantly different (P > 0.05).

5.5.5 Lactic acid bacteria (LAB) elimination by the antimicrobial formulations: *in situ* test without and with irradiation

The effect of γ-irradiation and antimicrobials on LAB counts is presented in Table 5.4. An increase of LAB count from 1.2 to 1.5 log was observed during fermentation in all groups (Table 4). EOs did not affect the LAB growth at Time 0 and during the whole fermentation period and LAB were stable during drying. However, irradiation applied after drying reduced the LAB level by 1.5-log. In the presence of EOs free or encapsulated, irradiation reduced the level of LAB by 2.5 to 3 log. With the growth of LAB in all non-irradiated groups and 0.1-log reduction in CT+GI group, the EOs and EOs encapsulated all showed synergistic effects inhibiting LAB when combined with γ-irradiation. LAB count in control group was stable during 6 weeks storage. A mean of 1 log reduction was observed in all non-irradiated groups at 6th week storage while a 2-log reduction was observed for irradiated groups. At the end of storage, the level of LAB is similar in all groups showing a mean of 3 log of LAB except for CT and CT+GI where 4.3 and 3.8 log was observed. EO-Alginate-CNC group showed the lower level of LAB as compared to the control and sausages treated with free or alginate encapsulated EOs. A significant reduction of LAB was observed at 8th week of storage in all samples treated with EOs non-irradiated groups. All samples treated with EOs, and γ-irradiation showed a lower level of LAB during drying, and the lower level of LAB was observed in samples treated with EOs encapsulated in Alginate and CNC. A synergistic effect was observed between EOs and irradiation.

The LAB counts obtained at the beginning in this test are comparable with other research (Rubio *et al.*, 2008) or different form some research (Fernández-López *et al.*, 2008). This kind of variations can be explained by the different quantities (Ben Fadhel *et al.*, 2016) or types of starter cultures. LAB also decreased slowly during storage for non-treated groups and remained around 8 log CFU/g as the dominant microflora of sausages which may be considered that vacuum packaging delays the growth of LAB, due to the capability of tolerating anaerobic conditions (Drosinos *et al.*, 2005; Rubio *et al.*, 2007). Previous research showed chitosan coating containing encapsulated *Paulownia tomentosa* essential oil has decreased LAB counts 2.61 log CFU/g in chilled pork meat after 16 days stored at 4 °C (Zhang *et al.*, 2019). Chitosan and cumin essential oil nanoemulsion combined with low dose γ-irradiation has also showed significant decrease in LAB counts in beef loins during storage (Dini *et al.*, 2020). Khan *et al.* (2012) has reported 1.5 kGy γ-irradiation combined with antimicrobial formulas including oregano EO could reduce LAB counts by around 3 log CFU/g in fresh pork sausage meat after 13 days storage at 4 °C.

Table 5.4 **Reduction of *lactic acid bacteria* during sausage processing and storage at room temperature[1].**

	Concentration of LAB (log CFU/g)										
Samples	Time 0	Time F	End dry	1week	2weeks	4weeks	6weeks	8weeks	12weeks	16weeks	20weeks
CT	7.2 ± 0.7^{deA}	8.6 ± 0.2^{fgA}	9.0 ± 0.4^{gE}	8.6 ± 0.3^{fgD}	8.2 ± 0.0^{fE}	7.7 ± 0.4^{eC}	6.9 ± 0.3^{dF}	6.6 ± 0.2^{dE}	6.0 ± 0.1^{cC}	5.4 ± 0.6^{bD}	4.3 ± 0.0^{aC}
EO	6.9 ± 0.5^{eA}	8.2 ± 0.3^{fA}	8.2 ± 0.1^{fD}	8.0 ± 0.3^{fC}	8.0 ± 0.2^{fDE}	7.4 ± 0.5^{eC}	6.0 ± 0.2^{dDE}	5.0 ± 0.2^{cCD}	4.2 ± 0.4^{bB}	3.7 ± 0.0^{bB}	3.0 ± 0.0^{aAB}
AE	7.0 ± 0.6^{cdA}	8.2 ± 0.7^{eA}	8.3 ± 0.4^{eD}	8.2 ± 0.3^{eC}	8.1 ± 0.0^{eDE}	7.6 ± 0.2^{deC}	6.4 ± 0.2^{cEF}	5.1 ± 0.3^{bD}	4.4 ± 0.4^{bB}	3.7 ± 0.0^{aB}	3.3 ± 0.0^{aAB}
ACE	6.8 ± 0.6^{eA}	8.3 ± 0.1^{gA}	8.1 ± 0.1^{fgD}	7.9 ± 0.1^{fgC}	7.6 ± 0.4^{fD}	7.0 ± 0.5^{eC}	5.8 ± 0.1^{dD}	4.8 ± 0.2^{cCD}	4.5 ± 0.4^{cB}	3.7 ± 0.0^{bB}	3.0 ± 0.0^{aAB}
CT+GI	7.2 ± 0.7^{dA}	8.6 ± 0.2^{eA}	7.1 ± 0.3^{dC}	5.5 ± 0.4^{cB}	5.4 ± 0.3^{cC}	4.6 ± 0.1^{bB}	4.6 ± 0.7^{bC}	4.5 ± 0.7^{bC}	4.7 ± 0.5^{bB}	4.5 ± 0.2^{bC}	3.8 ± 0.4^{aBC}
EO+GI	6.9 ± 0.5^{eA}	8.2 ± 0.3^{fA}	5.4 ± 0.2^{dAB}	4.6 ± 0.3^{cA}	3.9 ± 0.4^{bAB}	3.8 ± 0.5^{bA}	3.7 ± 0.3^{bB}	3.7 ± 0.4^{bAB}	3.0 ± 0.4^{aA}	2.6 ± 0.3^{aA}	2.9 ± 0.3^{aA}
AE+GI	7.0 ± 0.6^{fA}	8.2 ± 0.7^{gA}	5.7 ± 0.3^{eB}	4.8 ± 0.3^{dA}	4.1 ± 0.6^{cdB}	3.7 ± 0.6^{bcA}	3.7 ± 0.3^{bcB}	3.8 ± 0.2^{bcB}	3.4 ± 0.6^{bcA}	2.7 ± 0.2^{aA}	3.1 ± 0.6^{abAB}
ACE+GI	6.8 ± 0.6^{dA}	8.3 ± 0.1^{eA}	5.3 ± 0.3^{cA}	4.5 ± 0.3^{bA}	3.4 ± 0.5^{aA}	3.2 ± 0.2^{aA}	3.0 ± 0.5^{aA}	3.3 ± 0.0^{aA}	3.2 ± 0.5^{aA}	2.9 ± 0.4^{aA}	3.1 ± 0.0^{aAB}

[1]Numbers are expressed as means ± standard deviations. Within each row, means with the same lowercase letter are not significantly different (P > 0.05). Within each column, means with the same uppercase letter are not significantly different (P > 0.05).

5.5.6 Total mesophilic flora (TMF) elimination by the antimicrobial formulations: *in situ* test without and with irradiation

The effect of γ-irradiation and antimicrobials on TMF counts is presented in Table 5.5. The LAB count was around 8-log during storage that attributed to a high level of TMF during storage (Table 5). A 1-log reduction was observed in all non-irradiated samples and from 3.3 to 3.9 log reduction was observed in all samples treated by γ-irradiation at the end of storage. The only γ-irradiated sample had a 1-log reduction after one week of storage but when EOs was added, a 1.6-log reduction was observed at the end of drying showing a synergy between irradiation and EOs. Total mesophilic flora count of non-irradiated groups increased at the end of drying and reduced slowly during storage but not significantly. EOs treated groups showed significantly lower TMF counts starting at the end of drying and over whole storage period. Irradiation reduced by 2.1-log the level of TMF count compared to CT after drying and a significant reduction was observed until the end of storage. A 1.2 log and 2.6 log of reduction from end drying to the 20th week storage were observed for control and 1.5 kGy treated samples, respectively. In the presence of EOs free or encapsulated, the reduction of TMF count was between 1.4 to 1.8 log

after 20 weeks, as compared to 8.3 log CFU/g of control. The reduction of TMF count for irradiated samples was 3.5 log and between 4.1 and 4.4 log in the presence of EOs.

In one report, nutmeg EO was proved to be able to extend the shelf life of cooked sausages (Šojić *et al.*, 2015). Chitosan-cinnamon essential oil nano-formulation decreased the total mesophilic viable counts greatly and extended the shelf life of beef patties (Ghaderi-Ghahfarokhi *et al.*, 2017). Irradiation at 1.5 kGy was effective in reducing TMF level similar to results obtained on pork sausages (Khan *et al.*, 2012). Chitosan and cumin essential oil nanoemulsion combined with gamma irradiation had decreased TMF and extended the shelf life of beef loins from 12 days to 21 days (Dini *et al.*, 2020). Criado *et al.* (2019) have demonstrated a synergy of thyme EO loaded alginate-CNC microbeads and γ-irradiation in eliminating *L. innocua*, and extended the shelf life of ground pork. Marinated meat combined to 1.5 kGy irradiation increased 9 more days of shelf life (Ben Fadhel *et al.*, 2016).

EOs free and EOs encapsulated in Alginate-CNC have similar effects on LAB counts until 1 week storage and TMF until 8-week storage. From 2nd week to 8th week storage, the ACE+GI group showed less LAB counts than EO+GI group while from 12th week to 20th week EO+GI group showed less LAB and TMF counts. All three antimicrobials combined to 1.5 kGy γ-irradiation showed synergistic effects on LAB after drying until 6 weeks' storage except non-encapsulated EOs irradiated at 1.5 kGy at 6th week storage. Irradiation contributed significantly in the inhibition of LAB and TMF.

Table 5.5 Reduction of total mesophilic flora during sausage processing and storage at room temperature[1].

	Concentration of TMF (log CFU/g)										
Samples	Time 0	Time F	End dry	1week	2weeks	4weeks	6weeks	8weeks	12weeks	16weeks	20weeks
CT	8.1±0.3^{aB}	8.6±0.2^{bcdB}	9.5±0.4^{fE}	9.1±0.3^{eD}	8.8±0.2^{dE}	8.6±0.4^{bcdE}	8.6±0.2^{cdD}	8.4±0.4^{abcD}	8.5±0.1^{bcdE}	8.3±0.0^{abE}	8.3±0.1^{abD}
EO	7.8±0.3^{deA}	8.2±0.2^{fgA}	8.3±0.4^{gD}	8.1±0.1^{efgC}	7.9±0.2^{defCD}	7.8±0.6^{cdeD}	7.6±0.2^{cdC}	7.5±0.3^{cC}	7.00±0.3^{bD}	6.8±0.2^{bD}	6.5±0.1^{aC}
AE	7.7±0.3^{cdA}	8.4±0.2^{gB}	8.5±0.4^{gD}	8.3±0.3^{fgC}	8.1±0.3^{efD}	7.9±0.2^{deD}	7.8±0.3^{deC}	7.5±0.2^{bcC}	7.2±0.3^{bD}	6.9±0.2^{aD}	6.9±0.1^{aC}
ACE	7.7±0.4^{cA}	8.3±0.2^{dA}	8.3±0.3^{dD}	8.0±0.2^{dC}	7.7±0.2^{bcC}	7.5±0.0^{bcD}	7.4±0.4^{bcC}	7.4±0.3^{bC}	7.0±0.3^{aD}	7.0±0.1^{aD}	6.8±0.1^{aC}
CT+GI	8.1±0.3^{hB}	8.6±0.2^{iB}	7.4±0.3^{gC}	6.9±0.5^{fB}	6.5±0.4^{eB}	6.0±0.2^{dC}	5.8±0.4^{cdB}	5.5±0.3^{bcB}	5.4±0.2^{bC}	5.2±0.7^{bC}	4.8±0. 7^{aB}
EO+GI	7.8±0.3^{gA}	8.2±0.2^{gA}	6.2±0.1^{fA}	6.1±0.1^{fA}	5.8±0.1^{efA}	5.5±0.3^{deAB}	5.2±0.2^{cdA}	4.9±0.2^{bcA}	4.6±0.5^{bA}	4.0±0.7^{aA}	3.9±0.7^{aA}
AE+GI	7.7±0.3^{gA}	8.4±0.2^{hB}	6.7±0.1^{fB}	6.3±0.3^{efA}	6.0±0.2^{deA}	5.9±0.3^{cdBC}	5.5±0.4^{bcAB}	5.0±0.3^{bA}	5.0±0.5^{bBC}	4.2±0.5^{aAB}	4.2±0.7^{aAB}
ACE+GI	7.7±0.4^{gA}	8.3±0.2^{gA}	6.4±0.1^{fAB}	6.0±0.2^{efA}	5.8±0.1^{deA}	5.3±0.3^{cdA}	5.1±0.4^{bcA}	4.9±0.1^{bcA}	4.9±0.6^{bcAB}	4.6±0.7^{abB}	4.1±0.6^{aAB}

[1]Numbers are expressed as means ± standard deviations. Within each row, means with the same lowercase letter are not significantly different (P > 0.05). Within each column, means with the same uppercase letter are not significantly different (P > 0.05).

5.5.7 Physicochemical evaluation of sausages: pH, temperature and Aw results during ripening

Results of pH and temperature of sausages during fermentation and Aw for sausages at end drying before irradiation are presented in Table 5.6. Fermentation took 50.00 ± 0.00 h and drying took 117.83 ± 0.29 h. All groups containing antimicrobial formulations didn't affect the pH values after certain time of fermentation and drying. The pH, the temperature and the Aw of sausages with different treatments all didn't show significant differences. All values are considered in a normal range for this type of sausages (Lizaso *et al.*, 1999).

Table 5.6 pH, temperature and Aw of sausages during ripening before irradiation[1].

Samples	Time 0		Time F		After drying
	pH	temperature	pH	temperature	Aw
CT	5.91±0.14^{A}	25.16±0.13^{A}	5.24±0.04^{A}	25.27±0.03^{A}	0.84±0.01^{A}
EO	6.00±0.12^{A}	25.18±0.11^{A}	5.21±0.04^{A}	25.26±0.06^{A}	0.83±0.02^{A}
AE	5.89±0.10^{A}	25.11±0.07^{A}	5.18±0.02^{A}	25.24±0.06^{A}	0.83±0.01^{A}

ACE	5.94±0.03^{A}	25.14±0.14^{A}	5.22±0.04^{A}	25.22±0.02^{A}	0.83±0.01^{A}

[1] Numbers are expressed as means ± standard deviations. Within each column, means with the same uppercase letter are not significantly different (P > 0.05).

5.5.8 Color evaluation of sausages

For color values of the surface of sausages are presented in supplemental files (Table 5.7). The *L** values (lightness) of all samples varies between 37.4±1.6 to 42.2±0.4. The *L** value changed slightly during storage and between the treatments. The addition of EO slightly increased the lightness of the sausages. The *L** value of control group at end day was 38.1±0.6, which was increased to ~40 when encapsulated EO was added. Also, the *L** values of the sausages slightly increased during the storage time. However, there was no significant difference in the *L** values between non-irradiated and γ-irradiated groups. The *a** value (redness) of the surface of samples also changed slightly with addition of EO, γ-irradiation treatment, and storage time. Like lightness and redness, yellowness (*b** value) of the surface of the sausage also was not affected significantly by the addition of EOs, γ-irradiation treatment, and storage time. There is no clear trend in the change of yellowness among the samples with different treatments and storage time. The Hue angle is another parameter frequently used to characterize color in food products. An angle of 0° or 360° represents red Hue, while angles of 90°, 180° and 270° represent yellow, green and blue Hue respectively (Benković & Tušek, 2018). Irradiation didn't affect hue values however the addition of EOs reduced the value of hue angle slightly, which varied from -0.9 to -1.3 during the whole storage for all treatments. Results of ΔE* represent the total change of color which was also not greatly affected with treatments and storage time. The above results exhibited that the type of treatments and storage time had no serious effect of color parameters of the sausages.

The results on the color parameters of the inner surface of the sausages are presented in supplemental files (Table 5.8). Similar to the result for the color values for the outer surface of sausage, the *L** values of inner surface were also not affected by addition of EO, irradiation treatment, and storage time. The lightness of the interior surface was stable during storage that showed no significant difference at 20th week storage from end drying. All the groups have slightly decreased interior redness after 20th week storage except irradiated group which showed no significant changes after 20 weeks from end dry, However, EO treated groups showed a bit higher redness than redness at end drying. γ-irradiation did not affect yellowness values of inner surface significantly for 1st week, 4th, 12th and 16th week storage, but decreased between groups of AE and increased between groups of ACE at the beginning. All the groups showed no significant

changes on yellowness after 20th week storage compared to beginning of storage except EO treated group that showed significantly higher yellowness. All the groups exhibited lower hue values after 20th week storage except EO treated groups where hue values increased. However, irradiated groups showed no significant difference in hue values at 20th week compared to the beginning of storage. All groups showed no significant difference on total color changes at 12th week storage. Only non-treated groups showed non-noticeable difference of interior total color change at ΔE* value of 1.8, EO-Alginate-CNC and EO-1.5 kGy groups showed highest total color change at ΔE* value of 4.4 and 4.9, respectively after 20th week storage. The redness, yellowness, total color change, and hue angle values of inner surface were generally higher than outer surface. γ-irradiation did not affect the color values of the outer surface as well as inner surface of sausages except interior redness values which decreased after irradiation for all the control groups. This color change may be due to the intrinsic sensitivity of myoglobin molecules to the energy caused by γ-irradiation (Ben Fadhel *et al.*, 2016). Essential oils encapsulated in Alginate or Alginate-CNC have increased redness and yellowness significantly of inside color of sausages and have a significant decrease on total color change of surface of sausages at 1st week storage that shows the good protection of exterior color by encapsulation.

Table 5.7 Color attributes of surface of sausages during storage at room temperature[1].

Samples		End dry	1week	2weeks	4weeks	6weeks	8weeks	12weeks	16weeks	20weeks
CT	*L**	38.1±0.6^{aA,1}	38.6±1.4^{abA}	38.2±0.3^{aA}	37.4±1.6^{aA}	37.7±1.1^{aA}	40.0±0.3^{bcA}	40.4±0.2^{cA}	40.5±0.4^{cAB}	39.9±0.5^{bcA}
	*a**	-4.9±0.8^{abBC}	-3.3±0.3^{cdE}	-4.1±0.5^{bcA}	-4.7±0.8^{abA}	-2.5±0.5^{dE}	-4.2±0.4^{bcB}	-4.5±0.3^{abB}	-4.8±0.2^{abAB}	-5.2±0. 6^{aAB}
	*b**	1.5±0.4^{aA}	2.4±0.6^{bcB}	2.8±0.3^{cA}	1.4±0.1^{aA}	3.6±0.7^{BC}	1.8±0.1^{abAB}	2.4±0.3^{bcB}	1.9±0.2^{abA}	1.8±0.3^{abA}
	h	-1.3±0.1^{aAB}	-0.9±0.1^{bB}	-1.0±0.0^{bA}	-1.3±0.2^{aA}	-0.6±0.2^{cD}	-1.2±0.0^{aAB}	-1.1±0.1^{abB}	-1.2±0.0^{aAB}	-1.2±0.1^{aA}
	ΔE	-	2.3±0.6^{abcDE}	1.6±0.2^{aAB}	1.8±0.5^{abB}	3.3±0.8^{dB}	2.1±0.2^{abcBCD}	2.6±0.3^{cC}	2.5±0.4^{bcC}	2.0±0.4^{abcAB}
EO	*L**	38.7±0.5^{aAB}	38.4±0.4^{aA}	40.2±0.5^{cBC}	40.2±0. 5^{cBC}	39.4±0.5^{bB}	40.2±0.1^{cA}	40.3±0.3^{cA}	40.1±0.2^{cA}	40.4±0.4^{cAB}
	*a**	-5.7±0.7^{aAB}	-4.5±0.3^{bcBC}	-5.2±0.7^{abA}	-5.4±0.6^{aA}	-4.0±0.5^{cBC}	-4.9±0.1^{abA}	-5.5±0.4^{aA}	-5.5±0.2^{aA}	-5.6±0.2^{aA}
	*b**	1.5±0.4^{aA}	2.9±0. 5^{cB}	2.8±0.5^{bcA}	2.1±0.6^{abAB}	2.5±0.3^{bcA}	2.7±0.3^{bcBC}	1.4±0.3^{aA}	1.7±0.4^{aA}	2.1±0.4^{abA}
	h	-1.3±0.1^{aAB}	-1.0±0.1^{cB}	-1.1±0.0^{bcA}	-1.2±0.1^{abAB}	-1.0±0.1^{cAB}	-1.1±0.1^{bcBC}	-1.3±0.1^{aA}	-1.3±0.1^{aA}	-1.2±0.1^{aA}
	ΔE	-	2.0±0.2^{abCD}	2.3±0.5^{bBC}	1.8±0.2^{abB}	2.1±0.5^{abA}	2.1±0.3^{abBCD}	1.7±0.3^{abAB}	1.5±0.1^{aAB}	1.9±0.5^{abAB}
AE	*L**	40.5±0.7^{abCD}	40.3±0.8^{aB}	41.4±0.2^{cC}	40.6±0.5^{abBC}	41.6±0.6^{cD}	41.3±0.4^{bcB}	41.9±0.4^{cB}	41.7±0.3^{cCD}	41.5±0.4^{cC}

	*a**	-4.4±0.9^{aC}	-4.2±0.8^{aBCDE}	-4.3±0.3^{aA}	-4.7±0.4^{aA}	-4.2±0.2^{aAB}	-4.1±0.5^{aB}	-4.1±0.6^{aBC}	-4.6±0.5^{aAB}	-5.2±0.4^{aAB}
	*b**	3.0±0.6^{aB}	3.0±0.6^{aB}	2.6±0.4^{aA}	3.0±0.1^{aB}	4.1±0.2^{bC}	3.0±0.7^{aCD}	3.2±0.7^{aBC}	2.8±0.6^{aBC}	2.6±0.3^{aAB}
	h	-1.0±0.2^{aCD}	-0.9±0.2^{aB}	-1.0±0.1^{aA}	-1.0±0.1^{aBC}	-0.8±0.1^{aCD}	-0.9±0.1^{aCD}	-0.9±0.2^{aBC}	-1.0±0.2^{aBC}	-1.1±0.1^{aAB}
	ΔE	-	1.2±0.2^{bAB}	1.1±0.3^{bA}	0.7±0.2^{aA}	1.7±0.5^{cA}	1.2±0.3^{bA}	1.7±0.4^{cAB}	1.4±0.3^{bcA}	1.5±0.1^{bcA}
ACE	*L**	40.1±0.8^{aBCD}	40.4±0.5^{abB}	41.4±0.5^{bcdC}	41.2±0.7^{bcC}	41.6±0.4^{cdD}	42.3±0.3^{dC}	41.7±0.5^{cdB}	42.0±0.3^{cdD}	41.9±1.1^{cdC}
	*a**	-4.0±0.8^{aC}	-3.5±0.7^{aDE}	-4.5±0.5^{aA}	-3.5±0.5^{aB}	-3.6±0.5^{aBC}	-4.2±0.5^{aB}	-4.4±0.8^{aB}	-3.8±1.0^{aC}	-4.5±0.6^{aC}
	*b**	2.9±0.5^{aB}	3.9±0.3^{aCD}	2.9±0.5^{aA}	4.1±0.1^{aCD}	3.7±0.8^{aBC}	3.8±0.2^{aD}	3.0±0.7^{aBC}	3.6±0.6^{aD}	3.6±1.1^{aC}
	h	-0.9±0.1^{aD}	-0.7±0.1^{aC}	-1.0±0.0^{aA}	-0.7±0.1^{aD}	-0. 8±0.2^{aCD}	-0.8±0.1^{aD}	-1.0±0.2^{aBC}	-0.8±0.2^{aD}	-0.9±0.2^{aC}
	ΔE	-	1.4±0.4^{aB}	1.5±0.5^{abAB}	1.8±0.4^{abcB}	1.9±0.6^{abcA}	2.5±0.3^{cD}	2.0±0.5^{abcBC}	2.2±0.5^{bcBC}	2.4±0.6^{cB}
CT+GI	*L**	39.0±0.2^{aAB}	39.0±0.9^{aA}	39.4±1.2^{aAB}	39.3±0.72^{aB}	39.8±0.7^{aB}	40.2±0.5^{aA}	40.3±0.6^{aA}	40.5±0.4^{aAB}	40.2±0.2^{aA}
	*a**	-5.5±0.6^{aAB}	-4.0±0.3^{bCDE}	-4.2±0.8^{bA}	-4.7±0.91^{abA}	-2.8±0.5^{cDE}	-4.1±0.2^{bB}	-3.9±0.5^{bBC}	-4.2±0.4^{bBC}	-4.9±0.4^{abABC}
	*b**	1.3±0.2^{aA}	1.4±0.1^{abA}	2.3±0.2^{cA}	2.2±0.65^{cAB}	2.5±0.4^{cA}	2.1±0.4^{cA}	2.4±0.3^{cB}	2.4±0.4^{cAB}	2.0±0.2^{bcA}
	h	-1.3±0.1^{aA}	-1.2±0.0^{abA}	-1.1±0.1^{bcA}	-1.1±0.18^{bcABC}	-0.8±0.2^{dBC}	-1.1±0.1^{bcBC}	-1.0±0.1^{cB}	-1.1±0.1^{bcBC}	-1.2±0.1^{abcA}
	ΔE	-	1.7±0.3^{abBC}	2.1±0.6^{abcBC}	1.7±0.5^{abB}	3.2±0.6^{dB}	2.1±0.1^{abcBCD}	2.4±0.0^{cC}	2.3±0.5^{bcC}	1.6±0.2^{aA}
EO+GI	*L**	39.6±0.7^{abBC}	39.1±0.1^{abA}	38.4±2.1^{aA}	40.3±0.2^{bBC}	40.2±0.8^{bBC}	40.5±0.1^{bA}	40.3±0.5^{bA}	40.3±0.7^{bA}	39.9±0.6^{bA}
	*a**	-6.6±0.5^{aA}	-6.2±0.1^{abA}	-4.5±0.6^{dA}	-5.2±0.2^{cA}	-4.9±0.6^{cdA}	-5.3±0.1^{cA}	-5.6±0.5^{bcA}	-5.3±0.4^{cA}	-5.2±0.2^{cAB}
	*b**	1.3±0.3^{aA}	1.2±0.3^{aA}	2.5±0.7^{bA}	2.4±0.4^{bAB}	2.4±0.4^{bA}	1.6±0.2^{aAB}	1.3±0.2^{aA}	1.7±0.3^{aA}	1.8±0.3^{aA}
	h	-1.4±0.1^{aA}	-1.4±0.0^{aA}	-1.1±0.2^{dA}	-1.1±0.1^{bcdABC}	-1.1±0.1^{cdA}	-1.3±0.0^{aA}	-1.3±0.1^{aA}	-1.3±0.1^{abA}	-1.3±0.1^{abcA}
	ΔE	-	0.8±0.2^{aA}	3.3±0.9^{dD}	1.9±0.2^{bcB}	2.20±0.61^{cA}	1.6±0.1^{bcAB}	1.3±0.4^{abA}	1.6±0.5^{bcAB}	1.6±0.3^{bcA}
AE+GI	*L**	41.0±1.0^{aD}	40.8±0.1^{aB}	41.2±1.0^{aBC}	40.6±0.7^{aBC}	41.1±0.8^{aCD}	41.8±0.8^{aBC}	41.7±0.9^{aB}	41.1±0.5^{aBC}	41.2±0.7^{aBC}
	*a**	-5.7±0.7^{aAB}	-4.3±0.2^{bBCD}	-4.3±0.4^{bA}	-4.4±0.4^{bAB}	-4.0±0.3^{bcBC}	-4.0±0.5^{bcB}	-3.4±0.2^{cC}	-4.7±0.3^{bAB}	-4.7±0.3^{bBC}
	*b**	2.7±0.6^{abB}	3.2±0.6^{abBC}	4.8±0.2^{cC}	3.2±0.8^{abBC}	3.0±0.5^{abAB}	3.7±0.9^{bD}	3.4±1.0^{abC}	2.5±0.7^{aAB}	3.4±0.5^{abBC}

	h	-1.1±0.1^{aBC}	-0.9±0.1^{bcB}	-0.7±0.0^{dB}	-0.9±0.2^{bcC}	-0.9±0.1^{bcABC}	-0.8±0.1^{cdD}	-0.8±0.2^{cdC}	-1.1±0.1^{abABC}	-1.0±0.1^{bcBC}
	ΔE	-	1.6±0.2^{aBC}	2.7±0.1^{cCD}	1.7±0.4^{abB}	1.9±0.3^{abA}	2.4±0.7^{bcCD}	2.6±0.7^{cC}	1.2±0.4^{aA}	1.4±0.4^{aA}
ACE+GI	*L**	40.1±1.6^{aBCD}	42.2±0.4^{bC}	41.5±0.5^{bC}	41.5±0.5^{bC}	41.3±0.7^{bD}	41.7±0.3^{bBC}	41.0±0.7^{abAB}	42.1±0.4^{bD}	42.0±0.4^{bC}
	*a**	-4.1±0.5^{bcdC}	-5.1±0.3^{aB}	-5.0±1.1^{abA}	-3.6±0.4^{cdB}	-3.3±0.7^{dCD}	-3.6±0.6^{cdB}	-4.3±0.3^{abcB}	-4.6±0.4^{abAB}	-4.7±0.4^{abBC}
	*b**	3.2±0.9^{abB}	4.1±0.3^{bcD}	3.6±0.3^{abcB}	4.4±0.9^{cD}	3.3±0.2^{abBC}	3.0±0.6^{aCD}	2.7±0.6^{aBC}	3.3±0.4^{abCD}	3.1±0.2^{abBC}
	h	-0.9±0.2^{abD}	-0.9±0.1^{abcBC}	-0.9±0.2^{abA}	-0.7±0.1^{cD}	-0.8±0.1^{bcCD}	-0.9±0.1^{abcD}	-1.0±0.1^{aB}	-1.0±0.1^{abCD}	-1.0±0.1^{abBC}
	ΔE	-	2.5±0.4^{cE}	2.0±0.3^{bcBC}	2.1±0.3^{bcB}	1.6±0.5^{abA}	1.8±0.1^{abBC}	1.4±0.4^{aAB}	2.1±0.5^{bcBC}	2.0±0.4^{bcAB}

[1]Numbers are expressed as means ± standard deviations. Within each row, means with the same lowercase letter are not significantly different (P > 0.05). Within each column, means with the same uppercase letter are not significantly different (P > 0.05).

Table 5.8 Color attributes of inside of sausages during storage at room temperature[1].

Samples		End dry	1week	2weeks	4weeks	6weeks	8weeks	12weeks	16weeks	20weeks
CT	*L**	37.8±3.5^{bA,1}	35.8±2.7^{abA}	41.5±1.0^{cB}	32.6±1.2^{aA}	35.2±0.6^{abA}	35.9±0.4^{abA}	35.3±0.7^{abA}	36.8±3.8^{bA}	37.6±1.0^{bA}
	*a**	-1.5±0.3^{bBC}	-1.5±0.3^{bC}	-1.5±0.4^{bC}	-0.9±0.2^{cC}	-0.8±0.1^{cC}	-0.9±0.1^{cC}	0.1±0.0^{dE}	-1.8±0.5^{bAB}	-3.0±0.3^{aAB}
	*b**	4.0±0.7^{abA}	3.1±0.7^{aA}	3.9±0.3^{abA}	4.0±0.2^{bA}	3.5±0.3^{abA}	4.5±0.1^{bcB}	5.1±0.8^{cA}	4.0±0.7^{abA}	3.9±0.4^{abA}
	h	-0.4±0.1^{bB}	-0.5±0.1^{bB}	-0.4±0.1^{bB}	-0.2±0.0^{cC}	-0.2±0.0^{cC}	-0.2±0.0^{cC}	0.1±0.0^{dE}	-0.4±0.1^{bA}	-0.7±0.1^{aAB}
	ΔE	-	3.3±0.4^{cBC}	3.7±1.1^{cBC}	5.3±1.2^{dC}	2.8±0.7^{bcA}	2.1±0.4^{abABC}	3.3±0.3^{cA}	3.3±0.1^{cBC}	1.8±0.2^{aA}
EO	*L**	39.5±3.5^{bcdA}	40.7±2.9^{cdB}	35.6±0.9^{aA}	37.5±0.8^{abcB}	37.1±0.7^{abB}	38.1±0.2^{abcdB}	37.0±0.1^{abAB}	40.7±3.1^{cdBC}	41.2±0.5^{dB}
	*a**	-2.1±0.5^{aB}	-0.9±0.1^{cdD}	-0.6±0.1^{defD}	-0.4±0.1^{efD}	-0.3±0.1^{fD}	-1.3±0.1^{bcB}	-0.8±0.1^{deC}	-2.1±0.6^{aA}	-1.6±0.1^{bCD}
	*b**	4.8±0.5^{aAB}	5.0±0.4^{aB}	4.8±0.3^{aA}	5.3±0.6^{abBC}	5.1±0.2^{abB}	5.0±0.1^{aB}	5.7±0.3^{bcA}	6.2±0.6^{cdB}	6.7±0.2^{dCD}
	h	-0.4±0.1^{aB}	-0.2±0.0^{cdC}	-0.1±0.0^{deC}	-0.1±0.0^{eD}	-0.1±0.0^{eD}	-0.3±0.0^{bcB}	-0.2±0.0^{deC}	-0.3±0.1^{bB}	-0.2±0.0^{cD}
	ΔE	-	2.9±0.4^{bAB}	4.3±0.8^{cC}	2.8±0.4^{bA}	3.1±0.6^{bAB}	1.6±0.1^{aA}	3.0±0.0^{bA}	3.2±1.0^{bBC}	2.6±0.4^{bAB}
AE	*L**	42.7±4.5^{aA}	42.3±0.7^{aB}	41.1±0.2^{aB}	40.7±0.6^{aC}	41.8±1.2^{aD}	41.8±0.1^{aD}	40.0±1.4^{aC}	43.7±0.7^{aC}	42.0±1.1^{aBC}
	*a**	1.9±0.4^{gE}	0.5±0.1^{cdeE}	0.6±0.1^{defEF}	0.9±0.2^{fF}	0.2±0.1^{cdE}	0.2±0.0^{cF}	0.6±0.2^{efF}	-1.7±0.1^{aAB}	-1.1±0.3^{bD}

	*b**	7.3±0.5^{abcC}	6.6±1.0^{aC}	6.6±0.8^{aBC}	7.2±0.4^{abcE}	6.8±0.8^{abCD}	7.1±0.2^{abcDE}	8.2±0.9^{cB}	6.8±0.5^{abBC}	7.9±0.4^{bcE}
	h	0.2±0.1^{fD}	0.1±0.0^{cdD}	0.1±0.0^{deDE}	0.1±0.0^{eFG}	0.1±0.0^{cE}	0.1±0.0^{cF}	0.1±0.0^{dF}	-0.3±0.0^{aB}	-0.1±0.0^{bD}
	ΔE	-	1.9±0.5^{aA}	2.2±0.3^{aA}	2.2±0.6^{aA}	2.3±0.5^{aA}	1.9±0.1^{aABC}	3.3±0.9^{bA}	3.8±0.3^{bC}	3.2±0.1^{bB}
ACE	*L**	41.7±2.4^{bcdA}	41.5±2.1^{bcdB}	41.1±1.7^{bcB}	37.4±1.4^{aB}	39.6±1.0^{abC}	41.4±0.1^{bcdD}	42.3±2.0^{bcdD}	43.1±1.3^{cdC}	44.1±0.7^{dD}
	*a**	1.7±0.5^{dE}	0.6±0.1^{cE}	0.3±0.1^{cE}	0.2±0.1^{cE}	0.2±0.0^{cE}	-0.5±0.1^{bD}	-0.5±0.1^{bD}	-1.0±0.2^{bCD}	-1.9±0.5^{aC}
	*b**	7.3±1.1^{bcC}	6.9±0.4^{abC}	7.7±0.9^{bcC}	5.9±0.1^{aCD}	6.9±0.2^{abCD}	6.8±0.2^{abCD}	8.1±0.5^{cB}	7.7±0.7^{bcCD}	7.7±0.4^{bcE}
	h	0.2±0.0^{fD}	0.1±0.0^{eD}	0.1±0.0^{deD}	0.1±0.0^{deE}	0.1±0.0^{dE}	-0.1±0.0^{bcD}	-0.1±0.0^{cD}	-0.1±0.0^{bC}	-0.3±0.1^{aD}
	ΔE	-	2.0±0.5^{aA}	2.2±0.6^{abA}	4.7±1.2^{cBC}	2.7±0.7^{abA}	2.3±0.1^{abBC}	2.9±0.2^{abA}	3.2±0.7^{bBC}	4.4±0.8^{cC}
CT+GI	*L**	40.7±2.7^{bcA}	39.3±0.5^{abAB}	42.8±0.2^{cB}	38.9±3.6^{abBC}	38.2±0.8^{abBC}	37.5±0.6^{aB}	37.4±1.3^{aB}	38.1±1.9^{abAB}	38.5±0.6^{abA}
	*a**	-3.0±0.6^{abA}	-3.6±0.5^{aA}	-2.7±0.3^{bcA}	-2.3±0.6^{cA}	-1.4±0.2^{dB}	-1.6±0.2^{dA}	-1.5±0.2^{dB}	-1.3±0.3^{dBC}	-3.5±0.5^{aAB}
	*b**	3.7±0.8^{aA}	3.1±0.3^{aA}	4.5±1.1^{aA}	3.7±0.7^{aA}	3.5±0.4^{aA}	3.8±0.4^{aA}	4.7±0.8^{aA}	4.4±0.4^{aA}	3.5±0.5^{aA}
	h	-0.7±0.2^{abA}	-0.9±0.1^{aA}	-0.6±0.1^{bcA}	-0.5±0.0^{bcA}	-0.4±0.0^{cdB}	-0.4±0.1^{cdA}	-0.3±0.1^{dB}	-0.3±0.1^{dB}	-0.8±0.1^{aA}
	ΔE	-	1.7±0.2^{aA}	2.5±0.4^{abcAB}	3.5±0.9^{cdAB}	2.9±0.8^{bcdA}	3.5±0.5^{cdD}	3.9±0.9^{dA}	3.4±1.0^{bcdBC}	2.4±0.5^{abAB}
EO+GI	*L**	37.0±2.4^{aA}	42.7±1.0^{dB}	41.3±0.9^{cdB}	39.0±0.2^{bBC}	39.2±0.5^{bC}	36.5±1.0^{aA}	40.5±0.5^{bcCD}	40.6±0.7^{bcBC}	41.3±1.0^{cdB}
	*a**	-1.9±0.1^{bcB}	-2.4±0.5^{bB}	-2.2±0.2^{bcB}	-1.6±0.4^{cB}	-2.4±0.3^{bA}	-1.0±0.1^{dC}	-2.2±0.2^{bcA}	-2.0±0.3^{bcA}	-3.6±0.7^{aA}
	*b**	4.0±0.4^{abA}	5.0±1.0^{bcB}	4.7±0.9^{bcA}	4.9±0.5^{bcB}	3.6±0.3^{aA}	5.0±0.5^{bcB}	5.5±0.3^{cA}	5.7±0.6^{cB}	4.9±0.8^{bcB}
	h	-0.4±0.0^{bcB}	-0.5±0.1^{bcB}	-0.5±0.1^{bcB}	-0.3±0.1^{cdB}	-0.6±0.1^{abA}	-0.2±0.0^{dC}	-0.4±0.0^{cA}	-0.3±0.1^{cdAB}	-0.6±0.2^{aB}
	ΔE	-	5.9±1.0^{cD}	4.5±0.8^{bC}	2.3±0.4^{aA}	2.3±0.4^{aA}	1.8±0.5^{aAB}	3.8±0.4^{bA}	4.1±0.8^{bC}	4.9±0.9^{bC}
AE+GI	*L**	42.3±1.1^{aA}	41.8±3.8^{aB}	40.4±2.9^{aB}	40.0±1.4^{aBC}	39.2±1.7^{aC}	40.1±0.3^{aC}	40.1±0.0^{aC}	43.5±0.4^{aC}	43.2±1.6^{aCD}
	*a**	-1.0±0.1^{bC}	1.4±0.3^{dF}	1.5±0.4^{dG}	1.2±0.2^{dF}	1.6±0.3^{dF}	-0.1±0.0^{cE}	0.1±0.0^{cE}	-1.0±0.2^{bCD}	-2.8±0.6^{aB}
	*b**	6.0±0.7^{aB}	7.5±0.8^{aC}	7.2±0.5^{aBC}	6.9±0.4^{aE}	6.3±0.6^{aC}	6.3±0.4^{aC}	7.3±0.2^{aB}	6.7±0.1^{aBC}	6.3±1.0^{aC}
	h	-0.2±0.0^{bC}	0.2±0.1^{deD}	0.2±0.0^{deF}	0.2±0.0^{dG}	0.3±0.1^{eF}	-0.1±0.0^{cE}	0.1±0.0^{cE}	-0.1±0.0^{bC}	-0.4±0.1^{aC}
	ΔE	-	4.3±0.7^{cC}	4.1±1.0^{cC}	3.4±1.0^{bcAB}	4.2±1.2^{cB}	2.4±0.2^{abC}	2.8±0.1^{bA}	1.4±0.3^{abA}	2.6±0.7^{abAB}
ACE+GI	*L**	44.4±1.7^{cA}	41.1±1.1^{aB}	41.5±1.4^{aB}	40.3±1.1^{aC}	41.5±0.2^{aD}	41.5±0.4^{aD}	42.1±0.6^{abD}	43.5±1.7^{bcC}	44.5±0.3^{cD}
	*a**	0.9±0.2^{eD}	0.8±0.2^{deE}	0.9±0.1^{eF}	0.4±0.1^{cdE}	0.2±0.1^{cE}	0.1±0.0^{cF}	-0.4±0.^{bD}	-0.6±0.1^{bD}	-2.0±0.4^{aC}
	*b**	8.6±0.7^{bD}	6.6±0.4^{aC}	6.3±0.6^{aB}	6.6±0.5^{aDE}	7.4±1.4^{abD}	7.6±0.3^{abE}	8.0±0.9^{bB}	8.3±1.1^{bD}	7.5±0.5^{abDE}

h	0.1±0.0^{deD}	0.1±0.0^{deD}	0.1±0.0^{eEF}	0.1±0.0^{cdEF}	0.1±0.0^{cE}	0.1±0.0^{cEF}	-0.1±0.0^{bD}	-0.1±0.0^{bC}	-0.3±0.1^{aD}
ΔE	-	3.9±1.1^{bcBC}	3.8±0.9^{bcBC}	4.6±1.2^{cBC}	3.3±0.5^{abcAB}	3.1±0.4^{abD}	2.8±0.7^{abA}	2.4±0.4^{aAB}	3.2±0.5^{abB}

[1]Numbers are expressed as means ± standard deviations. Within each row, means with the same lowercase letter are not significantly different (P > 0.05). Within each column, means with the same uppercase letter are not significantly different (P > 0.05).

5.5.9 Texture evaluation of sausages

The results of the max stress and Young's modulus of sausages are presented in Table 5.9. The EO treatment increased the max stress around 18% at the end of storage compared to control. However, γ-irradiation treatment did not change the max stress of samples during storage. Max stress of γ-irradiated samples in the presence of EOs was similar with values of non-irradiated samples that increased 21% than CT samples at 20th week. γ-irradiation didn't affect the modulus significantly initially but the hardness increased during storage. The modulus of sausages increased by 21-22% was observed for CT and AE. The CT+GI is the most stable group over time and only 10% difference at 20th week was observed. The ACE and ACE+GI showed the lowest hardness after drying but very high value after 20 weeks storage with a value of 8.32 for ACE+GI and 9.10 for ACE. Research showed the differences of stress and Young's modulus were less with longer storage weeks (Houben & van 't Hooft, 2005).

Table 5.9 Max stress (MPa) and Young's modulus (MPa) of sausages measured during storage at room temperature[1].

Samples	End dry	1week	2weeks	4weeks	6weeks	8weeks	12weeks	16weeks	20weeks
	Max stress (MPa)								
CT	0.26±0.03^{bcB,1}	0.29±0.04^{bcA}	0.17±0.02^{aAB}	0.20±0.05^{abA}	0.24±0.02^{abcA}	0.23±0.01^{abcAB}	0.25±0.06^{abcAB}	0.31±0.09^{cA}	0.28±0.06^{bcA}
EO	0.21±0.03^{aAB}	0.21±0.05^{aA}	0.23±0.02^{aBC}	0.27±0.01^{abA}	0.25±0.05^{aA}	0.28±0.03^{abBC}	0.33±0.06^{bcBC}	0.36±0.07^{cA}	0.34±0.08^{bcA}
AE	0.23±0.01^{aB}	0.27±0.04^{aA}	0.28±0.04^{aCD}	0.29±0.04^{aA}	0.27±0.01^{aA}	0.31±0.06^{aC}	0.33±0.06^{aBC}	0.33±0.04^{aA}	0.30±0.03^{aA}
ACE	0.20±0.01^{aAB}	0.23±0.07^{abA}	0.34±0.09^{bcD}	0.28±0.07^{abcA}	0.29±0.08^{abcA}	0.31±0.07^{abcC}	0.38±0.10^{cC}	0.39±0.10^{cA}	0.38±0.10^{cA}
CT+GI	0.27±0.04^{cB}	0.38±0.04^{dB}	0.10±0.03^{aA}	0.24±0.01^{bcA}	0.19±0.00^{bA}	0.17±0.04^{bA}	0.21±0.05^{bcA}	0.23±0.06^{bcA}	0.27±0.01^{cA}
EO+GI	0.21±0.00^{aAB}	0.27±0.00^{abcA}	0.25±0.03^{abBC}	0.30±0.08^{bcdA}	0.27±0.05^{abcA}	0.31±0.04^{bcdC}	0.33±0.03^{cdBC}	0.35±0.04^{dA}	0.35±0.05^{dA}

AE+GI	0.27±0.06^{aB}	0.26±0.04^{aA}	0.27±0.06^{aCD}	0.25±0.03^{aA}	0.25±0.05^{aA}	0.30±0.03^{aC}	0.31±0.06^{aBC}	0.34±0.04^{aA}	0.31±0.03^{aA}
ACE+GI	0.15±0.02^{aA}	0.23±0.05^{abA}	0.34±0.05^{cD}	0.31±0.07^{bcA}	0.27±0.05^{bcA}	0.28±0.06^{bcBC}	0.33±0.06^{cBC}	0.34±0.07^{cA}	0.34±0.07^{cA}
	Young's modulus (MPa)								
CT	7.01±0.97^{cdC,1}	8.64±0.45^{dD}	3.19±0.47^{aAB}	5.15±0.97^{abcB}	4.27±0.72^{abAB}	5.59±1.11^{bcA}	6.77±0.28^{cdAB}	7.12±1.88^{cdBC}	8.82±2.33^{dA}
EO	5.52±0.77^{abcBC}	3.69±1.03^{aA}	6.3±0.98^{bcdD}	5.37±0.24^{abcB}	5.51±0.76^{abcBC}	5.54±0.16^{abcA}	4.65±0.76^{abA}	7.21±1.41^{cdBC}	7.99±2.01^{dA}
AE	6.13±0.84^{abcC}	3.80±0.75^{aA}	5.46±0.56^{abCD}	7.18±1.33^{bcCD}	7.24±0.90^{bcC}	5.42±0.05^{abA}	8.02±1.87^{bcB}	8.48±2.24^{cC}	7.43±2.11^{bcA}
ACE	4.44±0.71^{aAB}	4.40±0.64^{aAB}	6.00±1.11^{abD}	7.96±1.28^{bcD}	6.88±1.86^{abcC}	5.27±1.19^{aA}	7.74±1.78^{bcB}	8.76±1.40^{cC}	9.10±2.26^{cA}
CT+GI	6.00±0.42^{dC}	5.08±1.36^{dC}	2.29±0.62^{aA}	3.59±0.38^{abA}	2.88±0.79^{aA}	4.44±0.20^{bcA}	6.21±0.30^{dAB}	3.83±0.57^{abA}	5.88±1.60^{cdA}
EO+GI	4.46±0.27^{abAB}	6.81±1.19^{bcdC}	4.21±0.49^{aBC}	7.12±0.99^{cdCD}	6.71±1.70^{bcdC}	5.79±1.18^{abcA}	7.54±1.76^{cdB}	7.49±1.68^{cdBC}	8.57±1.65^{dA}
AE+GI	6.45±1.06^{bcC}	5.99±0.75^{bC}	2.97±0.66^{aAB}	6.00±0.34^{bBC}	6.13±0.91^{bBC}	5.39±0.32^{bA}	8.71±2.40^{cdB}	8.47±1.13^{cdC}	9.84±2.25^{dA}
ACE+GI	3.28±0.57^{aA}	5.53±0.85^{bBC}	8.02±1.74^{cdE}	5.78±0.86^{bBC}	6.54±1.32^{bcdC}	5.59±0.57^{bA}	6.06±1.46^{bcAB}	5.73±1.15^{bAB}	8.32±1.72^{dA}

[1]Numbers are expressed as means ± standard deviations. Within each row, means with the same lowercase letter are not significantly different (P > 0.05). Within each column, means with the same uppercase letter are not significantly different (P > 0.05).

5.6 Conclusion

EOs, EOs encapsulated in alginate, and EOs encapsulated in alginate-CNC in combination with 1.5 kGy γ-irradiation synergistically inhibited the growing of a resistant *E. coli* O157:H7 to the dry and acid condition of dry fermented sausage and lactic acid bacteria present in dry fermented sausages at certain storage time period. Synergistic effects were not observed for *L. monocytogenes*, molds and yeasts, and TMF, however, the strong inhibition was found by combined treatments. Also, the combined treatments did not cause significant changes on texture and results showed the encapsulation contributed to the color protection of sausages. This research can be of great interests to food industry for food preservation and this work presents a new method of combined treatments applied on DFS during whole ripening and 20 weeks storage.

5.7 Declaration of competing interest

The authors declare that the research was conducted in the absence of any conflict of interest.

5.8 Acknowledgments

The authors are grateful for the financial support from Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery no: RGPIN-2017-05947, NSERC grant with Amsellem no. RDCPJ 531475-18, Ministère de l'Économie et de l'Innovation (MEI) of Germany no: PSO-I-2-44530, a chair granted by Quebec Ministry of Agriculture, Fishery and Food (MAPAQ) no: PPIA 12, and International Atomic Energy Agency (IAEA) Agreement No: 24359.

6 ARTICLE 4 – *IN SITU* TESTS WITH GAMMA RAY AND X-RAY

Combined effects of microencapsulated essential oils and irradiation from gamma and X-ray sources on microbiological and physicochemical properties of dry fermented sausages during storage

Effets combinés des huiles essentielles microencapsulées et de l'irradiation des sources de rayons gamma et X sur les propriétés microbiologiques et physico-chimiques des saucisses fermentées et séchées pendant le stockage

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Title of the journal:

LWT-Food Science and Technology

Submitted on 21-09-2021

Manuscript Number: LWT-D-21-06352

Contribution of authors:

ML is the research responsible and coordinator of the project. She has planned the research experiment. JJ conceived the design of the experiments, performed the experiments, organized the database, performed the statistical analysis and wrote the first draft of the manuscript. Z. Allahdad, S. Salmieri and ML contributed to manuscript revision, read, and approved the submitted version.

6.1 Abstract

Essential oils (EOs) or EOs encapsulated in alginate and alginate-CNC combined with 1.5 kGy X-ray (0.76 kGy/h) or γ-ray (6.37 kGy/h) irradiation were applied on dry fermented sausages (DFS). Microbiological quality was tested in terms of the reduction of *Escherichia coli* O157:H7 cocktail, *Listeria monocytogenes*, molds and yeasts, lactic acid bacteria (LAB), and total mesophilic bacteria (TMF) during storage at room temperature (20 ± 1 °C) for 8 weeks. Physicochemical quality was tested by following the changes of texture and color of sausages. Synergistic effects were observed with combined treatments with γ-irradiation on inhibiting *Escherichia coli* O157:H7 cocktail and LAB and with X-ray on inhibiting *E. coli* O157:H7 cocktail. Extensive inhibition of *L. monocytogenes*, molds and yeasts, and TMF was also noticed during storage. Antimicrobial formulations combined with γ-irradiation didn't show adverse effects on texture and color of sausages while when combined with X-ray, a reduction of redness and an increase of hardness were noticed. However, the differences of texture were eliminated during storage.

Keywords: Essential oils; Microencapsulation; Gamma and X-ray irradiation; Dry fermented sausage; Microbiological and physicochemical properties

6.2 Résumé

Des huiles essentielles (HE) ou des HE encapsulées dans de l'alginate et de l'alginate en présence de nanocrystal cellulose (alginate-CNC) associées à une irradiation aux rayons X de 1.5 kGy (0.76 kGy/h) ou aux rayons γ (6.37 kGy/h) ont été appliquées sur des saucisses fermentées et séchées (SFS). La qualité microbiologique a été testée en termes de réduction du cocktail *Escherichia coli* O157:H7, *Listeria monocytogenes*, moisissures et levures, bactéries lactiques (LAB) et bactéries mésophiles totales (TMF) pendant le stockage à température ambiante (20 ± 1 °C) pendant 8 semaines. La qualité physico-chimique a été testée en suivant les changements de texture et de couleur des saucisses. Des effets synergiques ont été observés avec des traitements combinés avec irradiation sur l'inhibition du cocktail *Escherichia coli* O157:H7 et LAB et avec des rayons X sur l'inhibition du cocktail *E. coli* O157:H7. Une inhibition importante de *L. monocytogenes*, des moisissures et des levures, et une réduction importante du TMF a également été observée pendant le stockage. Les formulations antimicrobiennes combinées à l'irradiation γ n'ont pas montré d'effet néfaste sur la texture et la couleur des saucisses, tandis que lorsqu'elles sont combinées aux rayons X, une réduction de la couleur rouge et une augmentation de la fermeté des saucissons ont été observées. Cependant, les différences de texture entre les groupes ont été éliminées pendant le stockage.

Mots-clés : Huiles essentielles; Microencapsulation; Irradiation gamma/rayons X; Saucisson sec fermenté; Propriétés microbiologiques et physico-chimiques

6.3 Introduction

Food irradiation has been historically in use for more than 100 years and is increasingly being accepted and widely recognized as a part of overall good manufacturing practice (GMP) and hazard analysis critical control points (HACCP) systems (Diehl, 2002; Shah, Mir, & Pala, 2021). Food irradiation is a process of exposing food to the controlled amounts of ionizing radiations such as γ rays, X-rays and accelerated electrons, to reduce food-borne pathogens, spoilage microorganisms and parasites, extend shelf-life, disinfect insects, detoxify toxic substances and maintain nutrition (Indiarto, Pratama, Sari, & Theodora, 2020; Singh & Singh, 2020). It is a non-thermal method that can be used without affecting the sensory properties and product qualities (Pedreschi & Mariotti-Celis, 2020). Therefore, irradiation is particularly useful for the decontamination for foods that are sold without thermal treatments such as raw poultry, meat, and seafood (Shah *et al.*, 2021). γ rays and X rays are short wavelength radiations with very high associated energy levels (Lacroix, 2014). Cobalt-60 is the most commonly used radionuclide for food in the form of γ rays. The emitted energy can be used as high as 95% (Indiarto & Qonit, 2020). X-rays that have high penetrating power and no left radiation hazards are raising interests as low risk-significant radioactive sources (Indiarto & Qonit, 2020). However, less studies have been done in use of X-ray for food pasteurization (Begum *et al.*, 2020). The mechanism of ionizing radiation is mainly damage to nucleic acids, interruption of chemical bonds in DNA, or direct or indirect damage caused by oxidative free radicals generated by water radiation decomposition (Lacroix, 2014).

Essential oils (EOs) are aromatic volatile oily liquids extracted from plant materials such as leaves, seeds, flowers, roots, peel, fruits and wood and are generally recognized as safe (GRAS) as food additives (Burt, 2004). Due to different biosynthetic chains, EOs are a complex mixture of natural compounds divided into two groups: the terpene group and the aromatic and aliphatic group (Falleh, Ben Jemaa, Saada, & Ksouri, 2020). The main constituents of EOs are phenolic compounds which are one of the most important molecules determining the biological properties of EOs (Varghese, Siengchin, & Parameswaranpillai, 2020). The antimicrobial activity of EOs has been widely recognized by previous research against foodborne pathogens and food spoilage fungi such as *Aspergillus* spp., *Penicillium* spp. (Faleiro, 2011; Ji, Shankar, Royon, Salmieri, & Lacroix, 2021). Antimicrobial compounds can accumulate on the membrane that result in fluidifying effect on the membrane, leakage of intracellular constituents and cell death through various mechanisms depending on different components of EOs and target microorganisms (Ji *et al.*, 2021; Pateiro *et al.*, 2021). The use of EOs or their constituents as food preservatives is

often limited due to a required higher concentration in food models causing negative organoleptic effects. Food components such as fat, starch, protein can interact with hydrophobic compounds that reduce the antimicrobial activity of EOs (Hyldgaard, Mygind, & Meyer, 2012). Applying several EOs synergistically or in combination with organic acids is one good alternative (Calo, Crandall, O'Bryan, & Ricke, 2015). Encapsulating EOs into biopolymers as films, coatings or sachets allows a protection of the bioactivity during the process and during the storage, can assure a controlled release of EOs to action sites, and it is a promising technique that avoids the intense aroma of EOs (Castro-Rosas *et al.*, 2017).

Dry fermented sausages (DFS) are defined as sausages that have a final pH ranging between 5.2 and 5.8, which is consistent with the lower lactic acid content (0.5% – 1.0%), the moisture lower than 30%, a_w ranges from 0.85 to 0.91, and an moisture:protein ratio lower than 2.3:1 (Vignolo, Fontana, & Fadda, 2010). The production of DFS consists of three clearly-defined steps: ingredients mixing, fermentation and drying (Fernández-López, Sendra, Sayas-Barberá, Navarro, & Pérez-Alvarez, 2008). During the three steps, the physical, chemical and microbiological diversifications are closely related to the raw material characteristics and to the process conditions. These characteristics determine the shelf life and also the organoleptic properties of the final product (Fernández-López *et al.*, 2008; Houben & van't Hooft, 2005). Because of the relative high level of fat and distinctive processing characteristics such as the use of diverse raw materials, absence of thermal treatment, fermented sausages are highly subjected to quality deterioration, which mainly includes lipid oxidation and microbial deterioration (Tomović *et al.*, 2020). Many studies have reported that some pathogens like *Listeria monocytogenes*, *Salmonella* and *Escherichia coli,* can survive in DFS and cause many cases of foodborne disease outbreaks in many countries (Lindqvist & Lindblad, 2009).

In this study, free EOs and encapsulated EOs in alginate or alginate-CNC combined with irradiation at a dose of 1.5 kGy using cobalt-60 γ-rays (1.17 and 1.33 MeV) with a dose rate of 6.37 kGy/h as well as low-energy (125 keV) X-rays with a dose rate of 0.76 kGy/h were applied on DFS to compare the effects of γ-ray at high dose rate of irradiation with X-ray irradiation at low dose rate of irradiation on microbial and physicochemical properties of DFS during storage. The results are expected to provide a useful reference for reasonable application of two types of irradiation in combination with EOs, and encapsulation on fermented and dry sausage.

6.4 Materials and methods

6.4.1 Bacterial culture preparation

Before tests, cultures from stock at − 80 °C were propagated through three successive growth cycles at 37°C for 24 h in Tryptic Soy Broth (TSB; Becton-Dickinson, Sparks, MD, USA) for *E. coli* cocktail (mixture of five *E. coli* O157:H7 strains of RM1239, RM1931, RM1933, RM1934, 380-94) and *L. monocytogenes* (LM 1045) to obtain a concentration of approximately 10^{12} CFU mL^{-1} and 10^{9} CFU mL^{-1} respectively.

6.4.2 Antimicrobial formulations

The formulations were prepared based on the method of Huq *et al.* (2015) with some modifications. EO mixtures of cinnamon bark and cinnamomum cassia (3% w/v, Zayat-Aroma, Canada) were used in the preparation. Finally, four formulations were prepared following this procedure including non-encapsulation (EO), alginate encapsulated EO (AE), alginate-CNC encapsulated (ACE) and non-EOs as a control (CT).

6.4.3 Sausage manufacture

Manufacturing protocol and materials (beef, spices, casing, and ferments) were provided by Usine Amsellem. *E. coli* cocktail 4% and 0.1% *Listeria monocytogenes* were inoculated to ground meat to obtain around 7.5 log and 5 log separately before manufacturing. Three prepared formulas were mixed to meat to obtain a final concentration of 0.45% EO before casing. Samples of 50-g sausage were cased by Tre Spade sausage stuffing (Mod. 10 Deluxe; P/N 21100/L; FACEM SpA, Turin, Italia). Final products were obtained after a 48-h fermentation (25±0.3 °C, 90 ± 2% RH) with an ending pH of about 5.20, and a 5-d drying (141 °C, 70 5% RH) with an ending a_w of about 0.85. Sausages were then vacuum packed by using a Sipromac vacuum packaging machine (model 350; Sipromac II Inc., St-Germain, QC, Canada) and stored at room temperature (20 ± 1 °C).

6.4.4 Sample irradiation

Samples were sealed under 96% vacuum in eight transparent bags (Winpak Ltd., Vaudreuil-Dorion, QC, Canada) separately using a packaging machine (model 250 Single Chamber, Sipromac Inc., St-Germain-de-Grantham, QC, Canada). The γ-irradiation procedure was done at the Canadian Irradiation Centre (CIC, Laval, QC, Canada) in a cobalt-60 Underwater

Calibrator UC-15A (energy level: 1.25 MeV; Nordion Canada Inc., Ottawa, ON, Canada) at a dose rate of 6.37 kGy/h. X-ray irradiation was realized in INRS-Armand Frappier Health Biotechnology Centre (Laval, QC, CANADA) using X-ray machine (Philips appliance-model MG160, 125 keV) at a dose rate of 0.76 kGy/h. Samples were irradiated to 1.5 kGy by X- or γ-ray. Four groups of CT, EO, AE, ACE treated with γ-irradiation were CT+GI, EO+GI, AE+GI, ACE+GI. Four groups treated with X-ray irradiation were CT+XI, EO+XI, AE+XI, ACE+XI.

6.4.5 Microbiological analysis

Each 10 g sausage sample was mixed in 90 mL of peptone water (0.1%) in sterile Whirl-Pak sampling bags (Fisher Scientific) and homogenized at 260 rpm for 1 min in a Seward 400 Circulator Stomacher® (Fisher Scientific). Tryptic soy agar, Man Rogosa Sharpe agar and potato dextrose agar (Alpha Biosciences Inc., Baltimore, MD, USA) were used for aerobic mesophilic bacteria (37 ± 1 °C, 48 h), lactic acid bacteria (30 ± 1 °C, 72 h), molds and yeasts (25 ± 1 °C, 72 h) separately. Palcam agar supplemented with antibiotics acriflavine (5 mg/mL), polymyxin B (10 mg/mL) and ceftazidime (8 mg/mL), macconky sorbital agar (Oxoid Unipath Ltd. Nepean, Ottawa, Canada) were used for L. monocytogenes (37 ± 1 °C °C, 48 h) and *E.coli* O157:H7 (37 ± 1 °C, 24 h) respectively. Sampling was performed at the end of drying before irradiation and after irradiation, at 4th week and 8th week during storage. The detection limit was 10 CFU/g.

6.4.6 Color

Color was measured using a Minolta Colorimeter Color reader CR10 (Konica Minolta Sensing, Inc, Mahwah, NJ, USA) (Ben Fadhel *et al.*, 2016). The outer color was measured on the surface of sausages; the interior color was measured on the tangent plane. Color was expressed in CIE-LAB system, *L** for lightness, *a** for redness, and *b** for yellowness. The total change of color ΔE* was calculated according to Equation (11).

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad (11)$$

6.4.7 Texture

Texture was measured according the method of Houben & van't Hooft (2005) with some modifications. Samples were prepared by cutting sausages into 2 cm thick slices with a flat tangent plane. Tests were performed at room temperatures with a Universal Tensile Machine (Model H5KT, Tinius-Olsen Inc., Horsham, PA, USA,) equipped with a 100 N-load cell (type FBB). Compression from position model was applied and a distance of 20% of slice height was

compressed on each sample. Stress-strain curves were determined from force-distance recordings. Maximum stress and Young's modulus (maximum slope of the stress-strain curve between the origin and the yield point) were recorded indicating the hardness of samples.

6.4.8 Statistical analysis

For all results, a one-way analysis of variance (ANOVA), Tamhane's test for unequal variances and Duncan's multiple-range test for equal variances were performed by PASW Statistics 18 software (IBM Corporation, Somers, NY, USA). Differences between means were considered significant when the confidence interval was lower than 5% ($P \leq 0.05$). Experiments were done in triplicate (n = 3). For each replicate, 2 samples from each treatment group were analyzed for microbial tests and 3 samples were analyzed for color and texture during storage.

6.5 Results and discussion

6.5.1 *E. coli* elimination

The reduction of the tested microorganisms during the whole process is shown in Table 6.1. The EOs, EOs encapsulated in alginate, and EOs encapsulated in alginate-CNC treatments showed no difference in *E. coli* count at the end of drying ($p > 0.05$) but contributed similarly to eliminate *E. coli* at the 4th week of storage while the control group had 2.63 log CFU/g *E. coli* after 4 weeks of storage. **γ-**irradiation showed no significant effect ($p > 0.05$) at the end of drying when applied individually but reduced *E. coli* from 4.06 log CFU/g to non-detected level at 4th week compared to the control. X-ray irradiation (CT+XI sample) also showed no significant effect ($p > 0.05$) at the end of drying but a 2.09-log reduction was observed within 4 weeks of storage. The reduction amount was comparable with the control group without irradiation indicating that *E. coli* is more resistant to X-ray than γ-ray. Synergistic effects were observed when γ-irradiation was used in combination with the free EOs, EOs encapsulated in alginate, and EOs encapsulated in alginate-CNC. The combined treatments of EO-GI, AE-GI and ACE-GI eliminated *E. coli* at the end of drying. But the synergistic effect of both free EOs and encapsulated ones when combined with X-ray irradiation was not as great as the similar treatments with γ-irradiation at the end of drying so that *E. coli* was around 2.5 log CFU/g and was not eliminated as what happened in γ-irradiation treatments.

Previous research has demonstrated the inhibitory effects of EOs and irradiation to *E. coli.* Sage EO applied in minced pork significantly reduced the growth of *E. coli* during storage (Danilović *et al.*, 2021). Edible coatings based on agar/sodium alginate containing ginger EO

applied on sliced fresh beef were proved to extend shelf-life well by inhibiting *E. coli*, molds and yeasts and total viable counts during refrigerated storage (B. Zhang *et al.*, 2021). Cho & Ha (2019) studied the effects of X-ray for the inactivation of foodborne pathogens in ready-to-eat sliced ham. *E. coli* O157:H7 was eliminated to a non-detective level when irradiated at ≥ 0.6 kGy. Begum *et al.* (2020) tested γ- and X-ray sources at different dose rates combined with oregano/thyme EO against *E. coli* O157:H7 in rice. The radiosensitivity was significantly affected ($p > 0.05$) by dose rates and EOs and synergistic effects were observed against *E. coli* with the combined treatments. *E. coli* O157:H7 was observed to be more resistant to X-ray than γ-ray in rice due to the lower dose rates which are in agreement with our test.

Table 6.1 Reduction of microbial counts during storage at room temperature[1].

	Concentration of *E. coli* cocktail (log CFU/g)			Concentration of *L. monocytogenes* (log CFU/g)			Concentration of molds and yeasts (log CFU/g)			Concentration of LAB (log CFU/g)			Concentration of TMF (log CFU/g)		
Samples	After drying	4weeks	8weeks	After drying	4weeks	8weeks	After drying	4weeks	8weeks	After drying	4weeks	8weeks	After drying	4weeks	8weeks
CT	4.84±0.09 ^{Cc}	2.63±0.32 ^{Bb}	ND ^{Aa}	3.94±0.69 ^{Cb}	ND ^{Aa}	ND ^{Aa}	6.87±0.48 ^{Cc}	4.00±0.54 ^{Bb}	ND ^{Aa}	8.94±0.31 ^{Gc}	7.62±0.31 ^{Fb}	6.61±0.18 ^{Ea}	9.28±0.28 ^{Fc}	8.64±0.12 ^{Gb}	8.30±0.30 ^{Da}
EO	4.13±0.75 ^{Cb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	8.34±0.18 ^{Fc}	7.53±0.39 ^{Fb}	4.99±0.13 ^{CDa}	8.33±0.27 ^{Ec}	7.66±0.39 ^{Fb}	7.10±0.70 ^{Ca}
AE	4.44±0.33 ^{Cb}	ND ^{Aa}	ND ^{Aa}	1.26±0.45 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	8.29±0.28 ^{Fc}	7.54±0.14 ^{Fb}	5.12±0.26 ^{Da}	8.48±0.25 ^{Ec}	7.73±0.27 ^{Fb}	7.02±0.84 ^{Ca}
ACE	4.32±0.24 ^{Cb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	8.19±0.12 ^{Fc}	7.37±0.50 ^{Fb}	5.32±0.59 ^{Da}	8.32±0.17 ^{Ec}	7.59±0.14 ^{Fb}	6.93±0.64 ^{Ca}
CT+GI	4.06±0.31 ^{Cc}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	5.66±0.31 ^{Bb}	1.62±0.87 ^{Aa}	ND ^{Aa}	7.13±0.29 ^{DEb}	4.65±0.13 ^{Ca}	4.50±0.69 ^{BCa}	7.37±0.31 ^{Dc}	6.04±0.17 ^{DEb}	5.50±0.18 ^{Ba}
EO+GI	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	5.36±0.22 ^{Ab}	3.74±0.50 ^{Ba}	3.72±0.42 ^{Aa}	6.23±0.15 ^{Ac}	5.42±0.23 ^{ABb}	4.93±0.08 ^{Ba}
AE+GI	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	5.72±0.27 ^{Bb}	3.72±0.62 ^{Ba}	3.76±0.20 ^{Aa}	6.65±0.16 ^{BCc}	5.93±0.09 ^{CDEb}	4.99±0.26 ^{Ba}
ACE+GI	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	5.26±0.31 ^{Ab}	3.13±0.18 ^{Aa}	3.29±0.02 ^{Aa}	6.36±0.06 ^{ABc}	5.22±0.20 ^{Ab}	4.91±0.13 ^{Ba}
CT+XI	4.62±0.06 ^{Cc}	2.53±0.35 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	5.38±0.10 ^{Bc}	1.30±0.00 ^{Ab}	ND ^{Aa}	7.21±0.22 ^{Ec}	6.40±0.12 ^{Eb}	4.71±0.22 ^{CDa}	7.19±0.25 ^{Dc}	6.18±0.13 ^{Eb}	5.78±0.29 ^{Ba}
EO+XI	2.45±0.13 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	6.71±0.10 ^{Cc}	5.64±0.02 ^{Db}	3.35±0.07 ^{Aa}	6.71±0.38 ^{Cc}	5.57±0.11 ^{ABCb}	3.20±0.12 ^{Aa}
AE+XI	2.63±0.30 ^{Bb}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	6.95±0.38 ^{CDEc}	5.44±0.29 ^{Db}	3.94±0.21 ^{ABa}	6.7±0.47 ^{Cc}	5.70±0.00 ^{BCDb}	3.85±0.21 ^{Aa}
ACE+XI	2.56±0.49 ^{Ba}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	ND ^{Aa}	6.87±0.09 ^{CDc}	5.70±0.05 ^{Db}	3.68±0.03 ^{Aa}	6.74±0.18 ^{Cc}	5.68±0.15 ^{BCDb}	3.33±0.35 ^{Aa}

[1]Numbers are means ± standard deviations from triplicate samples. Within each row for each tested strain, means with the same lowercase letter are not significantly different (P > 0.05). Within each column, means with the same uppercase letter are not significantly different (P > 0.05).

6.5.2 *L. monocytogenes* elimination

The samples treated with EOs, EOs encapsulated in alginate-CNC and all irradiated with γ- and X-ray were devoid of *L. monocytogenes* at the end of drying (Table 6.1). But the samples formulated with EOs encapsulated in alginate reduced by 2.68 log CFU/g *L. monocytogenes* after drying when compared to control and were able to eliminate *L. monocytogenes* at the 4th week of storage.

In this test, *L. monocytogenes* was eliminated to below detection level with free EO or the ACE treatment without irradiation. The radiosensitivity of *L. monocytogenes* to X-ray or γ-irradiation cannot be compared in this study since the remaining bacteria in both treatments are below detection limit. However, it has been already observed that *L. monocytogenes* is more resistant to X-ray and γ-irradiation than *E. coli* O157:H7 (Cho & Ha, 2019; Tawema, Han, Vu, Salmieri, & Lacroix, 2016). According to Begum *et al.* (2020), *L. monocytogenes* was found to have higher D_{10} values with X-ray at dose rate of 0.76 kGy/h than γ-ray treatments applied at dose rates of 9.1, 3.93 and 0.22 kGy/h and synergistic effects were observed with the combination of X-ray/γ-ray irradiation with oregano/thyme EOs. It has been also reported the synergistic effects of 1.5 kGy γ-irradiation with oregano or cinnamon EO and nisin microencapsulated in alginate-CNC against *L. monocytogenes* in ready-to-eat ham (Huq *et al.*, 2015).

6.5.3 Molds and yeasts elimination

The EOs with or without encapsulation treatments all eliminated molds and yeasts upon completing drying storage (Table 6.1). X-ray and γ irradiation reduced significantly ($p \leq 0.05$) molds and yeasts count around 1.49 log and 1.21 log CFU/g, respectively, at the end of drying. More loss was observed at 4th week of storage, with respective values by 2.7 log and 2.38 log CFU/g for X-ray and γ irradiation treatments as compared to the control group. Therefore, X-ray and γ-ray irradiation have similar effects on molds and yeasts when used irradiation alone.

However, molds and yeasts showed to be more resistance to X-ray and γ-ray irradiation than *L. monocytogenes* and *E. coli* O157:H7 cocktail in this study. But the introduction of EO into the product made increased the sensitivity of molds and yeasts implying more susceptibility of molds and yeasts to the applied EOs than irradiation. Other literatures are in parallel with our findings. *A. niger* showed more resistance to X-ray at 0.76 kGy/h than γ-ray at 0.085, 4.558 and 10.445 kGy/h (Shankar *et al.*, 2020). However, when X-ray (0.76 kGy/h) was combined with EOs, the lowest radio sensitivity was observed for *A. niger* similar to γ-ray (10.445 kGy/h) combined with EOs.

6.5.4 Lactic acid bacteria (LAB) elimination

According to the results (Table 6.1), although applying EOs reduced the LAB in the product, the encapsulation had no tangible effect on the LAB compared to free EOs. X-ray and γ-ray reduced LAB approximately by 1.73 and 1.81 log CFU/g, respectively, at the end of drying demonstrating the similar effects of X-ray and γ-ray in deactivation of LAB. However, their effectiveness became different during storage after 4 weeks. X-ray and γ-ray reduced by 1.22 and 2.97 log CFU/g of LAB, respectively, compared to control. Samples treated with X-ray had significantly higher level in LAB showing that LAB was more resistant to X-ray than γ-ray. After 8 weeks, the groups treated with X-ray and γ-ray showed similar LAB loss. A synergistic effect was observed when γ-irradiation was combined with EOs and their encapsulated forms (ACE and AE) after drying and during 4 weeks of storage. At 8^{th} week of storage, following total release of active compounds in the product the similar antimicrobial activity was observed for EOs, AE, and ACE. The more efficient role of γ-irradiation in comparison with X-ray in reducing LAB was proved in the combined treatments. Regarding X-ray, the presence of EOs increased the radiosensitivity of LABs, but the type of encapsulation material had no effect on this parameter during the entire storage period.

The resistance of LAB during storage and its presence as dominant microflora were also demonstrated in vacuum packed sausages by Rubio *et al.* (2007). Combined effect of active chitosan-based films containing cumin EO nanoemulsion and 2.5 kGy γ-irradiation was observed to reduce significantly total mesophilic bacteria and LAB and extend shelf-life of beef loins during chilled storage (Dini, Fallah, Bonyadian, Abbasvali, & Soleimani, 2020). Gelatin-CMC films incorporated with chitin nanofiber and higher concentration of *Trachyspermum ammi* EO (1%) reduced most total viable counts, LAB and molds and yeasts in raw beef (Azarifar, Ghanbarzadeh, Sowti khiabani, Akhondzadeh basti, & Abdulkhani, 2020).

6.5.5 Total mesophilic flora (TMF) elimination

The decrease in the number of TMF during the storage of irradiated meat is due to the post-irradiation effect so that the surviving cells damaged by γ-rays cannot adapt to the surrounding environment and gradually die (D. H. Kim *et al.*, 2000). The impact of EOs in the free state was similar to that of their encapsulated forms regardless of the type of wall material and reduction value was approximately 1 log CFU/g after drying. It is clear that the storage time had pronounced decreasing effect for the treatments of EOs, AE, and ACE. X-ray and γ-ray reduced by 1.91 and 2.09 log CFU/g of TMF, respectively, at the beginning of storage and these values increased to

2.46 and 2.6 log CFU/g, respectively, at the 4th week of storage and to 2.52 log and 2.8 log CFU/g at 8th week after drying. Similar trends of X-ray and γ-ray in combination with EO, whether free or encapsulated, were observed on TMF at the end of drying and 4th week of storage but more reduction was observed for combined treatments of X-ray over those of γ-irradiation at 8th week of storage. No synergistic effect ($p > 0.05$) was observed for X-ray and γ-ray combined with EO, AE and ACE at the beginning of drying and at the 4th week of storage after drying. However, the synergistic effect was observed only for X-ray in combination with EO, AE, and ACE at 8th week.

Ionizing radicals originated from the radiolysis of water, can damage to cell by destroying the structure and function of cellular components such as DNA, pigments, fatty acids, and membrane lipids therefore causing chromosomal abnormalities, errors in cell division, and inactivation of endogenous enzymes (Ahn, Kim, & Lee, 2013; Cho & Ha, 2019; S. Y. Kim *et al.*, 2018). Thus, irradiation may enhance the contact between antimicrobial molecules and cell membranes increasing the inhibitory effects of EOs and radio sensitivity of microorganisms (Turgis, Han, Caillet, & Lacroix, 2009; Turgis, Vu, Dupont, & Lacroix, 2012). In the present study, X-ray irradiation reduced TMF by around 2 log CFU/g compared to control which is in a good agreement with the results observed in previous research of X-ray irradiated beef (S.Y. Kim *et al.*, 2018).

6.5.6 Color evaluation of sausages

In all treated and untreated samples, storage time had no significant impact ($p > 0.05$) on interior and exterior *L** parameter. It is clear from the Table 6.2 that all treatments applied in this study has increased the color coordinate of *L** in a significant way whether on the surface or within the sausages. Apparently, the changes made by γ-irradiation is more tangible than X-ray as compared to the control sample. The introduction of EO into the product raised the exterior lightness but its encapsulation with polymers created more notable increment in the surface lightness. By comparing the lightness of EO, AE, and ACE formulated samples simultaneously treated with X- and γ-ray, it seems that the increase of lightness is resulted from the EO presence not from irradiation as no significant difference was observed between the *L** values of EO, AE, and ACE formulated samples and respective values to the same samples treated with X- and γ-irradiation ($p > 0.05$). Regarding interior lightness, such effectiveness was observed when EO was added to the sausage formulation although its encapsulation enhanced lightness more effectively. Similarly, interior lightness increment under the effect of γ-irradiation was more pronounced than X-irradiation. But AE in combination with X-ray as well as ACE in combination with γ-ray showed better effect in lightness improvement compared to other formulations. Among

non-irradiated samples, AE and the control sample adopted respectively the highest and the lowest values of *L**.

As can be seen in Table 6.2, all treatments applied in this study has decreased the color coordinate of *a** in a significant way on the surface of the sausages. Apparently, the change made by X-ray is more than γ-ray as compared to the control sample. The introduction of EO into the product didn't affect the exterior and interior redness but its encapsulation with polymers created notable increments. By comparing the redness of EO, AE, and ACE formulated samples simultaneously treated with X- and γ-ray, the EO and its encapsulation further decreased the exterior and interior redness, and even more decrease was observed among the *a** values of EO, AE, and ACE formulated samples treated with X-ray and respective values to the same samples treated with -irradiation. By considering the common phenomenon of decreasing redness during storage time, the redness was noticed to be stable for surface of non-irradiated groups and inside of X-ray treated samples.

In all treated and untreated samples, storage time had no strong impact on interior and exterior *b** parameter. The introduction of EO into the product didn't affect the exterior or interior yellowness but its encapsulation with polymers created more notable increment of the surface and inside yellowness. X- or γ-irradiation didn't change significantly the exterior or interior yellowness. By comparing the yellowness of EO, AE, and ACE formulated samples simultaneously treated with X- and γ-ray, a significant difference was observed between the *b** values of EO, AE, and ACE formulated samples and the values respective to the same samples treated with X- and γ- irradiation except for exterior yellowness after drying. Regarding interior yellowness, wider range of *b** values was observed. Interior yellowness increment under the effect of γ-irradiation was more pronounced than X-irradiation. EO in combination with γ-ray showed similar yellowness while ACE in combination with γ-ray showed the greatest *b** values compared to control samples.

The incorporation of EO and its encapsulated forms retained the exterior color difference of samples unchanged during 2 months of storage. Regarding interior of the products, total color difference within the samples of EO and AE notably reduced during one month but the application of CNC in encapsulation of EO prevented color changes although there was no difference among all formulated samples with free EO and encapsulated EO and control at 8th week. X- and γ-ray did not affect the exterior total color changes but interior total color change was adversely affected at the end of storage. Apparently, the changes made by X-ray are more tangible than γ-ray as compared to the control sample. By comparing the total color changes of EO, AE, and ACE

formulated samples simultaneously treated with X- and γ- ray, the increase was observed mostly on interior total color change at 4th week and ΔE values of EO and its encapsulated forms combined to X-ray were higher than respective values to the same samples treated with γ-irradiation. The values of exterior ΔE reduced for combined treatments with X- and γ- ray during storage and the reduction was much more obvious for interior color change. Also, the total color change reduction under the effect of X-ray was more pronounced than γ-ray compared to the only irradiated samples. The color of samples treated with only γ- or X-ray was less affected by the applied treatment compared to samples in presence of formulations. AE and ACE combined with X-ray showed the highest surface total color change and the lowest interior total color change at the end of storage.

Generally, the color of irradiated meat products can vary depending on the radiation source, radiation dose, animal species of raw meat, muscle type, packaging type, and myoglobin concentration (Ham *et al.*, 2017). Nitrosohemoglobin is usually the main pigment that causes redness in meat products containing nitrite (Ham *et al.*, 2017). The effect of irradiation on the color change of fermented sausages has not been determined. It is thought that the reduction in redness during storage may be due to the destruction of nitrosoheme by irradiation (I. S. Kim *et al.*, 2012). On the other hand, phenolic compounds can interrupt the oxidation reaction of irradiated meat by providing hydrogen atoms or quenching free radicals, thereby avoiding the color and texture changes caused by irradiation (Ahn, Kim, & Lee, 2013). Ben Fadhel *et al.* (2016) observed that 1 kGy γ-irradiation caused a reduction in *a** value which was indicative of a significant greenness of fresh pork meat. But the green pigments were not stable when the irradiation doses increased to 1.5 and 3 kGy, while the red color related to the formation of heme pigment-CO ligand was more stable. These results are consistent with what was found with 1.5 kGy γ-irradiation on redness of DFS. According to I. S. Kim *et al.* (2012), redness of beef sausage patties was not affected by γ-irradiation lower than 1 kGy but decreased during storage when irradiation increased to 2 and 4 kGy. Song *et al.* (2017) observed similar *a** values after γ- and X-ray irradiation for low-salt sausages although they significantly lowered at the end of refrigerated storage.

Samples irradiated with X-ray exhibited a reduction in redness accompanied by more greenness compared to the control and γ-irradiated samples. Color changes may be due to the intrinsic sensitivity of myoglobin molecules to the energy caused by irradiation (Ben Fadhel *et al.*, 2016). Myoglobin can be bound to oxygen to form bright red oxygenated myoglobin. After irradiation, free binding sites can react with free radicals such as hydroxyl (-OH) and sulphuryl (-

SH) radicals to form metmyoglobin (brown) and thiomyoglobin (green), respectively (Ouattara, Giroux, Smoragiewicz, Saucier, & Lacroix, 2002). Besides, meat color stability depends on the residual enzymatic activity in meat which controls myoglobin oxygenation, oxidation and reduction (Rodrigues *et al.*, 2020). Rodrigues *et al.* (2020) observed the reduction of *a** values in parallel with oxymyoglobin loss and metmyoglobin increase at high doses of γ-irradiation, owing to the increased lipid oxidation and reduced metmyoglobin reducing capacity and oxygen consumption rates due to irradiation. Hydroxyl radicals produced by ionizing radiation are considered to be a factor in accelerating lipid oxidation, which adversely affects the color, flavor, texture and nutritional value of meat (Ham *et al.*, 2017; Park *et al.*, 2010). Therefore, the lipid oxidation reinforces meat discoloration (Faustman, Sun, Mancini, & Suman, 2010). Ham *et al.* (2017) studied the effects of three different irradiation sources of γ-ray, electron-beam, and X-ray at 0, 2.5, 5, 7.5, and 10 kGy on the quality of cooked beef patties and pork sausages during 10 days of storage at 30±1°C. X-ray irradiation resulted in significantly higher TBARS values than γ-ray, while X-ray resulted in lower *a** values for all doses applied on pork sausages than γ-ray. The *a** value of beef was observed initially lower in X-ray irradiated samples, but the difference was disappeared with extended storage (S. Y. Kim *et al.*, 2018).

However, further research should be conducted clearly to determine the effect of different irradiation sources and dose levels on nitrosyl hemochrome and endogenous enzyme stability and free radical generation in dry fermented and irradiated meat products.

Table 6.2 Color attributes of outside and inside of sausages during storage at room temperature[1].

		Exterior color			Interior color		
Samples		After drying	4weeks	8weeks	After drying	4weeks	8weeks
CT	*L**	38.03±0.55 ^{Aa}	37.33±1.53 ^{Aa}	39.98±0.29 ^{ABb}	37.80±3.49 ^{Aa}	32.67±1.14 ^{Aa}	35.90±0.36 ^{Ba}
	*a**	-4.90±0.78 ^{BCa}	-4.67±0.76 ^{DEa}	-4.18±0.36 ^{DEFa}	-1.50±0.26 ^{CDEa}	-0.83±0.15 ^{Fa}	-0.90±0.10 ^{Ea}
	*b**	1.50±0.36 ^{Aa}	1.47±0.14 ^{ABa}	1.82±0.04 ^{Aa}	3.93±0.67 ^{Aa}	4.00±0.10 ^{Ca}	4.50±0.10 ^{ABa}
	∆E	-	1.70±0.51 ^{CDE}	2.13±0.18 ^{BC}	-	5.18±1.14 ^{D}	2.08±0.33 ^{AB}
EO	*L**	38.65±0.42 ^{ABDa}	40.13±0.4 ^{CDEb}	40.25±0.06 ^{ABb}	39.50±3.41 ^{Aa}	37.50±0.71 ^{BCa}	38.13±0.12 ^{CDa}
	*a**	-5.68±0.66 ^{Ba}	-5.40±0.56 ^{CDa}	-4.93±0.10 ^{BCa}	-2.03±0.40 ^{BCDa}	-0.38±0.09 ^{FGc}	-1.33±0.12 ^{CDb}

	*b**	1.45±0.37 ^{Aa}	2.07±0.55 ^{BCDa}	2.63±0.30 ^{BCa}	4.73±0.40 ^{ABa}	5.25±0.57 ^{EFa}	5.00±0.10 ^{BCa}
	ΔE	-	1.78±0.09 ^{DE}	2.13±0.21 ^{BC}	-	2.74±0.42 ^{A}	1.57±0.07 ^{AB}
AE	*L**	40.45±0.67 ^{DEa}	40.55±0.44 ^{DEFGa}	41.25±0.37 ^{BCDa}	42.63±4.39 ^{Aa}	40.70±0.58 ^{Ea}	41.87±0.06 ^{Fa}
	*a**	-4.35±0.94 ^{Ca}	-4.70±0.41 ^{DEa}	-4.12±0.46 ^{DEFa}	1.90±0.42 ^{Fc}	0.86±0.21 ^{HIb}	0.20±0.00 ^{Ga}
	*b**	2.93±0.57 ^{Ca}	2.95±0.10 ^{DEa}	3.02±0.64 ^{CDa}	7.30±0.44 ^{Da}	7.16±0.32 ^{Ga}	7.10±0.10 ^{EFa}
	ΔE	-	0.63±0.15 ^{A}	1.12±0.30 ^{A}	-	2.23±0.56 ^{A}	1.88±0.03 ^{AB}
ACE	*L**	40.03±0.78 ^{CDEa}	41.13±0.67 ^{EFGa}	42.3±0.26 ^{DEb}	41.65±2.39 ^{Aa}	37.40±1.32 ^{BCa}	41.33±0.06 ^{EFa}
	*a**	-4.03±0.74 ^{Ca}	-3.45±0.43 ^{Fa}	-4.17±0.45 ^{DEFa}	1.63±0.44 ^{Fc}	0.23±0.06 ^{GHb}	-0.53±0.06 ^{Fa}
	*b**	2.95±0.49 ^{Ca}	4.08±0.14 ^{Fb}	3.80±0.17 ^{Eb}	7.35±1.09 ^{Da}	5.87±0.06 ^{Fa}	6.73±0.21 ^{EFa}
	ΔE	-	1.78±0.38 ^{DE}	2.46±0.22 ^{C}	-	4.74±1.14 ^{CD}	2.27±0.06 ^{BC}
CT+GI	*L**	38.97±0.23 ^{ABCa}	39.28±0.68 ^{BCa}	40.17±0.45 ^{ABa}	40.63±2.68 ^{Aa}	37.20±0.92 ^{BCa}	37.43±0.54 ^{BCa}
	*a**	-5.53±0.55 ^{Ba}	-4.70±0.86 ^{DEa}	-4.07±0.12 ^{DEFa}	-2.93±0.55 ^{Ba}	-2.40±0.35 ^{Da}	-1.55±0.17 ^{Cb}
	*b**	1.30±0.20 ^{Aa}	2.23±0.56 ^{BCDa}	2.17±0.32 ^{ABa}	3.70±0.72 ^{Aa}	3.73±0.64 ^{Ca}	3.80±0.39 ^{Aa}
	ΔE	-	1.62±0.47 ^{CDE}	2.13±0.14 ^{BC}	-	3.54±0.83 ^{ABC}	3.56±0.50 ^{E}
EO+GI	*L**	39.63±0.65 ^{BCa}	40.30±0.17 ^{CDEFa}	40.50±0.08 ^{ABCa}	37.03±2.38 ^{Aa}	39.03±0.12 ^{CDEa}	36.48±0.99 ^{BCa}
	*a**	-6.53±0.40 ^{Aa}	-5.27±0.15 ^{CDb}	-5.25±0.06 ^{Bb}	-1.85±0.07 ^{CDEa}	-1.63±0.31 ^{Ea}	-1.00±0.10 ^{DEb}
	*b**	1.33±0.25 ^{Aa}	2.43±0.31 ^{CDEb}	1.55±0.13 ^{Aa}	4.00±0.35 ^{Aa}	4.93±0.42 ^{DEa}	5.02±0.50 ^{BCa}
	ΔE	-	1.82±0.20 ^{DE}	1.63±0.05 ^{AB}	-	2.25±0.30 ^{A}	1.71±0.47 ^{AB}
AE+GI	*L**	41.02±1.04 ^{EFa}	40.63±0.71 ^{DEFGa}	41.77±0.81 ^{CDEa}	42.33±1.05 ^{Aa}	40.00±1.47 ^{DEa}	40.10±0.20 ^{EFa}
	*a**	-5.65±0.63 ^{Ba}	-4.43±0.36 ^{Eb}	-3.98±0.40 ^{DEFb}	-1.00±0.00 ^{Ea}	1.20±0.18 ^{Ic}	-0.10±0.00 ^{Gb}
	*b**	2.67±0.57 ^{Ca}	3.25±0.85 ^{Ea}	3.68±0.82 ^{DEa}	6.00±0.70 ^{BCa}	6.83±0.42 ^{Ga}	6.30±0.35 ^{Da}
	ΔE	-	1.70±0.41 ^{CDE}	2.31±0.63 ^{BC}	-	3.47±0.98 ^{ABC}	2.40±0.20 ^{BCD}
ACE+GI	*L**	40.10±1.54 ^{CDEa}	41.43±0.45 ^{FGa}	41.70±0.26 ^{CDEa}	44.40±1.60 ^{Aa}	40.35±1.08 ^{Ea}	41.50±0.30 ^{EFa}
	*a**	-4.13±0.53 ^{Ca}	-3.67±0.31 ^{Fa}	-3.67±0.50 ^{EFa}	0.97±0.15 ^{Fc}	0.43±0.10 ^{Hb}	0.10±0.00 ^{Ga}
	*b**	3.23±0.88 ^{Ca}	4.47±0.86 ^{Fa}	3.00±0.53 ^{CDa}	8.67±0.67 ^{Ec}	6.55±0.48 ^{Ga}	7.57±0.25 ^{EFb}
	ΔE	-	2.04±0.32 ^{E}	1.80±0.14 ^{ABC}	-	4.60±1.17 ^{BCD}	3.17±0.33 ^{DE}

CT+XI	*L**	39.54±1.25 ^{BCDa}	38.73±0.80 ^{Ba}	39.23±0.81 ^{Aa}	39.75±2.39 ^{Ab}	36.85±0.64 ^{Bb}	34.10±1.12 ^{Aa}
	*a**	-6.55±0.80 ^{Aa}	-6.40±0.36 ^{ABa}	-4.57±0.91 ^{CDb}	-5.51±1.02 ^{Aa}	-4.35±1.2 ^{Ca}	-3.53±0.17 ^{Aa}
	*b**	1.45±0.31 ^{Ab}	1.00±0.24 ^{Aa}	2.57±0.06 ^{BCc}	3.77±0.51 ^{Aa}	4.40±0.14 ^{CDa}	4.23±0.39 ^{ABa}
	ΔE	-	1.20±0.31 ^{BC}	2.43±0.73 ^{C}	-	3.33±0.11 ^{AB}	6.02±1.08 ^{F}
EO+XI	*L**	40.44±0.74 ^{DEa}	39.83±0.79 ^{CDa}	40.58±0.43 ^{ABCa}	40.48±2.30 ^{Aa}	40.5±0.76 ^{Ea}	39.83±2.75 ^{DEa}
	*a**	-7.30±0.30 ^{Aa}	-6.80±0.32 ^{Ab}	-6.08±0.22 ^{Ac}	-2.10±0.29 ^{BCDb}	-6.65±0.33 ^{Aa}	-2.35±0.35 ^{Bb}
	*b**	1.90±0.33 ^{ABa}	1.35±0.21 ^{ABa}	2.05±0.13 ^{ABa}	5.36±0.77 ^{BCb}	1.70±0.40 ^{Aa}	5.63±0.56 ^{CDb}
	ΔE	-	1.28±0.11 ^{BCD}	1.30±0.22 ^{A}	-	5.89±0.20 ^{DE}	3.00±0.04 ^{CDE}
AE+XI	*L**	41.85±1.13 ^{Fa}	41.55±0.24 ^{Ga}	42.98±2.33 ^{Ea}	42.10±2.11 ^{Aa}	39.05±1.77 ^{CDEa}	43.93±0.38 ^{Ga}
	*a**	-6.70±0.47 ^{Aa}	-5.95±0.29 ^{BCb}	-3.55±0.35 ^{Fc}	-1.30±0.17 ^{DEb}	-5.73±0.51 ^{Ba}	-1.28±0.05 ^{CDEb}
	*b**	2.97±0.50 ^{Ca}	2.90±0.27 ^{DEa}	5.20±0.14 ^{Fb}	6.18±1.00 ^{CDb}	2.48±0.22 ^{Ba}	6.80±1.12 ^{EFb}
	ΔE	-	0.88±0.25 ^{AB}	4.73±1.38 ^{D}	-	6.70±0.64 ^{E}	2.12±0.61 ^{B}
ACE+XI	*L**	41.20±0.80 ^{EFa}	39.88±0.15 ^{CDa}	41.30±0.89 ^{BCDa}	40.13±1.78 ^{Aa}	38.15±1.97 ^{BCDa}	40.40±1.15 ^{EFa}
	*a**	-6.65±0.40 ^{Aa}	-6.38±0.29 ^{ABa}	-4.28±0.21 ^{DEb}	-2.30±0.10 ^{BCb}	-5.63±0.75 ^{Ba}	-2.43±0.71 ^{Bb}
	*b**	2.48±0.50 ^{BCa}	1.90±0.26 ^{ABCa}	2.60±0.33 ^{BCa}	5.41±0.85 ^{BCb}	2.25±0.52 ^{ABa}	5.50±0.46 ^{CDb}
	ΔE	-	1.51±0.10 ^{CDE}	2.52±0.22$^{B\ C}$	-	5.31±0.60 ^{D}	1.18±0.33 ^{A}

[1]Numbers are means ± standard deviations from triplicate samples. Within each row for each tested type, means with the same lowercase letter are not significantly different (P > 0.05). Within each column, means with the same uppercase letter are not significantly different (P > 0.05).

6.5.7 Texture evaluation

The textural characteristics including maximum stress and Young's modulus of the sausages processed with EOs, encapsulated EOs and combined treatments of EO and irradiation are presented in Table 6.3. Maximum force during the compression showed no difference in treatments of EO, AE and ACE during storage although these values insignificantly increased in the AE and ACE samples ($p \leq 0.05$). Young's modulus was not also affected by the storage time like maximum stress. After drying, ACE samples were more elastic than other samples, but all samples adopted the similar values of elasticity after 8 weeks indicating that the antimicrobial formula treatments had no effects on hardness compared to control. The γ-irradiation had no

effect on maximum stress and young's modulus during storage. It should be noted there were some fluctuations in elasticity for the irradiated samples of ACE and the control.

X-ray irradiation showed an increase of young's modulus during storage compared to the control group and also γ-irradiated samples. This effect was also observed for breaking stress. But it decreased for the X-ray irradiated samples containing encapsulated EOs. It is observed in this study that the presence of encapsulating polymers contributed to the softness of the texture. Interestingly, all samples irradiated with X-ray adopted the highest hardness in the middle of their storage (4^{th} week) and after one month they followed a decreasing trend which is indicative of vital role of aging for processed sausages. EO, AE and ACE samples treated with γ-ray groups had no difference ($p > 0.05$) on maximum stress and modulus compared to non-irradiated counterpart groups at 8^{th} week of storage after drying. AE-XI and ACE-XI showed higher modulus values than AE and ACE groups at the end of drying. X-ray treated antimicrobial groups also showed no difference on modulus at the 8^{th} week compared to control. Non-irradiated groups all showed stable maximum stress and young's modulus during storage. Groups treated with γ-ray and X-ray also showed stable maximum stress during storage.

The hardness of X-ray treated samples were significantly higher ($p \leq 0.05$) than control and γ-ray treated samples initially, but differences reduced during storage. In a study conducted by Houben & van't Hooft (2005), the role of storage time in reducing of maximum stress and Young's modulus values was confirmed. Irradiation of beef can induce oxidative conditions and promote protein oxidation, leading to myofibril protein denaturation and aggregation, and loss of proteolytic enzyme activity (S. Y. Kim *et al.*, 2018). It has been also observed that calpain-1 (a proteolytic enzyme found in meat that is the major enzyme for the degradation of myofibrillar proteins and contributes to the development of meat softness) has a low degree of autolysis, and the enzyme activity is less extensive in samples irradiated by X-rays (S. Y. Kim *et al.*, 2018). Also, degradation fragments of calpain-14-like were found in prok muscles at 3 kGy γ-irradiation and a complete inactivation of calpains may occur at higher dose of γ-irradiation (≥ 5 kGy) (M. Zhang *et al.*, 2020). In present test, X-ray irradiation may affect more calpain autolysis activity than γ-ray that resulted in higher hardness. It may also relate to higher lipid oxidation induced by X-ray compared to γ-ray. The products of the biochemical reactions directly responsible for myoglobin oxidation and lipid oxidation can further accelerate the oxidation in a mutual manner (Faustman, Sun, Mancini, & Suman, 2010). Protein oxidation in muscles is related to many factors, including transition metal ions and oxidases (Jia, Shi, Zhang, Shi, & Chu, 2021). Compared with the primary products of lipid oxidation (such as hydroperoxides), amino acids are more susceptible to damage by

secondary products of lipid oxidation (such as aldehydes). Secondary products from lipid oxidation can interact with amino acid residues of proteins which can affect protein structure and function (Zhang, Xiao, & Ahn, 2013). The irradiation of vacuum-packaged beef was able to reduce more lipid oxidation and discoloration, which avoids the unacceptable radiation changes due to the presence of oxygen during the irradiation process (Rodrigues *et al.*, 2020). As known, oxidative stress caused by irradiation is very obvious in meat products. However, few studies have been conducted about the effect of irradiation on the oxidation of muscle proteins from the perspective of proteomics and its potential contribution to the development of softness during further storage (M. Zhang *et al.*, 2020). Also, the effect of low-to-medium-dose irradiation on the quality of dry fermented beef products is not yet clear, because most studies have been conducted on ground beef, which responds differently to irradiation in terms of lipid oxidation and color changes.

Table 6.3 Max stress (MPa) and Young's modulus (MPa) of sausages measured during storage at room temperature[1].

	Max stress (MPa)			Young's modulus (MPa)		
Samples	After drying	4weeks	8weeks	After drying	4weeks	8weeks
CT	0.26±0.03 ^{CDEa}	0.21±0.05 ^{Aa}	0.23±0.01 ^{Ba}	7.04±0.91 ^{DEa}	5.18±0.94 ^{ABa}	5.57±1.07 ^{ABa}
EO	0.21±0.03 ^{ABCDa}	0.27±0.01 ^{ABCa}	0.28±0.03 ^{BCDa}	5.55±0.73 ^{BCDa}	5.4±0.19 ^{ABa}	5.57±0.10 ^{ABa}
AE	0.24±0.01 ^{BCDEa}	0.29±0.04 ^{BCa}	0.31±0.06 ^{DEa}	6.16±0.80 ^{BCDa}	7.20±1.31 ^{BCa}	5.45±0.04 ^{ABa}
ACE	0.20±0.01 ^{ABa}	0.28±0.07 ^{ABCa}	0.31±0.07 ^{DEa}	4.47±0.65 ^{ABa}	7.98±1.24 ^{CDb}	5.30±1.15 ^{ABa}
CT+GI	0.27±0.04 ^{CDEa}	0.24±0.01 ^{ABa}	0.18±0.04 ^{Aa}	5.97±0.36 ^{BCDc}	3.56±0.34 ^{Aa}	4.48±0.14 ^{Ab}
EO+GI	0.21±0.00 ^{ABCa}	0.30±0.07 ^{BCa}	0.31±0.04 ^{CDEa}	4.46±0.27 ^{ABa}	7.09±0.93 ^{BCa}	5.76±1.15 ^{ABa}
AE+GI	0.27±0.06 ^{DEa}	0.25±0.03 ^{ABCa}	0.30±0.02 ^{CDEa}	6.70±0.64 ^{CDEa}	5.97±0.31 ^{BCa}	5.43±0.26 ^{ABa}
ACE+GI	0.15±0.01 ^{Aa}	0.31±0.06 ^{Ca}	0.28±0.06 ^{BCDa}	3.31±0.51 ^{Aa}	5.80±0.82 ^{BCb}	5.63±0.52 ^{ABb}
CT+XI	0.30±0.01 ^{Ea}	0.41±0.05 ^{Db}	0.23±0.03 ^{Ba}	10.43±1.18 ^{Fb}	14.85±0.21 ^{Ec}	8.18±1.55 ^{Ca}
EO+XI	0.37±0.05 ^{Fa}	0.50±0.02 ^{Eb}	0.46±0.02 ^{Fb}	10.05±1.85 ^{Fa}	13.35±3.06 ^{Ea}	6.68±0.85 ^{BCa}
AE+XI	0.25±0.03 ^{BCDEa}	0.45±0.02 ^{DEc}	0.36±0.02 ^{Eb}	8.22±1.38 ^{Eb}	12.82±2.42 ^{Ec}	4.46±0.32 ^{Aa}
ACE+XI	0.21±0.04 ^{ABCa}	0.29±0.01 ^{BCc}	0.25±0.01 ^{BCb}	4.97±0.60 ^{ABCa}	9.45±0.61 ^{Db}	5.97±0.68 ^{ABa}

[1]Numbers are means ± standard deviations from triplicate samples. Within each row for each tested type, means with the same lowercase letter are not significantly different (P > 0.05). Within each column, means with the same uppercase letter are not significantly different (P > 0.05)

6.6 Conclusion

Encapsulation of alginate and alginate-CNC didn't show any considerable effect on microbial quality of dry fermented sausages. Synergistic effects were observed for EOs or EOs encapsulated combined with 1.5 kGy γ-irradiation against *E. coli* O157:H7 cocktail and lactic acid bacteria, and when combined with 1.5 kGy X-ray against *E. coli* O157:H7 cocktail. Combined treatments all showed strong inhibition on *L. monocytogenes*, molds and yeasts, and total mesophilic flora. Encapsulation contributed to the sausage color protection during storage. The combined treatments with γ-irradiation didn't affect the physicochemical quality of sausages. When EO formulas combined with X-ray, a reduction of redness was observed and higher hardness was noticed at initial time of storage but aging diminished the observed differences. Therefore, this research provides new methods of combining EOs or EOs encapsulated and γ-ray (6.37 kGy/h) or X-ray (0.76 kGy/h) irradiation on the preservation of dry fermented sausages for food industries.

6.7 Declaration of competing interest

All authors state that there is no conflict of interest.

6.8 Acknowledgments

The authors are grateful for the financial support from Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery no: RGPIN-2017-05947, NSERC grant with Amsellem no. RDCPJ 531475-18, Ministère de l'Économie et de l'Innovation (MEI) of Germany no: PSO-I-2-44530, a chair granted by Quebec Ministry of Agriculture, Fishery and Food (MAPAQ) no: PPIA 12, and International Atomic Energy Agency (IAEA) Agreement No: 24359. Nordion is also acknowledged for irradiation treatment.

7 GENERAL DISCUSSION AND CONCLUSION

In the present study, eight EOs (mustard, thyme thymol, garlic, oregano compact, Chinese cinnamon, cinnamon bark, Red bergamot, Winter Savory) against nine bacteria (*E. coli* O157:H7 RM1239, *E. coli* O157:H7 RM 1931, *E. coli* O157:H7 RM 1933, *E. coli* O157:H7 RM 1934, *E. coli* O157:H7 380-94, *Listeria monocytogenes* LM 1045, *Listeria innocua* ATCC 51742, *Salmonella* typhimurium SL 1344, *Salmonella enterica Newport* ATCC 6962) and two molds (*Penicillium chrysogenum* ATCC 10106, *Aspergillus niger* ATCC 1015) were firstly tested the antimicrobial activity by MIC. Then five EOs showing good inhibition to bacteria or fungi were selected for conducting the factorial design. The FIC method was conducted based on the selected formulas from the step of factorail design to verify the synergism and optimize the concentrations of formulas. The final formula developed was then encapsulated in Alginate-CNC microbeads applied in dry fermented sausages combined to γ-irradiation for enhancing the pathogen inhibition and extending the shelf life stored at room temperature. The effects of different irradiation sources of γ-ray and X-ray on microbial and physicochemical properties were also invested.

7.1 Research discussion

The meat industry is the largest sector of food processing in Canada. According to Statistics Canada, the meat and poultry market is estimated at more than $ 4.6 billion in 2013, including $ 390 million spent on processed products such as processed meats. Meat product manufacturing is a sub-sector accounting for more than 26% of total income from manufactured goods. It is also the largest sub-sector in terms of labor and value-added. According to Agriculture and Agri-Food Canada, there are more than 1,000 meat industries in Canada. Exports of meat products also exceed $ 6.9 billion and 48% of products are exported to the United States. A 12% increase in sales has been observed over the last 8 years and shows an annual consumption / person in Canada of 200 pounds / year.

The COVID-19 pandemic has generated a new era in the world and the consequences in different aspects affect our daily life (Rizou *et al.*, 2020). Food insecurity, a well-recognized determinant of chronic disease morbidity and mortality, is highly far-flung in the United States and has increased sharply due to the social-distancing policies and economic disruption brought on by the coronavirus 2019 (COVID-19) pandemic (Leddy *et al.*, 2020). This pandemic poses several major threats to food access, food availability and stability including declined food production, supply chain disruptions, trade restrictions, and intake of diets and nutrition (Laborde *et al.*, 2020).

To be noticed, meat is one of the nutrient-dense food kinds that reduced in household food consumption. This decreased micronutrient consumption, which has a lasting adverse effect on human health and development. Compared with less damaged staple foods, the supply of fruits, vegetables, milk and meat products is more disrupted, which exacerbates the reduction in food consumption associated with these incomes, especially for poor households (Laborde *et al.*, 2020). A severe pandemic causing more than a 25% reduction in labor availability can lead to significant food shortages across the globe (Galanakis, 2020). The lockdown limits the hampered food transportation across provinces and countries, perishable foods consumption and food supply chain (Rizou *et al.*, 2020). This situation forces more measures on food safety and reduction of food loss. Global food losses and waste estimated at 1.3 billion tons per year (FOOD, 2016). Global foodborne and waterborne diarrheal diseases kill about 2.2 million people annually and foodborne zoonotic diseases is a significant and widespread global public health threat with more than 320,000 human reported cases each year only in the European Union (Ben Fadhel *et al.*, 2016).

EOs are the secondary metabolites originated from aromatic and medicinal plants (Burt, 2004). EOs are also perfect for meeting the recent consumer's demand for all-natural and clean-label additives for food safety and quality (Aminzare *et al.*, 2016). A variety of active constituents (e.g., terpenes, terpenoids, carotenoids, coumarins, curcumins) in EOs have determined the great antibacterial, bactericidal, antifungal and food preservative properties of EOs (Hyldgaard *et al.*, 2012). Therefore, the various properties of EOs propose the potential of using EOs as natural, safe, eco-friendly, cost-effective, renewable, and easily biodegradable antimicrobials for future food commodity preservation (Pandey *et al.*, 2017).

Encapsulation is a technology that protects EOs by action of one or more wall materials that could avoid direct interaction with food components and increase the effectiveness of EOs (Barbosa *et al.*, 2021; Gómez *et al.*, 2018), conduct a control release and mask unpleasant odors to decrease the sensory impact on foods (Gulin-Sarfraz *et al.*, 2021; Nazzaro *et al.*, 2012). Not much research was published on microencapsulation of natural antimicrobial compounds as potential natural additives in food. However, the significant food preservative properties arouse the worldwide interests and possibility of more commercial use of microencapsulated antimicrobial in controlling microorganism activity in foods (Castro-Rosas *et al.*, 2017).

The concept of hurdle technology (often referred to combined methods, combination preservation, combined processes, barrier technology or combination techniques) has become a promising technology that can simultaneously maintain nutritional and sensory quality and

improve food safety (Khan *et al.*, 2017). Therefore, the hurdle technology aims to improve the overall quality of food and reduce the high processing intensity (Leistner, 2000). In addition, the hurdle technology shows a synergistic effect, using multiple mechanisms to inhibit or inactivate target microorganisms (Arya *et al.*, 2018).

Emerging decontamination technologies are expected to provide safe, high-quality food. Many problems have been discovered that have delayed the widespread application of these technologies, including huge investment costs and lack of appropriate regulations. Novel food processing technologies such as pulsed electric field, food irradiation, electrolyzed water, ohmic heating, high pressure processing, organic acids, antimicrobials can reduce emissions, reduce energy consumption, improve reliability, productivity and product quality (Arya *et al.*, 2018; Khan *et al.*, 2017).

The combination of two or more scaffolding technologies can reduce processing costs, the amount of disinfectant, minimize the impact of processing on food, and most importantly extend the shelf life (Ishaq *et al.*, 2021). Methods of using combination strategies in the food industry have been explored, however, further work is needed to establish appropriate combinations of different processing methods to reduce the intensity of individual unit processes and improve the overall performance of the food (Khan *et al.*, 2017).

In this study, the EO formula development based on a factorial design method. Design of Experiments (DOE) is a method used to study any response that varies with one or more independent variables or knobs. By observing the response under the planned knob setting matrix, a statistically valid mathematical model of the response can be determined. The resulting model can be used for multiple purposes: select the best level for the knob; focus on key knobs and eliminate the interference caused by smaller or insignificant knobs; provide response prediction under various knob settings; identify the response sensitivity to tricky knobs and the interaction between knobs; and many more. Obviously, DOE is an indispensable tool for studying complex systems. It is a common practice for studying one variable at a time (OVAT) (Mathews, 2005; N. Politis *et al.*, 2017). In the past 20 years, the application of DOE in manufacturing and non-manufacturing has been growing rapidly. It is the most popular tool in science fields such as medicine, engineering, biochemistry, physics, computer science, etc. (Durakovic, 2017).

From the DOE design, the most potential synergistic EO combinations were selected from a large number of combinations for verification by FIC method, which maximized the quantity of EOs and EO combinations but minimized the time period and amount of combinations for FIC as FIC is a time consuming method (Hyldgaard *et al.*, 2012). This allows the study of larger amount

of compounds in a shorter time compared to traditional method. Thus, the use of "design of experiment" (DOE) is a revolutionary method for optimizing and screening experimental parameters. Simple experimental design and statistical tools for data analysis can provide a wealth of information about the system under study in just a few experiments. This information is important to further experimental decision-making, and can develop robust and reliable protocols for chemical synthesis, analytical methods, or biological analysis (Tye, 2004).

In the *in vitro* research, by comparing both the factorial effects and FIC index of selected interactions, some interactions were not significantly efficient in factorial effects but still showed an addition in the FIC test. Also, the significant factorial effects could show no interactive effects in the checkerboard. For example, pregano*cinnamon bark didn't present significance against *L. monocytogenes* but showed additive effect while no interaction to *E. coli* O157:H7 RM 1931, which showed 99.9% confidence interval significance. This actually offers proof that there are no strict corresponding relationships between factorial effects and synergy effects. However, all the selected interactions showed additive effects to all the targeted pathogens except oregano*cinnamon bark which presented no interactive effects against three *E. coli* strains, but these FIC values are still as low as close to 1. This fact gives the factorial design a great potential to largely reveal the synergy effects of two- and more than two-factor interactions as a synergistic screening method. In summary, the potential synergistic or additive combinations from Factorial design showed mostly additive effects in the checkerboard method, which allocates the feasibility of using factorial design for rapidly synergistic screening on various antimicrobial mixtures for formula development.

Gamma irradiation is an efficient food decontamination method. A dose of 3 kGy γ-irradiation can be applied to meat without alterations of sensory properties (Lacroix, 2014). Combining irradiation with other food preservation methods can lower the dose of irradiation, keep the stability of organoleptic and nutritional properties of food, increase radiosensitization of microorganisms and extend the shelf life of food (Ben Fadhel *et al.*, 2016; Lacroix, 2014). X-rays that have high penetrating power and no left radiation hazards are raising interests as low risk-significant radioactive sources (Indiarto & Qonit, 2020). However, less studies have been done in use of X-ray for food pasteurization (Begum *et al.*, 2020). The mechanism of ionizing radiation is mainly damage to nucleic acids, interruption of chemical bonds in DNA, or direct or indirect damage caused by oxidative free radicals generated by water radiation decomposition (Lacroix, 2014).

In the *in situ* research combined with γ-irradiation, results showed that EOs, EOs encapsulated in alginate and EOs encapsulated in alginate-CNC have similar inhibition effects to the resistant *E. coli* cocktail under dry and acidity condition. EOs and EOs encapsulated in alginate-CNC showed stronger inhibition effects to *L. monocytogenes* than EOs encapsulated in alginate without CNC. Antimicrobials combined with 1.5 kGy γ-irradiation have inhibited all three microorganisms below the detection limit at the end of drying. All three antimicrobials also showed synergetic effects when combined with 1.5 kGy γ-irradiation against *E. coli* O157:H7 cocktail right after irradiation and until 1-week storage. EOs combined with 1.5 kGy group has also showed synergistic effects until 2-weeks storage. EOs free and EOs encapsulated in alginate-CNC have similar effects on LAB counts until 1-week storage and TMF until 8-week storage. From 2nd week to 8th week storage, the ACE+GI group showed less LAB counts than EO+GI group while from 12th week to 20th week EO+GI group showed less LAB and TMF counts. All three antimicrobials combined to 1.5 kGy γ-irradiation showed synergistic effects on LAB after drying until 6 weeks' storage except non-encapsulated EOs irradiated at 1.5 kGy at 6th week storage. Irradiation contributed significantly in the inhibition of LAB and TMF. Also the combined treatments did not cause significant changes on texture and results showed the encapsulation contributed to the color protection of sausages. This research can be of great interests to food industry for food preservation and this work presents a new method of combined treatments applied on DFS during whole ripening and 20 weeks storage.

Several research have reported synergistic effects when combined encapsualted EOs with γ-irradiation. Research on microencapsulation by Huq *et al.* (2015) revealed synergistic antimicrobial effect on ready-to-eat meat products during storage by combing microencapsulated essential oils-nisin and γ-irradiation. Microencapsulated cinnamon and nisin in alginate-CNC combined with γ-irradiation at 1.5 kGy reduced 0.14 ln CFU/g/day growth rate of *L. monocytogenes* that significantly ($P \leq 0.05$) improved the radiosensitivity of *L. monocytogenes*. Microencapsulated oregano and cinnamon essential oil with nisin showed the highest bacterial radiosensitization of 2.89 and 5 times to the control separately. The combination treatments of gamma irradiation at doses of 1, 3, and 5 kGy and edible rosemary essential oil coating improved the quality and safety of silver carp fish fillets through by eliminating bacteria and extending the refrigerated shelf life up to 24 days compared to 6 days for uncoated control samples without affecting chemical and sensory properties of fillets negatively (Abdeldaiem *et al.*, 2018). Dini *et al.* (2020) has reported the combination of chitosan (Ch), cumin essential oil nanoemulsion (CNE), gamma irradiation (GI) was the most effective treatment to control the population of microbial flora and inoculated pathogens, slow

down some certain physicochemical changes, and therefore extend the storage period of beef loins. Criado *et al.* (2019) observed that thyme loaded alginate beads and gamma irradiation showed synergistic effect against *L. innocua* and mesophilic total flora. Irradiation at 3 kGy with the active alginate beads had a complete inhibition of Listeria from day 0. Synergistic effect was also observed when the irradiated at 1 kGy combined with thyme EO loaded alginate beads applied in ground meat, which extended the shelf-life of meat 12 days more than the control.

In the study of comparision of γ-ray and X-ray, free EOs and encapsulated EOs in alginate or alginate-CNC combined with irradiation at a dose of 1.5 kGy using cobalt-60 γ-rays (1.17 and 1.33 MeV) with a dose rate of 6.37 kGy/h as well as low-energy (125 keV) X-rays with a dose rate of 0.76 kGy/h were applied on DFS to compare the effects of γ-ray at high dose rate of irradiation with X-ray irradiation at low dose rate of irradiation on microbial and physicochemical properties of DFS during storage. The results are expected to provide a useful reference for reasonable application of two types of irradiation in combination with EOs, and encapsulation on fermented and dry sausage.

Molds and yeasts showed to be more resistance to X-ray and γ-ray irradiation than *L. monocytogenes* and *E. coli* O157:H7 cocktail in this study. But the introduction of EO into the product made increased the sensitivity of molds and yeasts implying more susceptibility of molds and yeasts to the applied EOs than irradiation. Other literatures are in parallel with our findings. *A. niger* showed more resistance to X-ray at 0.76 kGy/h than γ-ray at 0.085, 4.558 and 10.445 kGy/h (Shankar *et al.*, 2020). However, when X-ray (0.76 kGy/h) was combined with EOs, the lowest radio sensitivity was observed for *A. niger* similar to γ-ray (10.445 kGy/h) combined with EOs.

Generally, the color of irradiated meat products can vary depending on the radiation source, radiation dose, animal species of raw meat, muscle type, packaging type, and myoglobin concentration (Ham *et al.*, 2017). Nitrosohemoglobin is usually the main pigment that causes redness in meat products containing nitrite (Ham *et al.*, 2017). The effect of irradiation on the color change of fermented sausages has not been determined. It is thought that the reduction in redness during storage may be due to the destruction of nitrosoheme by irradiation (I. S. Kim *et al.*, 2012). On the other hand, phenolic compounds can interrupt the oxidation reaction of irradiated meat by providing hydrogen atoms or quenching free radicals, thereby avoiding the color and texture changes caused by irradiation (Ahn, Kim, & Lee, 2013). Ben Fadhel *et al.* (2016) observed that 1 kGy γ-irradiation caused a reduction in *a** value which was indicative of a significant greenness of fresh pork meat. But the green pigments were not stable when the

irradiation doses increased to 1.5 and 3 kGy, while the red color related to the formation of heme pigment-CO ligand was more stable. These results are consistent with what was found with 1.5 kGy γ-irradiation on redness of DFS. According to I. S. Kim *et al.* (2012), redness of beef sausage patties was not affected by γ-irradiation lower than 1 kGy but decreased during storage when irradiation increased to 2 and 4 kGy. Song *et al.* (2017) observed similar *a** values after γ- and X-ray irradiation for low-salt sausages although they significantly lowered at the end of refrigerated storage.

Samples irradiated with X-ray exhibited a reduction in redness accompanied by more greenness compared to the control and γ-irradiated samples. Color changes may be due to the intrinsic sensitivity of myoglobin molecules to the energy caused by irradiation (Ben Fadhel *et al.*, 2016). Myoglobin can be bound to oxygen to form bright red oxygenated myoglobin. After irradiation, free binding sites can react with free radicals such as hydroxyl (-OH) and sulphuryl (-SH) radicals to form metmyoglobin (brown) and thiomyoglobin (green), respectively (Ouattara, Giroux, Smoragiewicz, Saucier, & Lacroix, 2002). Besides, meat color stability depends on the residual enzymatic activity in meat which controls myoglobin oxygenation, oxidation and reduction (Rodrigues *et al.*, 2020). Rodrigues *et al.* (2020) observed the reduction of *a** values in parallel with oxymyoglobin loss and metmyoglobin increase at high doses of γ-irradiation, owing to the increased lipid oxidation and reduced metmyoglobin reducing capacity and oxygen consumption rates due to irradiation. Hydroxyl radicals produced by ionizing radiation are considered to be a factor in accelerating lipid oxidation, which adversely affects the color, flavor, texture and nutritional value of meat (Ham *et al.*, 2017; Park *et al.*, 2010). Therefore, the lipid oxidation reinforces meat discoloration (Faustman, Sun, Mancini, & Suman, 2010). Ham *et al.* (2017) studied the effects of three different irradiation sources of γ-ray, electron-beam, and X-ray at 0, 2.5, 5, 7.5, and 10 kGy on the quality of cooked beef patties and pork sausages during 10 days of storage at 30±1°C. X-ray irradiation resulted in significantly higher TBARS values than γ-ray, while X-ray resulted in lower *a** values for all doses applied on pork sausages than γ-ray. The *a** value of beef was observed initially lower in X-ray irradiated samples, but the difference was disappeared with extended storage (S. Y. Kim *et al.*, 2018).

The hardness of X-ray treated samples were significantly higher ($p \leq 0.05$) than control and γ-ray treated samples initially, but differences reduced during storage. In a study conducted by Houben & van't Hooft (2005), the role of storage time in reducing of maximum stress and Young's modulus values was confirmed. Irradiation of beef can induce oxidative conditions and promote protein oxidation, leading to myofibril protein denaturation and aggregation, and loss of proteolytic

enzyme activity (S. Y. Kim *et al.*, 2018). It has been also observed that calpain-1 (a proteolytic enzyme found in meat that is the major enzyme for the degradation of myofibrillar proteins and contributes to the development of meat softness) has a low degree of autolysis, and the enzyme activity is less extensive in samples irradiated by X-rays (S. Y. Kim *et al.*, 2018). Also, degradation fragments of calpain-14-like were found in prok muscles at 3 kGy γ-irradiation and a complete inactivation of calpains may occur at higher dose of γ-irradiation (≥ 5 kGy) (M. Zhang *et al.*, 2020). In present test, X-ray irradiation may affect more calpain autolysis activity than γ-ray that resulted in higher hardness. It may also relate to higher lipid oxidation induced by X-ray compared to γ-ray. The products of the biochemical reactions directly responsible for myoglobin oxidation and lipid oxidation can further accelerate the oxidation in a mutual manner (Faustman, Sun, Mancini, & Suman, 2010). Protein oxidation in muscles is related to many factors, including transition metal ions and oxidases (Jia, Shi, Zhang, Shi, & Chu, 2021). Compared with the primary products of lipid oxidation (such as hydroperoxides), amino acids are more susceptible to damage by secondary products of lipid oxidation (such as aldehydes). Secondary products from lipid oxidation can interact with amino acid residues of proteins which can affect protein structure and function (Zhang, Xiao, & Ahn, 2013). The irradiation of vacuum-packaged beef was able to reduce more lipid oxidation and discoloration, which avoids the unacceptable radiation changes due to the presence of oxygen during the irradiation process (Rodrigues *et al.*, 2020). As known, oxidative stress caused by irradiation is very obvious in meat products. However, few studies have been conducted about the effect of irradiation on the oxidation of muscle proteins from the perspective of proteomics and its potential contribution to the development of softness during further storage (M. Zhang *et al.*, 2020). Also, the effect of low-to-medium-dose irradiation on the quality of dry fermented beef products is not yet clear, because most studies have been conducted on ground beef, which responds differently to irradiation in terms of lipid oxidation and color changes.

The innovation of the research is the development of formulation that can eliminate resistant *E. coli* to low Aw and high salt concentration; the mode of encapsulation who permits to prolong the activity of the formulations during storage, and then the development of new combined treatments of irradiation including low dose rate with less energy of irradiation.

7.2 Limits of work and future aspects

The scanning methodology was developed for checking potential synergy of a larger amount EOs. In this study as results observed in the first article, the EOs selected according to lower MIC

values for factorial design are all with strong antimicrobial activities, however due to the interaction effects of factorial design, the significant effects that inhibit most microorganisms were concentrated in binary combinations. It could be possible that single EO or binary combination always have the most significant effects when scanning quantities of EOs and microorganisms. Because an interaction of more than two EO combinations is harder to get synergistic effects according to the FIC index equation which calculates addition than binary compounds. The design method as a basic step for FIC in this study only verified with some certain binary combinations, the limit could be some other combinations left unverified that maybe not in close correspondence with results of FIC. The future research could be a further verification of the two methods and set up a sturdy link between the two methods for fast checking synergistic effects.

For the microencapsulation of developed EO formula applied in dry fermented sausages, the microbial results are showed high inhibitory to microbial counts especially when combined with irradiation. The interior and exterior color measures were hard to analyze the effects of different treatments. This could be due to the sausage model used in this study is smallest type in industry that the diameter is 18 mm. The area of cross section also the unhomogized meat particles of sausages such as fat, muscle fiber etc. may limit the accuracy of color tests. Also, the EOs in encapsulation didn't show significant higher inhibition during storage. This result could be related to the certain food model as DFS have specific manufacturing process with fermentation and drying. The high humidity during fermentation may affect the swelling of microbeads in sausages. For the future expects, the structure of EO-alginate-CNC microbeads could be studied, the releasing kineticism of EOs also worth to be identified, and the acting mechanism of microbeads within DFS is also unclear. Also, further research should be conducted clearly to determine the effect of different irradiation sources and dose levels on the decontamination efficiency, nitrosohemoglobin formation, and endogenous enzyme stability in dry fermented and irradiated meat products.

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