UNIVERSITY OF MODENA AND REGGIO EMILIA

Department of Life Sciences

PhD School in Food and agricultural science, technologies, and biotechnologies

Use of essential oils, bacteriocins and active edible coatings: an innovative and natural approach for the control of *Listeria monocytogenes* in fresh foods

XXXIV CYCLE

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- Titolo tesi in italiano (max 1800 caratteri, spazi compresi);
“Utilizzo di oli essenziali, batteriocine e coating commestibili attivi: un approccio innovativo e naturale per il controllo di Listeria monocytogenes in alimenti freschi”

- Titolo tesi in lingua inglese (max 1800 caratteri, spazi compresi);
“Use of essential oils, bacteriocins and active edible coatings: an innovative and natural approach for the control of Listeria monocytogenes in fresh food”

- Abstract in italiano (max 3800 caratteri, spazi compresi);
I coating commestibili sono un nuovo metodo di conservazione ecosostenibile basato sull’utilizzo di scarti generati dall’industria alimentare che, addizionati di sostanze naturali come oli essenziali e/o batteriocine, possono migliorare le proprietà igieniche e sensoriali e del prodotto. Le infezioni di origine alimentare dovute a batteri patogeni come Listeria monocytogenes, responsabile di oltre il 90% di tutti i casi di intossicazione alimentare, rimangono un grave problema clinico, e l'impiego di additivi chimici è sempre meno accettato dai consumatori e soprattutto limitato da leggi restrittive. L'innovazione consiste nell'incorporazione di oli essenziali (EOs) e/o batteriocine, nei materiali di imballaggio sia per il controllo degli agenti patogeni che per il mantenimento o l'estensione della durata di conservazione del prodotto.
Nel presente studio sono state valutate le proprietà anti-L. monocytogenes di entrambi i suddetti composti antibatterici naturali, scelti sulla base dei risultati ottenuti in precedenti studi. Gli EOs sono stati scelti tra quelli che si sono dimostrati più attivi nei confronti di patogeni alimentari isolati da alimenti o di collezione, mentre la nuova batteriocina bacLP17 utilizzata nel presente studio è stata prodotta dal ceppo batteriocinogenico LAB Enterococcus mundtii, isolato in una precedente indagine nel laboratorio di Microbiologia Applicata (Dipartimento di Scienze della Vita – Università di Modena e Reggio Emilia). Dopo approfondita valutazione dei composti lo studio è stato suddiviso in 2 fasi:
1. Valutazione “in vitro” dell’attività antimicrobica di EOs/ bacLP17, usati da soli o in associazione, nei confronti di L. monocytogenes sia in forma planctonica che sessile: il Disk Diffusion, MIC e Agar Well Diffusion sono stati utilizzati per valutare l'efficacia dei composti contro 12 ceppi di L. monocytogenes in forma planctonica, mentre l'attività anti-biofilm è stata determinata in densità ottica a 570 nm. I valori di MIC più bassi sono risultati per T. vulgaris e bacLp17 (0,5 ml/ml e 2 ml/ml). I migliori risultati, espressi come FIC-Index, sono stati osservati per T. vulgaris/S. officinalis EOs e EOs/bacLp17. Il miglior effetto anti-biofilm è stato osservato per bacLP17/S. officinalis e bacLP17/T. vulgaris, rispetto sia al controllo che all'uso singolo dei composti naturali.
2. Studio dell’attività anti-L. monocytogenes di un coating commestibile addizionato con composti naturali su campioni di gamberetti freschi artificialmente contaminati: Campioni di gamberetti (44) sono stati contaminati mediante l’inoculazione con una siringa Hamilton con circa 106 CFU/mL ed i coatings applicati per immersione su tutti i campioni.
Parte A. Valutazione dell’attività anti-L. monocytogenes di un coating commestibile addizionato di EOs: quattro oli essenziali (S. officinalis, M. piperita, C. limon, T. vulgaris), usati da soli e in combinazione, sono stati testati contro L. monocytogenes NCTC 10888 in gamberetti contaminati artificialmente. Tutti gli EOs sono risultati

attivi contro L. monocytogenes e la migliore attività è stata osservata per T. vulgaris. Inoltre, entro il periodo di shelf-life tutti i campioni hanno mostrato proprietà sensoriali accettabili fino a 5 e 10 giorni.
Parte B. Valutazione dell'attività anti-L. monocytogenes di EOs /bacLP17 addizionati ad un coating commestibile: i coatings commestibili con diverse concentrazioni dei quattro EO da soli e in combinazione, sono stati applicati su tutti i campioni. Tutti i composti naturali e le loro combinazioni hanno confermato la loro attività contro L. monocytogenes anche quando incorporati nei coatings e la migliore attività è stata osservata con la combinazione bacLP17/S. officinalis.
Saranno tuttavia necessari ulteriori studi per migliorare le prospettive di questi coating commestibili attivi per future applicazioni nell'industria alimentare.

- Abstract in lingua inglese (max 3800 caratteri, spazi compresi);

Edible coatings are a new eco-sustainable conservation method based on the use of waste generated by the food industry which, with the addition of natural substances such as essential oils and / or bacteriocins, can improve the hygienic and sensory properties of the product. Foodborne infections due to bacterial pathogens like Listeria monocytogenes, responsible for over 90% of all cases of food poisoning, remain a serious health concern, and the employment of chemical additives is less and less accepted by the consumers and limited by restrictive laws. The innovation is the incorporation of natural substances like EOs and/or bacteriocins in packaging materials for both the pathogens control and the maintenance and extension of the product shelf life. In the present study, the anti-L. monocytogenes properties of both of the aforementioned natural antibacterial compounds, chosen on the basis of the results obtained in previous studies, were evaluated. The EOs were selected from those that proved to be more active against foodborne pathogens isolated from food or of collection, while the new bacLP17 bacteriocin used in the present study was produced by the bacteriocinogenic LAB Enterococcus mundtii, isolated in a previous investigation in the Applied Microbiology laboratory (Department of Life Sciences - University of Modena and Reggio Emilia). After a thorough evaluation of the compounds, the study was divided into 2 phases:
1. "In vitro" evaluation of the antimicrobial activity of EOs / bacLP17, used alone or in combination, against L. monocytogenes in both planktonic and sessile form: the Disk Diffusion, MIC and Agar Well Diffusion assays were used to evaluate the effectiveness of the compounds against 12 L. monocytogenes in planktonic form, whereas the anti-biofilm activity was determined in optical density at 570 nm, with crystal violet staining method. The lowest MIC values resulted for T. vulgaris and bacLp17 (0.5 ml/ml and 2 ml/ml, respectively). The combinations with the best results, expressed as FIC-Index, were T. vulgaris/S. officinalis EOs and EOs/bacLp17. The best anti-biofilm effect was observed with the combination bacLP17/S. officinalis and bacLP17/T. vulgaris, compared to both control and the single use of the natural compounds.
2. Study of the anti-L. monocytogenes activity of edible coatings added with the natural compounds and carried out on artificially contaminated shrimp's samples
Shrimps samples (44) were inoculated with a Hamilton syringe with approximately 106 CFU/mL and coatings applied to all samples by dipping.

PART A Study of the anti-L. monocytogenes activity of edible coatings added with EOs: four essential oils (S. officinalis, M. piperita, C. limon, T. vulgaris), added to the coating alone and in combination, were tested against L. monocytogenes NCTC 10888 in artificially contaminated shrimps. All the EOs were active against L. monocytogenes, and the best activity was observed for T. vulgaris. Moreover, within the shelf-life period all samples showed acceptable sensory properties up to 5 and 10 days.
Part B Study of the anti-L. monocytogenes activity of edible coatings added with EOs/ bacLP17: edible coatings added with different concentrations of EOs/ bacLP17 were applied to all the artificially contaminated samples. All the natural compounds and their combinations confirmed their activity against L. monocytogenes even when incorporated into the coatings, and the best combination was observed for bacLP17/ S. officinalis mixture. Further studies will be however necessary to improve the perspectives of active edible coatings for future applications in the food industry.

- Lingua Tesi; Inglese
- Parole Chiave in italiano (5 parole di max 20 caratteri ciascuna e separate da uno spazio);
Batteriocine, alimenti pronti al consumo, oli essenziali, Listeria monocytogenes, Coating commestibile
- Parole Chiave in lingua inglese (5 parole di max 20 caratteri ciascuna e separate da uno spazio);
Bacteriocin, Ready-to-eat-food, Essential oil, Listeria monocytogenes, Edible coating
- Area e Settore Scientifico di attinenza della tesi; FOOD AND AGRICULTURAL SCIENCE, TECHNOLOGY AND BIOTECHNOLOGY
- Primo relatore (=tutor); Messi Patrizia
- Eventuale Correlatore (=co-tutor), da indicare solo se è stato ufficialmente nominato dal Collegio
Docenti del Corso: Sabia Carla
- Coordinatore del Corso di Dottorato: Ulrici Alessandro

1. INTRODUCTION

Foodborne diseases represent a global health threat besides the great economic losses encountered by the food industry (Heredia and García, 2018). These hazards necessitate the implementation of food preservation methods to control foodborne pathogens, the causal agents of human illnesses. Until now, most control methods rely on inhibiting the microbial growth or eliminating the pathogens by applying lethal treatments with antibiotics or chemical additives. The phenomenon of antibiotic resistance and the use of chemical additives with toxicity has prompted researchers to find natural solutions to counter the growth of pathogenic bacteria. The main bacterial pathogens in food are *Listeria monocytogenes, Staphylococcus aureus, Clostridium botulinum, Vibrio spp., Escherichia coli* O157:H7 and *Salmonella spp* (Schirone et al., 2019). To guarantee the quality and safety of food products, and to meet the consumers' requirements (e.g., for high quality, minimally processed and additive free-foods) the research has been oriented toward a bio-preservation approach. Bio-preservation is a technique of food preservation in which antimicrobial potential of selected microorganisms, their metabolites or other natural substances against spoilage and pathogenic bacteria are exploited. To meet the ever-growing skepticism of the consumer towards chemical additives, biocontrol and natural compounds for the prevention of the growth of spoilage and foodborne pathogens have emerged as novel preservation technologies. They mainly exploit the antimicrobial properties of biomolecules produced by lactic acid bacteria (bacteriocins) and of plant-derived compounds like essential oils (Kalogianni et al., 2020). Lactic Acid Bacteria (LAB) possess a major potential for use in bio-preservation. They are commonly used in food fermentation, may produce several metabolites with beneficial health effects and, thus, are generally recognized as safe (GRAS). LAB exert strong antagonist activity against many related and unrelated microorganisms, including food spoilage and pathogenic bacteria, such as *Listeria* and *Staphylococcus* spp. The antagonistic effect of LAB is due to a lowering of the pH of the food, to the competition for nutrients and to the production of inhibitory metabolites such as organic acid (lactic acid or acetic acid), hydrogen peroxide and antibacterial peptides like bacteriocins. Bacteriocins are antimicrobial peptides which act as antibacterial compounds against bacterial pathogens (Abdelhamid et al., 2020). Bacteriocins can inhibit Gram positive and Gram-negative bacteria such as *Listeria monocytogenes, Salmonella sp., Escherichia coli, Vibrio sp., Shigella sp.,*

Aeromonas sp. and *Pseudomonas sp*. Bacteriocins are good candidate as food bio-preservatives: they are resistant to heat and maintain activity in an acidic environment, and low temperature during storage does not affect bacteriocin activity. Bacteriocins can be damaged by degradation from proteolytic enzymes (Feli Feliatra et al., 2018). In recent years, there has been a growing interest in the potential use of Essential oils (EOs) in the food and cosmetics industries. Although EOs are often used in the industry as flavoring agents, these natural products possess a broad range of antimicrobial properties making them suitable for food preservation (Deyno et al., 2019). EOs are produced by plants and act in the defense against herbivores, and infections caused by microorganisms, as well as attracting pollinators, and in the plant–plant or plant–insect interactions. The discovery of inhibitory activity also against human pathogens has led to the development of numerous studies on their mode of action, and the interaction of EOs with microbial cell membranes is the main cause of the inhibition of the growth of some Gram-positive and Gram-negative bacteria, but with differences: Gram-positive bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus* are more susceptible to EOs than Gram-negative bacteria such as *Escherichia coli* and *Salmonella enteritidis* (Valdivieso-Ugarte et al., 2019). Moreover, several categories of natural antimicrobial compounds found in plant, spices, and herbs or produced by LAB have been incorporated into edible films and coatings, resulting in an improvement of their bioactive properties. Both companies and researchers have been working to develop new packaging strategies with environmentally friendly, abundant biodegradable packaging materials made from renewable natural polymers (Risch., 2000). Furthermore, the rapidly growing interest in the use of edible packaging can also be associated with a growing consumer demand for minimally processed fresh-like foods with an extended shelf life and trend in improving the quality of food with edible barriers (Gontard et al.,1994; Diab et al., 2001). Edible coatings or films may be a new green preservation method notably if based on of natural substances added to reduce the use of synthetic polymers. Therefore, bacteriocins, EOs and edible coating represent a natural promise in food preservation and in the food industry to reduce the addition of chemical preservatives, antibiotics and to reduce or eliminate the growth of pathogenic and spoilage bacteria, in particular in perishable foods like seafood products.

2. Seafood products

Fishery products are a source of valuable nutrients such as proteins, vitamins, minerals, omega-3 fatty acid, taurine etc. Fish constitutes a major part of protein consumption in many places in the world (Pilet et al., 2011). However, their nutritional composition furnishes an ideal environment for the growth and propagation of spoilage microorganisms and common food-borne pathogens (Ghanbari et al., 2013a).

2.1 Major Biological Hazard in Aquatic Food Products

The number of outbreaks attributed to seafood consumption is generally high (10-20% of the total food-borne outbreaks), but varies according to the quality of the surveillance system, the level of consumption and the consumers' habits (Pilet et al., 2011). The major bacterial hazards associated with aquatic food products is presented in Table 1. Pathogenic bacteria associated with seafood can be subdivided into three general groups: 1) indigenous bacteria that belong to the natural microflora of fish (*Aeromonas* spp., *Clostridium botulinum* and pathogenic *Vibrio* spp); 2) Enteric bacteria (non-indigenous bacteria) that are present due to faecal contamination (pathogenic *Escherichia coli*, *Salmonella* spp. and *Shigella* spp.) and 3) bacterial contamination during processing, storage or preparation for consumption (*Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, *Listeria monocytogenes*, *Staphylococcus aureus*) (Ghanbari et al., 2013). **Table 1** presents an overview on the major bacterial hazards associated with aquatic food products (from Ghanbari et al., 2013).

Bacteria	Product identified	References
Aeromonas spp.	Fish, shellfish	Fernandes, Flick, & Thomas, 1998; Isonhood & Drake, 2002
Clostridium botulinum Type E	Spores on surface, in intestine, on gills (trout, herring, salmon); vacuum packaged smoked fish products, cans, fermented fish, salted fish	Haagsma, 1991; Hatheway, 1995; Sramova & Benes, 1998; Johnson, 2000
Cl. perfringens	Cod, tuna salad, boiled salmon	Hewitt et al., 1986; Khatib et al., 1994; Aschfalk & Muller, 2002
Escherichia coli	Fresh fish, tuna paste, salted salmon roe, processed seafood	Ayulo, Machado, & Scussel, 1994; Calo-Mata et al., 2008; Asai et al., 1999; Mitsuda et al., 1998; Semanchek & Golden, 1998; Pierard et al., 1999;
Listeria monocytogenes	Ubiquitous, 3–10% human carriers; rarely in seawater or seawater fish, more frequently in freshwater and aquaculture fish, cold smoked products, salted fish products, hot smoked products, raw fish, prawns, mussels, oysters	Hoffman, Gall, Norton, & Wiedmann, 2003; Thimothe, Nightingale, Gall, Scott, & Wiedmann, 2004; Alves, De Martinis, Destro, Vogel, & Gram, 2005; Gudmundsdöttir et al., 2005; Miettinen & Wirtanen, 2005; Beaufort et al., 2007; Calo-Mata et al., 2008; Zunabovic, Domig, & Kneifel, 2011
Salmonella spp.	In intestine (tilapia and carp); prawns, mollusks, alaska pollack; eel and catfish, smoked eel, smoked halibut, dried anchovy	Heinitz, Ruble, Wagner & Tatini, 2000; Ling, Goh, Wang, Neo & Chua, 2002; Olgunoğlu, 2012
Staphylococcus aureus	Contamination from infected persons, fresh fish and fish fillets (*Cynoscion leiarchus*), smoked fish	Ayulo et al., 1994; Eklund, Peterson, Poysky, Paranjpye, & Pelroy, 2004
Vibrio parahaemolyticus	Shellfish, crustaceans on the skin, gills, intestine, fish-balls, fried mackerel (*Scomber scombrus*), tuna (*Thunnus thynnus*), and sardines (*Sardina pilchardus*),	Baffone, Pianei, Bruscolini, Barbieri, & Cierio, 2000; Calo-Mata et al., 2008; Daniels et al., 2000; IDSC, 1997
V. cholera Serovar O1 and O139	Prawns, shellfish, squid, seafood, uncooked fish marinade seviche (*Cilus gilberti*)	Kam, Leung, Ho, Ho, & Saw, 1995; Calo-Mata et al., 2008

Table 1: major bacterial hazards associated with aquatic food products (from Ghanbari et al., 2013).

2.1.1 *Aeromonas* spp.

Aeromonas are Gram-negative, rod-shaped, non-spore forming bacteria that are autochthonous and widely distributed in aquatic environments. Some strains are important fish pathogens in aquaculture, while others have been implicated in food-borne Disease (Reilly et al., 1998). Theese organisms are very frequently present in many food products, including fish, shellfish and also meats and fresh vegetables (Ghanbari et al., 2013).

2.1.2 Clostridium botulinum

Clostridium botulinum is a spore-forming, Gram-positive *bacillus* that is widespread in nature (Iwamoto et al., 2010). The bacterium produces a potent neurotoxin under anaerobic, low-acid conditions. Seven types of botulism toxin have been identified; toxin types A, B, and E cause most human illnesses (Iwamoto et al., 2010). Food-borne botulism is caused by the ingestion of food contaminated with preformed toxin produced by the spores of *C. botulinum* (Iwamoto et al., 2010). *C. botulinum* is ubiquitous in aquatic environments and has been isolated from water, ocean sediments, the intestinal tracts of fish, and the gills and viscera of crabs and other shellfish (Feldhusen, 2000). The spores can also adhere to the surface of fish. For these reasons *C. botulinum* can be found in the environment of most fish processors and cannot be totally eliminated using reasonable means (Feldhusen, 2000). In addition, even though a fish might be cleaned, gutted, and

air packaged, some risk will still exist because *C. botulinum* spores can find their way into muscle tissue during processing (Feldhusen, 2000). Muscle tissue below the surface of fish can provide an anaerobic environment where outgrowth and toxin production can occur if time and temperature permit (Feldhusen, 2000).

2.1.3 *Vibrio* spp.

Vibrio spp. are indigenous marine halophilic or halotolerant organisms that are commonly isolated from estuarine and coastal environments (Reilly et al., 1998). They are predominantly in tropical waters and can be isolated in temperate zones during the summer months (Feldhusen, 2000; Reilly et al., 1998). Three species of *Vibrio* dominate as food-borne pathogens, *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*. Other species, *V. hollisae*, *V. alginolyticus* and *V. fluvialis* have also been reported as the cause of gastroenteritis (Reilly et al., 1998). The main cause of this food poisoning is eating raw or undercooked seafood. The most affected by these pathogens are Japan, Taiwan, and other Asian coastal regions, though cases of disease have been described in many countries and on many continents. Cases of diseases caused by *V. parahaemolyticus* are occasional in Europe (Ghanbari et al., 2013).

2.1.4 Escherichia coli

Escherichia coli is often used as an indicator of fecal contamination. Some strains of *E. coli* are also capable of causing food-borne disease, ranging from mild enteritis to more serious illness and mortalities (Reilly et al., 1998). Shiga toxin-producing *E. coli* (STEC) O157:H7 infection, which can cause haemolytic uraemic syndrome and death, is a global public health concern (Feldhusen, 2000). The contamination fish-derived food with pathogenic *E. coli* probably occurs during handling of fish and during the production process (Ghanbari et al., 2013).

2.1.5 *Salmonella* spp.

Salmonella spp. are Gram-negative bacilli. Approximately 2,500 *Salmonella* serotypes have been identified, causing a variety of clinical syndromes ranging from asymptomatic carriage to invasive

disease (Iwamoto et al., 2010). *Salmonella* most commonly causes acute gastroenteritis, with symptoms including diarrhea, abdominal cramps, and fever. Other clinical manifestations can include enteric fever, urinary tract infections, bacteremia, and severe focal infections (Iwamoto et al., 2010). Fish cultured in coastal and brackish water environments can be exposed to contamination by bacteria of faecal origin resulting from disposal of sewage and land. Most outbreaks of food poisoning associated with fish derive from the consumption of raw or insufficiently heat treated fish and cross-contamination during processing.

2.1.6 Other toxin-forming bacteria

Bacillus cereus, *Clostridium perfringens* and *Staphylococcus aureus* can form enterotoxins that cause acute gastrointestinal illness. *B. cereus* and *C. perfringens* are found in the soil and are ubiquitous, but only a few reports of illness due to the presence of these organisms in seafood have been published. Certain strains of *S. aureus* produce a toxin that causes gastrointestinal illness. The main reservoir is humans, who carry the bacterium in their nasal passages, skin or wounds. *S. aureus* contamination of food, including seafood, is usually due to contamination by a food worker during food preparation (Iwamoto et al., 2010).

3. Listeria monocytogenes

Listeria species are Gram-positive bacteria widespread in both aquatic and terrestrial habitats. The genus *Listeria* is composed of six species, from which five species (*Listeria grayii*, *Listeria innocua*, *Listeria ivanovii*, *Listeria seeligeri*, and *Listeria welshimeri*) are not generally pathogenic for humans; whereas *Listeria monocytogenes* is an important opportunistic human pathogen (Razavilar et al., 1998). Listeriosis is a severe illness caused by an intracellular pathogen *L. monocytogenes* that contaminates dairy, meat, poultry, and seafood products. It is also a sporadic disease occurred mainly in pregnant women and their foetus, immunocompromised individuals, and the old people. The case-fatality rate of listeriosis (about 20–30%) is exceptionally high for a foodborne disease; and may reach more than 40% in susceptible populations (Painter et al., 2007; Todd et al., 2011). With the increased demand for lightly preserved and/or ready-to-eat (RTE) food products, the

prevalence of the foodborne pathogen *L. monocytogenes* has increased, becoming a major public health concern. Most strains of *L. monocytogenes* can tolerate or even grow at pH range of 4.1–9.6, and at salt concentrations up to 14%. *L. monocytogenes* is a psychrotrophic bacteria that can growth at refrigeration temperatures and can persist in food-processing plants and on equipment. Therefore, refrigerated ready-to-eat foods (RTEs) that do not need to be cooked or reheated before serving or that can be eaten without sufficient heat treatment are hazardous foods if contaminated with *L. monocytogenes*. These types of products include salads, vegetables, fruits, cooked meats, smoked fish, desserts, sandwiches, cheese and food that are previously cooked in order to be later served cold. The trend towards the consumption of minimally processed, ready-to-eat, chilled and frozen food products poses new problems in identifying and managing bacterial risks for consumers. For a psychrotrophic pathogen, like *L. monocytogenes*, the extended shelf-life of RTE foods in refrigerated environment provide time for multiplication at numbers above 100 CFU/g, bacterial load considered a potential health hazard particularly in susceptible populations (Ooi et al., 2005). With regard seafood products, although its natural niche is probably soil and vegetation, it can readily be isolated from fresh and marine waters. Therefore, these microorganisms are most likely present on the external surface of fish that swim in contaminated water (Jami et al., 2014). *L. monocytogenes* is prevalent in raw fresh fish in several countries, but the level of contamination tends to be low and varies between 0% and approximately 30% of the products (Jami et al., 2014). The rate of contamination of raw fish might vary among different geographical areas and processing plants (Jami et al., 2014). In ready-to-eat products, cooking, preservation ingredients, and storage atmosphere inhibit the Gram-negative organisms, resulting in a longer shelf-life. Such conditions favor the growth of psychrotropic pathogens such as *Listeria monocytogenes*, allowing them to grow to dangerous levels. Moreover, *L. monocytogenes* is halotolerant, resistant to freezing temperatures, can grow and multiply during refrigeration, where other competing organisms cannot, and is able to survive at low water activity (aw). Consequently, it may grow in many food products with extended shelf-life. Over the last decade, *L. monocytogenes* has been frequently isolated from ready-to-eat, including cold and hot-smoked salmon, gravad salmon, fermented fish, and fish salads (Jami et al., 2014). With regard the presence of *L. monocytogenes* in seafoods, light preservation processes such as marinating, curing, and cold-smoking may not be sufficient to eliminate that might be present on raw materials (Ben Embarek, 1994; Huss, 1997). Fish and shrimp captured from the waters contaminated with *L. monocytogenes* may carry this pathogen and *L. monocytogenes* is also

associated with shrimp and shrimp products, and the contamination ranges from low levels to 50% (Table 3). Cordano and Rocourt (2001) detected *L. monocytogenes* in 28% of the fresh shrimp in Chile. In Iceland, *Listeria* was observed in 20.9% of the fresh shrimp studied (Gudmundsdottir et al., 2006). Seafood products can be also contaminated during transportation and in the market environment (Ben Embarek, 1994; Norhana et al., 2010). In seafood processing industries, transient *L. monocytogenes* from raw materials may contaminate the final products. On the other hand, it is determined that persistent in-house strains of *L. monocytogenes* may also be the source of contamination for the final products (Huss et al., 2000; Norhana et al., 2010). The pathogen may enter the processing plant via contaminated water, utensils, staffs, and raw materials; hence contaminating the processing line and final products (Huss et al., 2000; Johansson et al., 1999). Table 2 shows the source of contamination in the fish processing environment.

Source of contamination	Direct/indirect food contact surface	Processed product	Country	N*	P [%]**	Reference
Conveyors	Direct	Smoked fish	Japan	7	5 (71.4)	Nakamura and others (2006)
	"	Catfish fillet	USA	36	6 (16.6)	Chen and others (2010b)
Spiral/Blast freezers	Direct	Blue crab	Australia	78	5 (6.4)	Pagadala and others (2012)
Personnel and their protection clothing e.g. white coats	Direct	Smoked rainbow trout	Denmark	2	2 (100)	Hansen and others (2006)
	"	Cold-smoked rainbow trout	Finland	19	6 (31.6)	Autio and others (1999)
	"	Smoked fish	USA	135	14 (10.4)	Thimothe and others (2004)
	"	Cooked shrimp. raw salmon or raw cod	Nordic countries	48	3 (6.3)	Gudbjornsdottir and others (2004)
	"	Blue crab	Australia	78	1 (1.3)	Pagadala and others (2012)
Packaging equipment	Direct	Cold smoked salmon Plant 1 .1998-1999	Denmark	472	131 (27.8)	Fonnesbech Vogel and others (2001)
	"	Cold-smoked rainbow trout	Finland	84	20 (23.8)	Autio and others (1999)
	"	Cold-smoked salmon	Norway	155	23 (14.8)	Klaeboe and others (2005)
	"	Smoked fish	Japan	101	9 (8.9)	Nakamura and others (2006)
	"	Cold-smoked salmon	USA	57	3 (5.3)	Hu and others (2006)
	"	Smoked fish	USA	125	6 (4.8)	Thimothe and others (2004)
	"	Cold smoked salmon Plant 2. 1998-1999	Denmark	346	9 (2.6)	Fonnesbech Vogel and others (2001)
	"	Cold-smoked salmon	USA	174	1 (0.6)	Hu and others (2006)
	"	Cold-smoked salmon	USA	113	1 (0.9)	Hu and others (2006)
Skinning. slicing. blending equipment and smoking area	Direct	Catfish fillet	USA	45	7 (15.6)	Chen and others (2010b)
Injection brines and other solutions	Direct	Cold-smoked rainbow trout	Finland	6	4 (66.7)	Autio and others (1999)
	"	Cold smoked salmon	Iceland	14	3 (21.4)	Gudbjornsdottir and others (2005)
	"	Cooked shrimp. raw salmon or raw cod	Nordic countries	23	2 (8.7)	Gudbjornsdottir and others (2004)
Drains	Indirect	Cold-smoked fish	USA	128	80 (62.5)	Hoffman and others (2003)
	"	Smoked fish	USA	131	31 (23.7)	Thimothe and others (2004)
	"	Cooked shrimp. raw salmon or raw cod	Nordic countries	166	37(22.3)	Gudbjornsdottir and others (2004)
	"	Catfish fillet	USA	9	2 (22.2)	Chen and others (2010b)
	"	Cold smoked salmon	Iceland	137	29 (21.2)	Gudbjornsdottir and others (2005)
Floors/gangways	Indirect	Cold-smoked rainbow trout	Finland	67	53 (79.1)	Autio and others(1999)
	"	Smoked fish	Japan	48	26 (54.2)	Nakamura and others (2006)
	"	Catfish fillet	USA	9	4 (44.4)	Chen and others (2010b)
	"	Cold-smoked fish	USA	96	31 (32.3)	Hoffman and others (2003)
	"	Smoked Rainbow trout	Denmark	29	7 (24.1)	Hansen and others (2006)
	"	Smoked fish	USA	162	20 (12.3)	Thimothe and others (2004)
	"	Blue crab	AUS	78	1 (1.3)	Pagadala and others (2012)
Cleaning equipment	Indirect	Cold-smoked rainbow trout	Finland	12	2 (16.7)	Autio and others(1999)

*Number of samples tested. **Number of positive samples (% positive).

Table 2: Source of contamination in the fish processing environment (from Jami et al., 2014).

In Table 3 the prevalence of *L. monocytogenes* in slightly preserved seafood products are reported

Reference	Country	Product type	N*	P**	[%]***
Gravad					
Jemmi and others (2002)	Switzerland	Marinated fish	125	48	38.4
Jorgensen and Huss (1998)	Denmark	Gravad fish	176	51	29.0
Loncarevic and others (1996)	Sweden	Gravad salmon and trout	58	12	20.7
Lambertz and others (2012)	Sweden	Gravad fish	200	28	14.0
Peiris and others (2009)	Sweden	Gravad salmon	31	4	12.9
Hartemink and Georgsson (1991)	Iceland	Gravad salmon	12	1	8.3
Kwiatek (2004)	Poland	Marinated fish	34	0	-
Seafood salad					
Van Coillie and others (2004)	Belgium	Seafood salad	45	16	35.6
Uyttendaele and others (1999)	Belgium	Fish and shrimp salad	362	98	27.1
Hartemink and Georgsson (1991)	Iceland	Seafood salad	29	7	24.1
Gombas and others (2003)	USA	Seafood salad	2446	115	4.7
Little and others (2007)	UK	Seafood salad	1418	54	3.8
Fish roe					
Handa and others (2005)	Japan	Fish roe	67	9	13.4
Miya and others (2010)	Japan	Salmon and cod roe	287	22	7.7
Miettinen and others (2003)	Finland	Fish roe	147	7	4.8
Kramarenko and others (2013)	Estonia	Caviar 2008-2010	44	0	-
Salted					
Basti and others (2006)	Iran	Salted mullet (*Liza aurata*)	40	24	60.0
Siriken and others (2013)	Turkey	Salted Anchovy	50	6	12.0
Kramarenko and others (2013)	Estonia	Salted fish 2008-2010	391	38	9.7
Kuzmanovic and others (2011)	Serbia	Salted fish	15	0	-
Cabedo and others (2008)	Spain	Salted herring and anchovies	27	0	-
Dried					
Miya and others (2010)	Estonia	Dried fish 2008-2010	89	0	-
Miya and others (2010)	Japan	Dried seafood	16	0	-
Dhanashree and others (2003a)	India	Dried fish and prawn	42	0	-
Miscellaneous					
El-Shenawy and others (2011)	Egypt	Cooked and fried seafood (sandwich)	71	10	14.1
Jamali and others (2013)	Malaysia	Fried and barbecue seafood	25	2	8.0
Kovacevic and others (2012)	Canada	RTE fish	40	2	5.0
Meloni and others (2009)	Italy	Cooked marinated products	42	2	4.8
Jorgensen and Huss (1998)	Denmark	Cured seafood[a]	191	8	4.2
Hosein and others (2008)	Trinidad and Tobago	RTE seafood	70	2	2.9
Kramarenko and others (2013)	Estonia	Heat-treated and non-heat treated fish products 2008-2010	596	17	2.9
Miya and others (2010)	Japan	Tuna block and sushi	74	1	1.4
Pagadala and others (2012)	Australia	Cooked crab meat	624	1	0.2
Fletcher and others (1994)	New Zealand	Marinated mussle	11	0	-
Kuzmanovic and others (2011)	Serbia	Heat-treated products and RTE fish	20	0	-
Thimothe and others (2002)	USA	Raw fish meat	78	0	-

*Number of samples tested, **Number of positive samples, ***Percent positive samples, RTE = Ready-to-eat; VP = Vacuum-packed; MAP = Modified atmosphere packaging.
[a] Cured seafood includes lightly preserved products such as brined shrimps and surimi, oil marinated shrimps, caviar and marinated herring, the latter being a semipreserved product.

Table 3: Prevalence of *L. monocytogenes* in slightly preserved seafood products (from Jami et al., 2014).

Hence, considering the significant public health implications of listeriosis and the importance of seafood products as a vehicle for *L. monocytogenes*, it is important to examine the incidence of diseases caused by this pathogen (Table 4)

Product	Country	Year	Number of cases	Serotype	Reference
Herring cutlet marinated in oil	Germany	2010	8 cases, 1 death[b]		Aichinger (2010)
Fish, vacuum-packed (suspected)[c]	Finland	1999 to 2000	10 cases, 4 deaths	1/2	Hatakka and others (2000)
Smoked rainbow trout	Finland	1999	5 cases	1/2a	Miettinen and others (1999)
Tuna-corn salad	Italy	1997	1566[d]	4b	Aureli and others (2000)
Imitation crab meat[e]	Canada	1996	2 cases	1/2b	Farber and others (2000)
Cold-smoked rainbow trout (suspected)	Sweden	1994 to 1995	9 cases, 2 deaths	4b	Ericsson and others (1997)
Smoked mussels	New Zealand	1992	3 cases[f], 1 death	1/2a	Brett and others (1998); McLauchlin and others (2004)
Smoked mussels	Australia (Tasmania)	1991	4 cases	1/2a	Misrachi and others (1991); Mitchel (1991)
Shrimp	United States	1989	2 cases	4b	Riedo and others (1994)
Smoked cod roe	Denmark	1989	1 case	4b	Rocourt (1991)
Fish[c]	Italy	1989	1 case	4	Facinelli and others (1989)
Fish or molluscan shellfish	New Zealand	1980	22 cases, 7 deaths[g]	1b-1/2a[h]	Lennon and others (1984); McLauchlin and others (2004)

[a] There was very limited epidemiological evidence available to confirm seafood as an etiological agent for these cases.
[b] Three of eight patients do not remember about the consumption of this particular fish; 1 fatal outcome could be directly linked to fish consumption 3 d before death.
[c] not specified in detail.
[d] Non-invasive listeriosis. Possible cross-contamination from other untreated foods.
[e] Artificially flavored Alaska pollock.
[f] DNA analysis using pulsed-field gel electrophoresis (PFGE) showed that the PFGE patterns of isolates from patients 1 and 2 were indistinguishable from the isolates from the mussels. Patient 3 had a history of consuming mussels and PFGE analysis of isolates of *L. monocytogenes* serogroup 1/2 revealed that the isolates were indistinguishable from the isolates of patients 1 and 2 (Brett and others 1998).
[g] The link was on the basis of recall of food consumption, not microbiological testing.
[h] While in the first report, the serovar 1b indicated for isolated *L. monocytogenes*, McLauchlin and others (2004) mentioned the serovar 1/2a for the pathogen.

Table 4: Survey of seafood products implicated in human listeriosis outbreaks (from Jami et al., 2014).

In this context, biopreservative methodologies based on natural compounds like bacteriocins by Lactic Acid Bacteria (LAB) and essential oils are promising strategies for the control of *L. monocytogenes* in RTE seafoods.

4. Lactic Acid Bacteria (LAB)

4.1 Background

Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore forming, cocci or rods, catalase-negative, and fastidious organisms, with high tolerance for low pH (Van Geel-Schuttená et al., 1998). LAB are among the most important microbes which are used in food fermentations, as well as in enhancing taste and texture in fermented food products (Kaban et al., 2008). They are characterized by the production of lactic acid as the main product from glucose and growth inhibition substances such as bacteriocins, hydrogen peroxide, diacyls, etc. which prevent the proliferation of food spoilage bacteria and pathogens (Hati et al., 2013). LAB are grouped into the *Clostridium* branch of Gram-positive bacteria which is related to bacilli, whereas *Bifidobacterium* belongs to *Actinomycetes*. The DNA of LAB has a low G + C content (Alakomi et al., 2000). Lactic acid bacteria ferment carbohydrates to obtain energy, using endogenous carbon sources as the final electron acceptor instead of oxygen. They are

aerotolerant and are protected against oxygen by-products such as hydrogen peroxide by peroxidases. LAB are usually non-motile, and cell division occurs in one plane, except in pediococci. Phenotypic methods have been most used for the identification of LAB, but more recently, molecular techniques such as 16S rDNA sequencing have been developed, enabling a more consistent and accurate identification of individual strains (König et al., 2009). Other promising identification tools include partial rRNA gene sequencing using the polymerase chain reaction, and the soluble protein patterns. The growth optimum for LAB is at pH 5.5–5.8, and these microorganisms have complex nutritional requirements for amino acids, peptides, nucleotide bases, vitamins, minerals, fatty acids, and carbohydrates. They are categorized into homofermentative and heterofermentative microorganisms, based on the products of the fermented carbohydrates. Homofermentative LAB mainly produce lactic acid from sugars, whereas heterofermentative LAB produce lactic acid, acetic acid or alcohol and carbon dioxide (Khalid, 2011). In addition, some species of LAB produce antimicrobial peptides known as bacteriocins. To date, several LAB isolates from the *Lactobacillus* genus and their bacteriocins have been applied in food preservation and in the control of human pathogens. Several authors have documented the ability of various LAB to inhibit growth of pathogenic microorganisms, their ability to degrade mycotoxins, their probiotic capabilities, as well as antimicrobial activities of cell-free extracts of the LAB isolates from different sources (Naidu et al., 1999).

4.2 Lactic Acid Bacteria: Classification, Distribution and Sources

LAB are found in decomposing plant material and fruits, in dairy products, fermented meat and fish, cereals, beets, pickled vegetables, potatoes, sourdough, silages, fermented beverages, juices, sewage and in cavities of humans and animals (Liu et al., 2014). In humans, they particularly inhabit the oral cavity, ileum, colon, and are the dominant organisms in the vagina (Devi et al., 2013). The LAB group is currently classified in the phylum *Firmicutes*, class Bacilli, and order *Latobacillales*. LAB are classified based on cellular morphology, mode of glucose fermentation, range of growth temperature, and sugar utilization patterns. LAB genera include *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Oenococcus, Tetragenococcus, Vagococcus* and *Weissella* (Djadouni et al., 2012), with *Lactobacillus* being

the largest genus, including more than 100 species that are abundant in carbohydrate-rich substances. Most *Lactobacillus* species have been isolated from the gastrointestinal tract of humans and animals. The second largest number of *Lactobacillus* species are from vegetables and their fermentation products, whereas species from the *Leuconostoc* genus are mainly isolated from chilled meats or clinical sources, although they are also obtained from plant material, fermented dairy products and wines (Quinto et al., 2014). Species of the genus *Pediococcus* are associated with spoilage of fermented beverages, especially beers. Although *Lactococcus* species have been isolated from plant material, they are most abundant in dairy products such as sour milk. Members of the genus *Lactobacillus* are also Gram-positive, non-motile and non-sporulating organisms. However, while they are acid-tolerant facultative anaerobes, they can be either homo- or heterofermentative (Alkema et al., 2016). Due to their health benefits, some LAB are used as probiotics. Probiotics are organisms such as bacteria or yeast that improve human or animal health and are available in supplements and fermented foods such as yoghurt, or as nutritional supplements that contain live bacteria for building up the intestinal microbiota. For an organism to be a probiotic, it must essentially be non-pathogenic, be generally regarded as safe (GRAS), tolerate low pH, tolerate high concentrations of conjugated and de-conjugated bile salts, be tolerated by the immune system, and should not result in the formation of antibodies (Belicova et al., 2013). In addition, such an organism must not confer antibiotic resistance genes to potential pathogens through horizontal gene transfer. The LAB used as probiotics require a careful safety assessment, and must adhere to strict selection guidelines (Degnan, 2008). Hence, the FDA has established a regulatory authority for probiotics production, manufacturers, labeling and safety of products, despite the GRAS status of these microorganisms. In the FDA, there are four regulatory categories informed by the intended use of the product and each of these has different requirements. These categories are (1) Drug or biological products; (2) Dietary supplements; (3) Food or food ingredient; and (4) Medical food (EFSA 2007). In Europe, probiotic mediated food is not regulated, but microbial feed additives are regulated by a safety assessment of these additives in animals and humans. According to the "Qualified Perception of Safety" (QPS) concept launched by the Scientific Committee on Animal Nutrition in Europe, the species that have adequate safety data can be marketed without extensive safety testing. The FAO and WHO collaborated to establish guidelines for probiotics in food (Pineiro et al., 2007).

4.3 Carbohydrate fermentation patterns

LAB does not possess a functional respiratory system; they must obtain energy by substrate-level phosphorylation. With the hexoses there are two basic fermentative pathways. The homofermentative pathway is based on glycolysis (or Embden-Meyerhof-Parnas pathway) and produces virtually only lactic acid. Heterofermentative or heterolactic fermentation (also known as pentose phosphoketolase pathway, hexose monophosphate shunt or 6-phosphogluconate pathway) produce, in addition to lactic acid, significant amount of CO_2 and ethanol or acetate. As a general rule, pentoses can only be fermented heterofermentatively by entering the pathway as either ribulose-5-phosphate or xylulose-5-phosphate, but then CO_2 is not produce (Lahtinen et al., 2012). Theoretically, homolactic fermentation produces 2 moles of ATP per mole of glucose consumed. In heterolactic fermentation the corresponding yield is only 1 mole of ATP if the acetyl phosphate formed as an intermediate is reduced in ethanol. However, if acetyl phosphate is converted to acetic acid in the presence of alternative electron acceptors, an extra ATP is formed (Lahtinen et al., 2012). Hexoses other than glucose (mannose, galactose and fructose) enter the major pathways outlined above after different isomerization and phosphorylation steps as either glucose-6-phosphate or fructose-6-phosphate (Lahtinen et al., 2012). Based on the fermentative characteristics, lactobacilli can be divided into three groups: obligatory homofermentative, obligatory heterofermentative and facultatively heterofermentative. Obligatory homofermentative lactobacilli degrade hexoses exclusively to lactic acid and do not ferment pentoses or gluconate. Obligatory heterofermentative lactobacilli degrade hexoses to lactic acid and additional products such as acetic acid, ethanol and CO_2 and pentoses to lactic and acetic acid. Facultatively heterofermentative lactobacilli ferment hexoses to lactic acid and may produce CO_2 from gluconate but not from glucose. They also ferment pentoses to produce lactic and acetic acid.

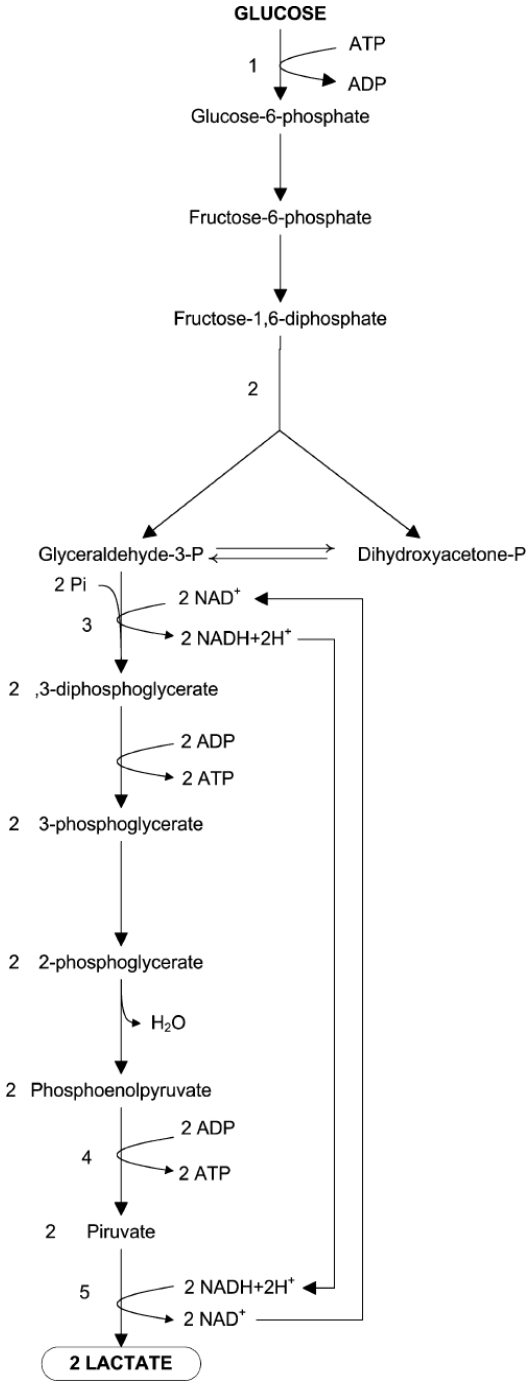

Figure 1: Homolactic fermentation (glycolysis, Embden-Meyerof-Parnas pathway); *1* glucokinase; *2* fructose-1,6-diphosphate aldolase; *3* glyceraldehyde-3-phosphate dehydrogenase; *4* pyruvate kinase; *5* lactate dehydrogenase (from Reis et al., 2012).

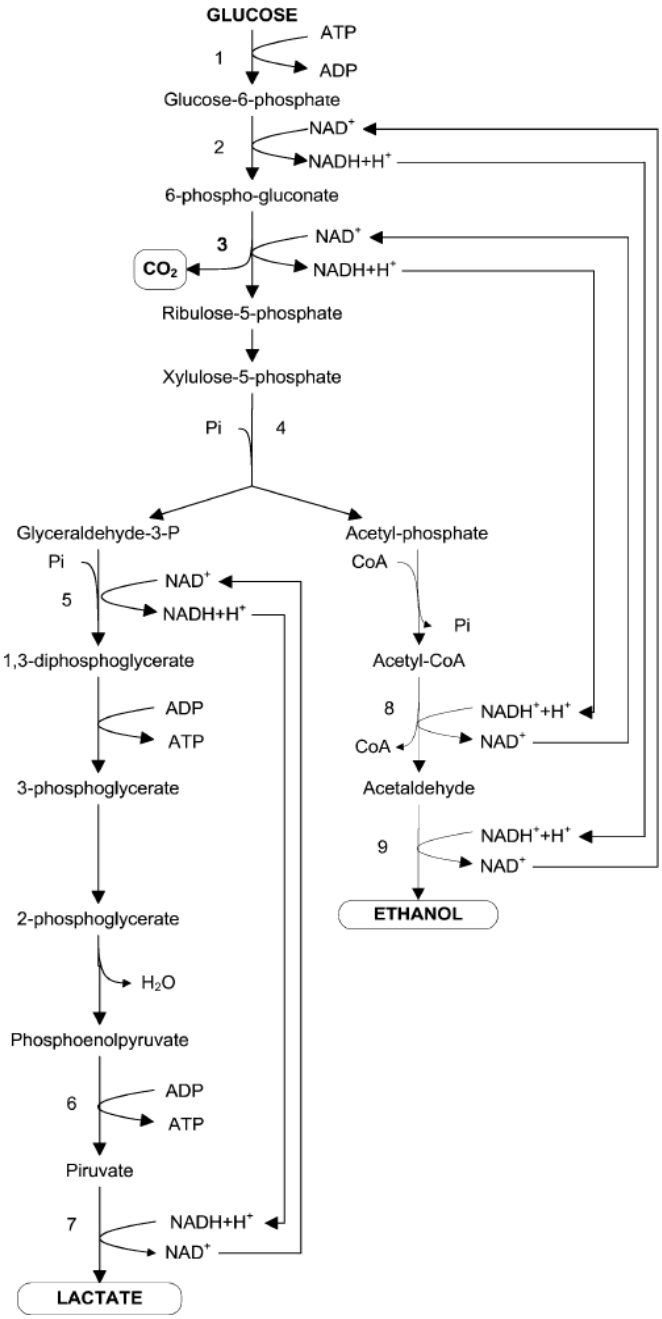

Figure 2: Heterolactic fermentation (6-phosphogluconate/phosphoketolase pathway); *1* glucokinase; *2* glucose-6-phosphatedehydrogenase;*3*-6-phosphogluconate dehydrogenase; *4* phosphoketolase; *5* glyceraldehyde-3-phosphate dehydrogenase; *6* pyruvate kinase; *7* lactate dehydrogenase; *8* acetaldehyde dehydrogenase; *9* alcohol dehydrogenase (from Reis et al., 2012).

4.4 Antimicrobial potential of LAB

The preservative effect of LAB is due to the production of one or more active metabolites, such as organic acids (lactic, acetic, formic, propionic, and butyric acids), carbon dioxide, hydrogen peroxide, diacetyl, and antibacterial peptides (bacteriocins).

4.4.1. Organic acid production

Organic acid production plays an important role in improving the shelf-life and the safety of the final product. Acidification is a highly used method of preservation during the production of many types of food, such as fermented milk, vegetables and sausages (Reis et al., 2012). Microorganisms display varied tolerances to acids. LAB are not only tolerant to weak lipophilic acids but also produce them as a by-product of their metabolism. Some acids, such as acetic acid, are critical to the metabolism of lactobacilli but inhibitory to bacilli (Reis et al., 2012). The types and levels of organic acids produced during the fermentation process depend on the LAB strains present, the culture composition and the growth conditions (Ghanbari et al., 2013). The antimicrobial effect of organic acids is due to the reduction of pH and in the action of undissociated acid molecules. It has been suggested that low external pH causes acidification of the cytoplasm. The lipophilic nature of the undissociated acid allows it to diffuse across the cell membrane collapsing the electrochemical proton gradient. Alternatively, cell membrane permeability may be affected, disrupting substrate transport systems (Ghanbari et al., 2013). LAB are able to reduce the pH to levels where putrefactive (e.g. clostridia and pseudomonads), pathogenic (e.g. *Salmonella* and *Listeria* spp.) and toxinogenic bacteria (*Staphylococcus aureus*, *Bacillus cereus*, *Clostridium botulinum*) will be either inhibited or killed (Ghanbari et al., 2013). Moreover, the undissociated acid, on account of its fat solubility, will diffuse into the bacterial cell, thereby reducing the intracellular pH and slowing down metabolic activities, and in the case of *Enterobacteriaceae* such as *E. coli* inhibiting growth at around pH 5.1 (Ghanbari et al., 2013).

4.4.2 Other antimicrobial substances

Hydrogen peroxide (H_2O_2) is produced from lactate by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or nicotinamide adenine dinucleotide (NADH) peroxidase (Ghanbari et al., 2013). The antimicrobial effect of H_2O_2 may result from the oxidation of sulfhydryl groups causing denaturing of enzymes, and from the peroxidation of membrane lipids thus increasing membrane permeability. Most undesirable bacteria such as *Pseudomonas* spp. and *S. aureus* are sensitive to H_2O_2 (Ghanbari et al., 2013). Carbon dioxide (CO_2) is generally produced by heterofermentative LAB. CO_2 plays a role in creating an anaerobic environment which inhibits enzymatic decarboxylations, and the accumulation of CO_2 in the membrane lipid bilayer may cause a dysfunction in permeability. CO_2 can effectively inhibit the growth of many food spoilage microorganisms, especially Gram-negative psychrotrophic bacteria (Ghanbari et al., 2013). Diacetyl is produced by strains within all genera of LAB by citrate fermentation. It is produced by heterofermentative lactic acid bacteria as a by-product along with lactate as the main product. Diacetyl is a high value product and is extensively used in the dairy industry as a preferred flavour compound. Diacetyl also has antimicrobial properties. Diacetyl was found to be more active against Gram-negative bacteria, yeasts, and molds than against Gram-positive bacteria. Diacetyl is thought to react with the arginine-binding protein of Gram-negative bacteria and thereby interfering with the utilization of this amino acid (Ghanbari et al., 2013).

4.4.3 Antimicrobial peptides (bacteriocins)

Bacteriocins are ribosomally synthesized peptides, that exert antimicrobial activity against either strains of the same species as the bacteriocin producer (narrow range), or to more distantly related species (broad range) (Ghanbari et al., 2013). It has been estimated that between 30% and 99% of all bacteria and archaea produce bacteriocins; their production by LAB is very significant from the point of view of their potential applications in food systems and thus, unsurprisingly, these have been most extensively investigated (Mozzi, 2016).

4.5 LAB in seafood products

4.5.1 LAB associated with seafood products

LAB have been associated to processed aquatic food products such as lightly preserved fish products (LPFP) and semi-preserved fish products (SPFP) (Ghanbari et al., 2013a).

The LPFP category includes uncooked or mildly cooked products, with low levels of preservatives and salt (<6%, w/w) (Ghanbari et al., 2013a; Leroi, 2010) This group consists of high-value delicacy products (cold-smoked, pickled, or marinated fish, brined shellfish, etc) that are typically consumed as ready-to-eat products, without any heat treatment. These highly perishable products are usually stored at chilled temperature under vacuum (VP) and modified atmosphere (MAP) to extend shelf-life (Leroi, 2010). LAB dominating vacuum-packaged cold-smoked fish products include the genera of *Lactobacillus, Lactococcus, Leuconostoc* and *Carnobacterium*. Many studies have shown that carnobacteria are quite common in chilled fresh and lightly preserved seafood, but at higher storage temperature (15°C-25°C) other species including *Enterococcus* could dominate the microbial spoilage community of seafood (Ghanbari et al., 2013a). Fish products with high salt content (>6% in aqueous phase) or with a pH below 5.0 and to which preservatives are added are defined as "semi-preserved" (Ghanbari et al., 2013a). Typically, the European products (e.g., salted and/or marinated herring, anchovies, caviar, etc) are distributed at cooled temperatures (<10°C). In marinated or dried fish, salted and fermented fish, the lactic acid microflora can be quite diverse, since the presence of lactobacilli and pediococci has been reported (Ghanbari et al., 2013a). Table 5 summarizes the most relevant species isolated from different ready-to-eat products.

Lactic acid bacteria	Product identified	References
Brine shrimp	*Aerococcus viridans*	Dalgaard and Jorgensen, 2000, Dalgaard et al., 2003
	Carnobacterium spp.	Dalgaard and Jorgensen, 2000, Dalgaard et al., 2003
	Carnobacterium divergens	Mejlholm et al., 2005
	C. maltaromaticum	Dalgaard and Jorgensen, 2000, Dalgaard et al., 2003, Mejlholm and Dalgaard, 2007
	Enterococcus faecalis	Dalgaard and Jorgensen, 2000, Dalgaard et al., 2003
	E. gallinarum	

	E. malodoratus	Mejlholm and Dalgaard, 2007
	Lactobacillus curvatus	Dalgaard and Jorgensen, 2000, Dalgaard et al., 2003
	Lactobacillus spp.	From and Huss, 1990
	Lactobacillus sakei	Mejlholm and Dalgaard, 2007
	Lactococcus garvieae	Dalgaard and Jorgensen, 2000, Dalgaard et al., 2003,
	Streptococcus spp.	From and Huss, 1990
Cold smoked fish	*C. divergens*	Leroi et al., 1998
	C. piscicola/maltaromaticum	Paludan-Müller et al., 1998, Leroi et al., 1998, González-Rodríguez et al., 2002, Olofsson et al., 2007
	E. faecalis	González-Rodríguez et al., 2002
	Enterococcus spp.	Lyhs et al., 1998
	Lb. alimentarius	Leroi et al., 1998
	Lb. casei ssp. tolerans	González-Rodríguez et al., 2002
	Lb. coryneformis	
	Lb. curvatus	Truelstrup Hansen and Huss, 1998, Lyhs et al., 1999, Jørgensen et al., 2000, Gonzalez-Rodriguez et al., 2002
	Lb. delbrueckii ssp. delbrueckii	Gonzalez-Rodriguez et al., 2002
	Lb. farciminis	Leroi et al., 1998
	Lb. homohiochii	Gonzalez-Rodriguez et al., 2002
	Lb. plantarum	Gancel et al., 1997, Truelstrup Hansen and Huss, 1998, Lyhs et al., 1999, Gonzalez-Rodriguez et al., 2002
	Lb. pentosus	Gancel et al., 1997
	Lb. sakei	Leroi et al., 1998, Truelstrup Hansen and Huss, 1998, Lyhs et al., 1999, Jorgensen et al., 2000, Gonzalez-Rodriguez et al., 2002
	Leuconostoc spp.	Paludan-Müller et al., 1998
	Leuconostoc carnosum	Truelstrup Hansen and Huss, 1998
	Leuconostoc citreum	Lyhs et al., 1999
	Leuconostoc gelidum	Truelstrup Hansen and Huss, 1998
	Leuconostoc mesenteroides	Truelstrup Hansen and Huss, 1998, Lyhs et al., 1999
	Weissella kandleri	Gonzalez-Rodriguez et al., 2002
Fermented fish	*Lb. brevis*	Lee et al., 2000
	Lb. pentosus	Paludan-Müller et al., 1998, Tanasupawat et al., 1998
	Lb. plantarum	Tanasupawat et al., 1998
	Lactococcus lactis	Lee et al., 2000
	Lc. lactis ssp. lactis	Paludan-Müller et al., 1998
	Leuconostoc citreum	Continue on next page
	Pediococcus pentosaceus	
Salted, marinated or dried fish	*Carnobacterium* spp.	Basby et al., 1998
	E. faecalis, E. faecium	Thapa et al., 2006
	Lb. alimentarius, Lb. buchneri	Lyhs et al., 2002
	Lb. delbrueckii ssp. lactis	
	Lb. plantarum	
	Lactococcus lactis	Thapa et al., 2006
	Leuconostoc mesenteroides	

	Pediococcus pentosaceus		
	Weisella confusa		
Seafood salad	*C. piscicola*		Andrighetto et al., 2009
	Enterococcus	*spp.,*	
	E. faecalis,		
	Lb. curvatus		
	Lb. malfermentans		
	Lb. paraplantarum		
	Lb. sanfranciscensis		
	Lactococcus lactis		
	Leuconostoc mesenteroides		
	Leuconostoc pseudomesenteroides		
	Pediococcus spp.		
	Streptococcus parauberis		
	Vagococcus spp.		
	Weisella spp.		
Sugar-salted (Gravad) fish	*C. divergens, C. piscicola*		Lyhs et al., 2002
	Lb. curvatus ssp. melibiosus		
	Lb. curvatus ssp. curvatus		
	Lb. curvatus, Lb sakei		Leisner et al., 1994
	Lb. sakei		Jeppesen and Huss, 1993, Lyhs et al., 2002
	Leuconostoc spp.		Leisner et al., 1994
	Weisella viridescens		

Table 5: Reports on LAB isolated from ready-to-eat seafood products (from Ghanbari et al., 2013a).

4.5.2 Spoilage potential of LAB

The use of LAB as protective culture in seafood implies that they do not have any spoiling capacity (Pilet et al., 2011). Currently, the role of LAB in fish spoilage is still controversial. They are not very competitive in refrigerated fresh fish and they produce fewer unpleasant odours compared to Gram-negative bacteria such as *Shewanella putrefaciens*, *Photobacterium phosphoreum* and *Pseudomonas* spp. (Pilet et al., 2011). Several authors have found no correlation between LAB and sensory spoilage (Leroi et al., 2001). However, Paludan-Müller et al. (1998) succeeded in increasing the shelf-life of cold-smoked salmon by inhibiting LAB with nisin, suggesting a possible spoiling effect of this bacterial group. According to various authors some *Lactobacillus* species found in cold-smoked salmon are very spoiling (*L. sakei*) while others had no effect (*L. alimentarius*). *L. sakei* generally produces sulphurous and acidic

odours (Nilsson et al., 1999; Stohr et al., 2001), associated with the production of H_2S, acetic acid and ethyl and n-propyl acetate (Joffraud et al., 2001), but some *L. sakei* strains do not affect the organoleptic quality of this product (Weiss et al., 2006). Moreover, *L. alimentarius* which does not spoil cold-smoked salmon has been identified as the main responsible for the sensory deterioration of marinated herring. Carnobacteria are microorganisms resistant to freezing, able to grow at refrigerated temperatures, in all packaging conditions and in the presence of many preservatives, explaining why this genus is very often found in refrigerated fish products. The role of thisgenus is still under debate. Many studies show that the inoculation of cold-smoked salmon by various strains of *C. maltaromaticum* and *C. divergens* leads to few or no changes in organoleptic quality (Nilsson et al., 1999). When the carnobacteria reach a high enough level, flavours of butter and plastic may be detected, due to the production of 2,3- butanedione (diacetyl) and 2,3-pentanedione (Stohr et al., 2001), but are not sufficient to reject the product (Brillet et al., 2005). In contrast, when strains of *C. maltaromaticum* and *C. divergens* were inoculated into arctic shrimp, a strong chlorine, malt, nuts and sour odours were generated and the samples were judged unfit for consumption (Laursen et al., 2006). Variability on the production of ammonia and numerous alcohols, aldehydes and ketones were also observed depending on the strains (Pilet et al., 2011). The interaction with other microorganisms should not be disregarded. Joffraud et al. (2001) demonstrate that the spoilage observed with *L. sakei* was weakened in the presence of *Serratia liquefaciens* even though the latter had also a spoiling effect in monoculture. In contrast, some associations appear to be much more spoiling than in pure culture (*Carnobacterium* with *Vibrio* or *Brochotrix thermosphacta*) due to de novo synthesis of total volatile basic nitrogen (Brillet et al., 2005).

5. Bacteriocins

5.1 Historical Background

The first description of antagonistic interactions between bacteria was reported in 1877, when Pasteur, together with Joubert, observed the ability of "common bacteria" (probably *Escherichia coli*) to interfere with the growth of co-inoculated anthrax bacilli, either in urine (used as a culture medium) or in experimentally infected animals (Jack et al., 1995). During the First World War in 1917, was isolated by Nissle a non-pathogenic *E. coli* strains with strong antagonistic activity against *Salmonella*, *Shigella* and other enteropathogens (Sonnenborn et al., 2009). The best strains were distributed commercially under the name Mutaflor, and their use was promoted for the treatment of constipation and dysentery and for typhoid carriers (Jack et al., 1995).The first clear documentation of the nature of antibiotic agent produced by *E. coli* was provided by Gratia, who demonstrated in 1925 that strain V (virulent in experimental infections) produced in liquid media a dialyzable and heat-stable substance (later referred to as colicin V) that inhibited in high dilution the growth of *E. coli* φ. This activity was found to be produced by various species of *Enterobacteriaceae* and for which the generic name "colicins" was proposed (Daw and Falkiner, 1996). With the discovery that the production of apparently similar agents is not limited to *Enterobacteriaceae*, Jacob et al. (1953) proposed that the general name "bacteriocins" should be used for highly specific antibacterial proteins, produced by certain strains of bacteria and active mainly against strains of the same species.

5.2 Bacteriocins vs Antibiotics

Bacteriocins are often confused in literature with antibiotics (Cleveland et al., 2001). The major difference between bacteriocins and antibiotics is that bacteriocins restrict their activity to closely related strains, while antibiotics have a wider activity spectrum. Each bacteriocin has its own immunity protein whose gene is linked to bacteriocin gene, whereas genetic determinants for antibiotic resistance are not linked to and are express independently of the genes encoding the antibiotic synthesis apparatus. Moreover, bacteriocins are ribosomally synthesized and produced during the growth phase, in contrast with antibiotics which are synthesized by unique enzymatic system and produced in stationary phase (Cleveland et al., 2001). Bacteriocins, which are clearly distinguishable from clinical antibiotics, should be safely

and effectively used to control the growth of target pathogens in foods (Cleveland et al., 2001). The main differences between bacteriocins and antibiotics are summarized in Table 6.

Characteristics	Bacteriocins	Antibiotics
Application	Food	Clinical
Synthesis	Ribosomal	Secondary metabolite
Activity	Narrow spectrum	Varying spectrum
Host cell immunity	Yes	No
Mechanism of target cell resistance or tolerance	Usually, adaptation affecting cell membrane composition	Usually genetically transferable determinant affecting different sites depending on the mode of action
Interaction requirements	Sometimes docking molecules	Specific target
Mode of action	Mostly pore formation	Cell membrane or intracellular targets
Toxicity/side effects	None known	Yes

Table 6: Bacteriocins vs antibiotics (from Cleveland et al., 2001).

5.3 Bacteriocins of Gram-positive bacteria

Bacteriocins produced by LAB are heterogeneous group of peptides, and because new bacteriocins are continuously being discovered their classification has been changing (Lahtinen et al., 2012). First, Klaenhammer (1993) classified bacteriocins of Gram-positive bacteria, including LAB bacteriocins, into four classes, according to their structures and characteristics. Class I bacteriocins or lantibiotics (lanthionine containg antibiotics), are small peptides (<5 kDa) that possess unusual post-translationally modified residues such as lanthionine or β-methyl-lanthionine. These unusual residues form covalent bonds between amino acids, which results in internal rings and give lantibiotics their characteristics structural features (Cotter et al., 2005; Perez et al., 2014). According to Jung (1991) lantibiotics are grouped into type-A and type-B peptides based on their structure and mode of action. In general, type-A lantibiotics are elongated, cationic peptides (for example, nisin), active through the formation of pores, leading to the dissipation of membrane potential and the efflux of small metabolites from sensitive cells. By contrast, type-B lantibiotics are globular peptides (for example, mersacidin) that have no net charge and were originally defined as those lantibiotics that acts through enzyme inhibition

(Figure 3). However, considering improved understanding of the mode of action of lantibiotics, the division based on functional features has become blurred. This is illustrated by the fact that nisin, in addition to being a pore former and therefore a type-A on that basis, also inhibits peptidoglycan synthesis in Gram-positive cells by binding the peptidoglycan precursor lipid II, and thus can also be classified as type-B (Rea et al., 2011). Class II bacteriocins or the non-lantibiotics, are the most naturally occurring bacteriocins. They are small (<10 kDa), heat-stable, non-lanthionine containing peptides, which, unlike lantibiotics, do not undergo extensive post-translational modification. This group can be further subdivided into three subclasses: "pediocin-like" bacteriocins (Class IIa), two-component bacteriocins (Class IIb) and thiol-activated bacteriocins (Class IIc) (Figure 3).

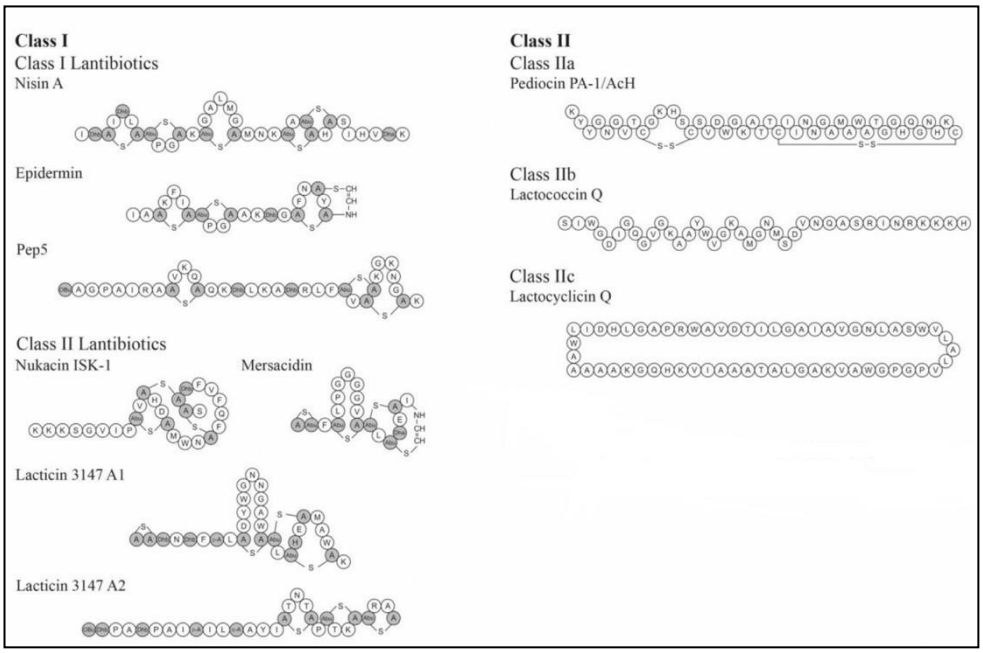

Figure 3: Primary structure of class I and II bacteriocins.

Class IIa bacteriocins, have a distinct conserved sequence (YGNGVXC) in the N-terminal region that is responsible for their high potency against the food pathogen *Listeria monocytogenes*. The designation "pediocin-like bacteriocins" refers to pediocin PA-1/AcH, which was the first class IIa bacteriocin characterized (Drider et al., 2006). The Class IIb bacteriocins are two-peptide bacteriocins that require both peptides to work synergistically to be fully active. Lactococcin Q is the representative class IIb bacteriocin (Perez et al., 2014). The Class IIc bacteriocins were originally proposed as thiol-activated bacteriocins required reduce cysteine residues for the activity but further revised as below. The Class III bacteriocins consist in large, heat-labile proteins, while class IV is defined as bacteriocins containing sugar or lipid moieties. Cotter et al. (2005) suggest some modification to Klaenhammer's classification scheme, resulting in only two principal categories: the

lanthionine-containing antibiotics (Class I) and non-lanthionine-containing antibiotics (Class II), involving IIa, IIb, IIc and IId subclasses and the bacteriolysins group. They changed Class IIc, from bacteriocins that are thiol-activated peptides to those are cyclic peptides, more specifically those that have linked C- and N-termini. In addition, the remaining bacteriocins that do not meet the requirements of the previous subclasses were grouped in Class IId.

The Class III bacteriocins are renamed bacteriolysins, whereas the complex bacteriocins of Class IV are removed. An update classification of bacteriocins was proposed by Rea et al. (2011). In this proposal, Class I bacteriocins are divided into three subclasses, namely, lantibiotics (Class Ia), labyrinthopeptines (Class Ib) and sactibiotics (Class Ic). The newly identified labyrinthopeptines, so name as a consequence on their "labyrinthine" structure, are distinguished by the presence of labionin, a previously unidentified carba-cyclic, post-translationally modified amino acid (Rea et al., 2011). The sactibiotics are another group of sulfur-bridged bacteriocins. Sactibiotics are characterized by the presence of a linkage between a cysteine thiol and the α-carbon of another residue (Lohans et al., 2014).A summary of the main classification schemes proposed is shown in Table 7.

Classes	Klaenhammer (1993)	Cotter et al. (2005)	Rea et al. (2011)
Class I	Lantibiotics, small membrane active peptides (<5 kDa) containing unusual amino acids	Lanthionine-containing antibiotics	Post-translationally modified bacteriocins **Ia**: lantibiotics (12 subclasses) **Ib**: labyrinthopeptines **Ic**: sactibiotics
Class II	Small heat stable non-lanthionine containg membrane-active peptides (<10 kDa) **IIa**: pediocin-like bacteriocins **IIb**: two-peptide bacteriocins **IIc**: thiol-activated peptides	Non-lanthionine-containing antibiotics **IIa**: pediocin-like bacteriocins **IIb**: two-peptide bacteriocins **IIc:** cyclic peptide **IId:** unmodified, linear, non-pediocin like bacteriocins	Unmodified bacteriocin **IIa**: pediocin-like bacteriocins **IIb**: two-peptide bacteriocins **IIc**: cyclic peptide **IId**: unmodified, linear, non-pediocin like bacteriocins
Class III	Large heat-labile proteins (>30 kDa)	Bacteriolysins group	Bacteriolysins group
Class IV	Complex bacteriocins, composed of protein plus one or more chemical moieties (lipid, carbohydrate)	-*	-*

Note: *, Class IV are removed.

Table 7: Classification of bacteriocins produced by Gram-positive bacteria.

5.4 Mode of action

Due to the great variety of their chemical structures, bacteriocins affect different essential functions on living cell, but most of them act by forming channels or pores through the membrane, that destroy the energy potential of sensitive cells (Oscáriz et al., 2001). In general, the elongated amphiphilic cationic lantibiotics (for example, nisin) inhibit target cell by forming pores in the membrane, depleting the transmembrane potential and the pH gradient, resulting in the leakage of cellular materials. By contrast, globular lantibiotics (for example, mersacidin) were originally defined as those latibiotics that act through enzyme inhibition. However, it has now been established that nisin possess both mechanisms of action. They can bind to lipid II, the main transporter of peptidoglycan subunits from the cytoplasm to the cell wall, and therefore prevent correct cell wall synthesis, leading to cell death. Furthermore, they can use lipid II as a docking molecule to initiate a process of membrane insertion and pore formation that lead to rapid cell death (Cotter et al., 2005) (Figure 4). The class II peptides have an amphiphilic helical structure, which allows them to insert into the membrane of the target cell, leading to depolarization and death. Class IIa bacteriocins show a pronounced anti-listerial specificity due to the presence of the sequence YGNGV in their N-terminal region. The current mechanistic hypothesis to explain the mode of action of bacteriocins belonging to this class includes electrostatic binding of the antibiotic to the target membrane mediated by a putative membrane-bound receptor molecule, although the necessity of this specific receptor is still controversial. The hypothetical receptor would be responsible for the recognition of the YGNGV anti-listerial motif present in these peptides (Oscáriz et al., 2001). The two-peptide bacteriocins require the combined activity of both peptides with a mechanism of action that again involves the dissipation of membrane potential, the leakage of ions and/or a decrease in intracellular ATP concentrations. These peptides display very low, if any, bacteriocin activity when tested individually. Large bacteriolytic proteins (called bacteriolysins, formerly class III bacteriocins) can function directly on the cell wall of Gram-positive targets, leading to death and lysis of the target cell (Cotter et al., 2005) (Figure 4).

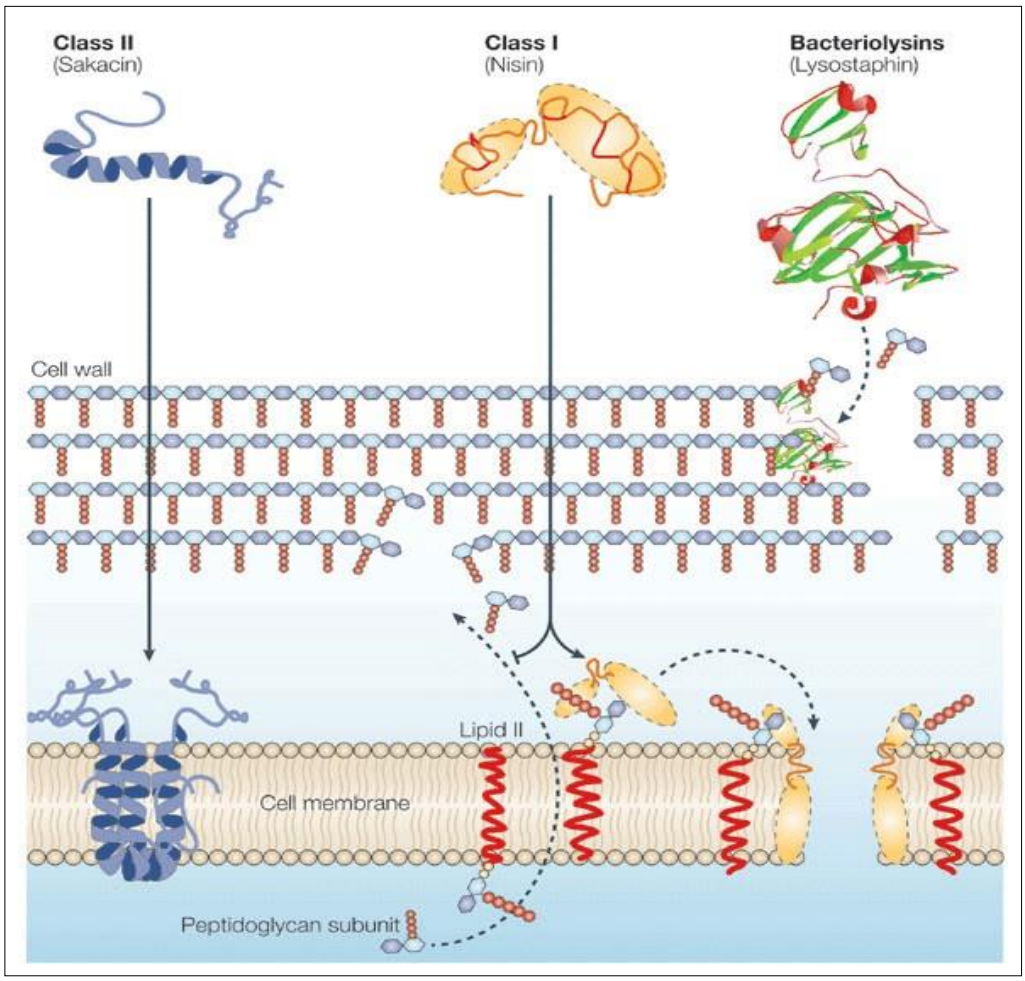

Figure 4: LAB bacteriocins mode of action. Some members of the class I (or lantibiotic) bacteriocins, such as nisin, have been shown to have a dual mode of action. They can bind to lipid II, the main transporter of peptidoglycan subunits from the cytoplasm to the cell wall, and therefore prevent correct cell wall synthesis, leading to cell death. The class II peptides have an amphiphilic helical structure, which allows them to insert into the membrane of the target cell, leading to depolarization and death. Large bacteriolytic proteins (called bacteriolysins, formerly class III bacteriocins), such as lysostaphin, can function directly on the cell wall of Gram-positive targets, leading to death and lysis of the target cell (from Cotter et al., 2005.)

5.5 Current and potential applications

The potential use of bacteriocins in various technological applications is related to their antimicrobial effect and a clear understanding on the value of this activity is needed to develop innovative strategies (Güllüce et al., 2013). In this regard, the increasing spread of multidrug-resistant bacteria state expressly the importance of the research studies purposing to find alternative methods combating of infections (Güllüce et al., 2013). Bacteriocins with broad inhibitory spectrum of activity can be thought as promising natural antimicrobials for many industrial applications in this manner.

Especially, human health and food industries have been dominated the related studies and many prosperous improvements have been done up to date (Güllüce et al., 2013).

5.5.1 Bacteriocins and human health applications

5.5.1.1 Application in pharmaceutical industry

The use of antibiotics for disease control, prophylactic agents and growth promotions has contributed to the emergence of resistant bacteria pathogenic to humans, animals and plants. The extensive use of antibiotics and the alarming nature of this antibiotic resistance problem have motivated to find alternatives (Lee, 2015). Numerous antibacterial agents, including bacteriocins, are now being considered as an alternative. The best-studied lantibiotic is undoubtedly nisin, produced by *Lactococcus lactis*. In addition to their use as a food preservative, nisin has been also considered for pharmaceutical applications. For example, it was suggested that nisin has potential in treating peptic ulcer disease by inhibiting *Helicobacter pylori* growth and colonization. Moreover, nisin was also used to inhibit growth of multi-drug resistant pathogens such as *Staphylococcus* and *Streptococcus* spp. (Gillor et al., 2005). Lantibiotics have also been investigated for use in animal health. The two-peptide lantibiotic, lacticin 3147, produced by *Lactococcus lactis* was found to be active against mastitis-causing bacteria streptococci and staphylococci. Mastitis is the most expensive disease in dairy cattle. It can be effectively treated with antibiotics, but the antibiotic residues found in the milk of treated cows may contribute to the selection for antibiotic resistance in humans who drink that milk. Thus, bacteriocins such as lacticin 3147 show considerable potential in the prevention of infectious disease in agricultural settings (Gillor et al., 2005).

5.5.1.2 Probiotics

The term “probiotic” is derived from the Greek “*probios”*, meaning “for life” or “in support of life”. The World Health Organization (WHO) defines probiotics as, “live microorganisms which, when administrated in adequate amounts, confer a health benefit on the host” (Yang et al., 2014). The characteristics of probiotics should include: a group of beneficial strains to the host animal that can stably survive and have metabolic activity in the intestinal environment, and being non-pathogenic and non-toxic, remain stable and viable for long periods of storage and harsh conditions (Yang et al., 2014). There are many ways for probiotics to control intestinal pathogens. Probiotics demonstrate the capabilities of antimicrobial substances production, competitive exclusion of pathogen binding, competition for nutrients, and modulation of the immune system. Many

antibacterial substances, such as bacteriocins, short chain fatty acids, and hydrogen peroxide, are produced by probiotics for inhibiting gastro-intestinal microorganisms or pathogens. Currently many probiotics are used in daily life, including LAB, non-pathogenic *E. coli*, bacilli and yeast (Yang et al., 2014).

5.5.2 Bacteriocins and Food Applications

To extend shelf-life, antibiotics or food preservatives are incorporated into foods. However, most commercial preservatives are chemical compounds, and long-term consumption of the synthetic preservatives may have an adverse impact on the human health. Moreover, it is illegal to use antibiotics in food products. Bacteriocins produced by LAB are natural food additives due to the bacteriocinogenic bacteria presence in many types of foods since ancient times (Yang et al., 2014). There are at least three ways in which bacteriocins can be introduced into a food to improve its safety: 1) by adding the purified or semi-purified bacteriocin directly into food; 2) by incorporating an ingredient previously fermented with a bacteriocin-producing strain and 3) by inoculating food with LAB strains that produce the bacteriocin *in situ* (Deegan et al., 2006; Gálvez et al., 2007; Rai et al., 2011). The use of purified/semi-purified bacteriocin is not always attractive to the food industry, as in this form they may have to be labelled as additives and require regulatory approval (Deegan et al., 2006). For that reason, the only LAB bacteriocins commercialized are nisin, produced by *Lactococcus lactis*, and pediocin PA-1, produced by *Pediococcus acidilactici*, marketed as Nisaplin® (product description-PD45003-7EN; Danisco, Copenhagen, Denmark) and ALTA® 2431 (Kerry Bioscience, Carrigaline, Co. Cork, Ireland), respectively (Deegan et al., 2006). Nisin is the first bacteriocin approved for utilization as a preservative in many foods by the U.S Food and Drug Administration (USFDA) and Nisaplin® is licensed as a food additive in over 45 countries (Yang et al., 2014) . Another commercially available bacteriocin is pediocin PA-1, which inhibits the growth of *Listeria monocytogenes* in meat products (Yang et al., 2014). However, nisin is the only bacteriocins currently approved as a food preservative, pediocin PA-1 are commercialized as food-ingredients or shelf-life extenders.The two other alternatives (fermented ingredient/starter culture) do not require regulatory approval or preservative label declarations. These options are frequently regarded as more attractive routes through which bacteriocins can be incorporated into a food (Deegan et al., 2006).

6. Essential Oils (EOs)

6.1 Background

Essential oils (EOs) are defined as volatile secondary metabolites of plants that give the plant a distinctive smell, taste, or both. EOs are produced by more than 17,500 species of plants from many angiosperm families, e.g., *Lamiaceae*, *Rutaceae*, *Myrtaceae*, *Zingiberaceae*, and *Asteraceae*, but only about 300 of them are commercialized (Mérillon et al., 2018). Compounds included in the EOs are synthesized in the cytoplasm and plastids of plant cells through the pathways of malonic acid, mevalonic acid, and methyl-d-erythritol-4-phosphate (MEP). They are produced and stored in complex secretory structures, such as glands, secretory cavities, and resin conduits, and are present as drops of liquid in the leaves, stems, flowers and fruits, bark, and roots of plants. Despite containing two or three main components at a level of 20–70%, EOs are very complex mixtures of mainly terpenes, terpenoids, and phenylpropanoids. They may also contain many other compounds, such as fatty acids, oxides, and sulfur derivatives (Stringaro et al., 2018). EOs are usually obtained as a result of hydrodistillation, steam distillation, dry distillation, or the mechanical cold pressing of plants. At the laboratory scale, the classical method is based on the use of the Clevenger steam distillation apparatus, discovered in 1928. Due to several disadvantages (i.e., placement of valve, fragility), this apparatus was modified by Jakub Deryng in 1951 (Deryng., 1951) and it is widely used in Central European countries. Modifications of the simultaneous distillation-extraction (SDE) equipment were described in the manuscript of Arora et al. (2016). The effectiveness of these modifications was described in detail by Baj et al. (2015). At the laboratory scale, modern methods also include processes supported by microwaves and extraction in supercritical fluids. EOs can also be isolated using fermentation, crushing, extraction, or hydrolysis. However, depending on the chosen method, the chemical composition of the obtained EO can unfortunately be different. Humans have used EOs for thousands of years, not only as ingredients of perfumes or as seasonings for the aromatization of food, but also in folk medicine, because of their many different biological properties, including antimicrobial properties (Brnawi et al., 2019). The antimicrobial qualities are essential in managing the rapidly growing issue of drug-resistant microorganisms. In 2016, about 6 million people died globally due to infections of the upper respiratory tract, tuberculosis, or diarrheal diseases. At the same time, the number of strains of microorganisms resistant to existing antibiotics is constantly increasing. Patients with infections caused by drug-resistant bacteria are, thus, exposed to an increased risk of worse clinical results and even death. Such patients also consume more healthcare resources than patients infected with non-resistant strains of the same bacteria. According to the WHO

report on drug resistance, the most serious problems include the resistance of *Klebsiella pneumoniae* to third generation cephalosporins and carbapenem, *Escherichia coli* to third generation cephalosporins and fluoroquinolone, *Staphylococcus aureus* to methicillin, *Streptococcus pneumoniae* to penicillin, and *Salmonella sp*. to fluoroquinolones. Among the fungal infections, the most common problem is candidiasis caused mainly by *Candida albicans* and less often by *C. glabrata* and *C. parapsilosis*, with more than 20 species of *Candida* that can cause human infection (Benzaid et al., 2019). Other examples of common fungal infections are aspergillosis, histoplasmosis, and skin mycosis (commonly known as ringworm) (Brun et al., 2019). In food production, inhibiting the growth of microorganisms using socially acceptable preservatives is a serious problem. Society's reluctance to use antibiotics and synthetic preservatives, such as benzoic acid, sorbic acid, lactic acid, propionic acid, acetic acid, and its derivatives, parabens or inorganic sulfites, nitrites, and nitrates, necessitates finding alternative solutions (Sharifi-Rad et al., 2018). This may be an application for EOs, especially since chemical preservatives cannot eliminate several pathogenic bacteria, such as *Listeria monocytogenes*, in food products or delay the growth of spoilage microorganisms. In addition, natural products are inherently better tolerated in the human body, usually with fewer side effects (Liu et al.,2017).

6.2 Antimicrobial, Antiviral, and Antibiotic effects

Essential oils are common natural products that can be used for various medical applications, and in combination with the emergence of antimicrobial resistance, essential oils have been studied as potential antimicrobials agents (Deyno et al., 2019). These naturally occurring compounds are linked to having bactericidal, virucidal, and fungicidal activity in clinical trials. It has also been suggested that these plant extracts might not only be used to fight cutaneous infections for example, but also serve a role in the preservation of food due to their antimicrobial activity combined with their antioxidant property (Valdivieso-Ugarte et al., 2019). Table 8 provides a brief summary of certain common essential oils and the organisms targeted. Bacterial infections remain a significant cause of mortality in the human population. This has triggered research into the exploration of alternative therapies against bacterial strains as the issue of antibiotic resistance has become more imminent even to the newest antibiotic drugs. The effect of antibacterial activity of essential oils may be bacteriostatic or bactericidal but is difficult to distinguish these actions therefore activity is commonly measured as the minimum bactericidal concentration (MBC) or the minimum inhibitory concentration (MIC) (Burt., 2004). The mechanism of antibacterial action is facilitated by a

succession of biochemical reactions within the bacterial cell that are dependent on the type of chemical constituents present in the essential oil. Due to these compounds being lipophilic, essential oils easily penetrate bacterial cell membranes and have been reported to disrupt critical processes of the cell membrane like nutrient processing, synthesis of structural molecules, emission of growth regulators, energy generation, and influences on the cell-cell communication quorum sensing network (Oussalah et al., 2006). The list of specific bacteria targeted by the essential oils is expanding and include, but are not limited to, *Listeria monocytogenes, Bacillus sphaericus*, *Enterobacter aerogenes, Escherichia coli* O157:H7, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella typhi, Shigella flexneri,* and *Yersinia enterocolitica* (Arora et al., 1999; Elgayyar et al., 2001; Ramos-Nino et al., 1996; Sakagami et al., 2000). Some of the essential oils commonly used come from garlic, ginger, clove, black pepper, green chile, cinnamon, clove, pimento, thyme, oregano, and rosemary. Similarly, to the effects on bacteria, essential oils have the ability to enter and interrupt the homeostasis of the fungal cell wall and cytoplasmic membranes, specifically the mitochondria (Akhtar MS., 2014). One of the mechanisms suggested involves the penetration of essential oils into the mitochondrial membranes and changing the electron flow through the electron transport system, which in return disrupts the lipids, proteins, and nucleic acid contents of the fungal cells (Arnal-Schnebelen., 2004). Another proposed mechanism is the depolarization of the mitochondrial membranes that decreases the membrane potential, affecting ion channels to reduce the pH and affect the proton pump leading to fungal cell apoptosis and necrosis (Yoon et al.,2000). Extracts from plants such as basil, clove, citrus, garlic, fennel, lemongrass, oregano, rosemary, and thyme have demonstrated their significant antifungal activity against a broad range of fungal human pathogens (Kivanç et al.,1991). Some of the fungal pathogens affected include *Candida acutus, C. albicans, C. apicola, C. catenulata, C. inconspicua, C. tropicalis, Rhodotorula rubra, Sacharomyces cerevisae, and Trignopsis variabilis, Aspergillus parasiticus*, and *Fusarium moniliforme* (Juglal et al., 2002). Since viral infections are still a problem for human health and only a narrow number of drugs are effective, it has prompted researchers to explore new antiviral molecules that can attack these human pathological viruses. Detailed insight on the antiviral action of essential oils still requires more research. Essential designed for entry into human host cells, synthesis of viral proteins, inhibition of the early gene expression process, glycosylation process of viral proteins, and inhibition of virus replication by hindering cellular DNA polymerase (Armaka et al., 1999). Some of the pathogens targeted include many DNA and RNA viruses, such as herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), dengue virus type 2, Junin virus, influenza virus adenovirus type 3, poliovirus, rhinovirus, and coxsackievirus B1 (Swamy et al., 2016). Activities of essential oils extracted from

Australian tea tree oil, eucalyptus oil, thyme oil, and many other medicinal and aromatic plants have been studied for their effect against viruses (Juglal et al., 2002; Akhtar., 2014).

Common Name	Plant	Major Essential Oil	Inhibited Microorganism
Thyme	*Thymus vulgaris*	Thymol	*S. aureus, V. parahaemolyticus, C. perfringens, L. monocytogenes*
Oregano	*Origanum vulgare*	Carvacrol	*Polio virus, Adeno virus, L. monocytogenes*
Garlic	*Allium sativum*	Isothiocynate	*Candida spp., Enterobacteriaceae*
Lemon	*Balm Melissa officinalis*	Linalool, myrcene, camphor	*HSV-2, avian influenza virus, L. monocytogenes*
Cinnamon	*Cinnamomum zelancium*	Cinnamaldehyde	*Enterobacteriaceae, P. mirabilis, S. pyogenes*
Lavender	*Lavandula angustifolia*	Linalool, Linalyl acetate	*E. coli, M. smegmatis*

Table 8: Common essential oils, plant of origin and microorganisms affected by the extracted active compounds.

6.3 The site of action of EOs

High-resolution microscopy, such as electron microscopy or atomic force microscopy (AFM), can reveal the most extreme consequences of exposure to an antimicrobial compound, i.e., deformation of cells occurring from lysis or from damages to the cell wall. An advantage of TEM is that ultra-thin cross sections can reveal ultrastructural changes in the interior of the cell. Scanning electron microscopy (SEM) and AFM only image the cell surface. AFM has one important advantage over electron microscopy, in that it allows measurements in liquid under physiological conditions, avoiding difficult sample preparation and the artifacts associated herewith (Alessandrini et al., 2005). A limitation of both AFM and electron microscopy is, however, that specific cellular structures must be identified according to morphology unless some form of labeling can be applied. While antibodies conjugated to metal nanoparticles have been used with TEM, no labeling techniques have been reported for SEM and AFM. It is, however, possible to combine AFM with optical microscopy and thus take advantage of the numerous options for fluorescent labeling of biomolecules. An important

site of action is the cell membrane, and indeed, many essential oil constituents have been proposed to act on the bacterial membrane. Interaction of antimicrobial compounds with the membrane can affect the transport of nutrients and ions, the membrane potential, and the overall permeability of the cell. These effects are investigated by measuring the efflux of intracellular ions like K+ and H+ (Ultee et al., 1999; Lambert et al., 2001). Efflux of small ions is not necessarily indicative of complete loss of membrane function and can be observed in viable cells where growth is inhibited because the cell uses energy for repair or survival rather than cell proliferation (Bouhdid et al., 2010). Effects on the cell membrane that lead to cell death is more accurately predicted by detecting the efflux of larger molecules like ATP or carboxyfluorescein diacetate (cFDA) after esterase reaction (Xu et al., 2008), or by influx of large polar organic DNA-binding stains like ethidium bromide (Lambert et al., 2001) and propidium iodide (Bouhdid et al., 2010). It is always good practice to validate the observed effects by combining several techniques. Monitoring the release of calcein encapsulated in membrane vesicles can for example be used as a complimentary technique to confirm the membrane as the site of action (Miron et al., 2000). If no effects are observed on cell structure and membrane functionality, it is assumed that the site of action is intracellular. The target can be proteins and enzymes in general, or it can be essential cellular processes involved in biosynthesis or energy generation. An intracellular site of action can for example be determined by incorporation of radioactively labeled substrates used in particular biosynthesis pathways (Schneider et al., 2010). Lack of or decreased incorporation is then taken as an indication of the process being affected by the antimicrobial compound. For example, radiolabeled nucleotides or amino acids can be used to detect if DNA replication or protein synthesis takes place, respectively (Schneider et al., 2010). Some compounds have multiple sites of action, and in that case, it can be difficult to pinpoint which one is ultimately responsible for cell death. For example, a compound that affects membrane permeability will also affect the membrane potential and thereby energy generation by respiration. It is thus difficult to distinguish direct effects on energy-generating processes from the indirect effect a permeable membrane has on these processes. At sublethal concentrations, changes to the transcriptome and proteome during exposure can reveal how the cell responds to the compound, and upregulation of genes involved in certain metabolic, or biosynthesis pathways can be indicative of which cell structures or processes that are affected (Burt et al., 2007; Rao et al., 2010).

6.4 Mode of Action of EOs

The probably most comprehensive approach to investigate the mode of action of a particular compound is to perform random transposon mutagenesis to search for mutations that compensate for the antimicrobial effect of a particular compound. In this way, it is possible to identify the mode of action of compounds that interact very specifically with, e.g., a single enzyme or with proteins or lipids in the membrane (Shapira et al., 2007; Van Hoang et al., 2011). The approach is, however, not suited for investigating antimicrobial compounds that act simultaneously on several components in the cell, as a single mutation is unlikely to facilitate compensation for the antimicrobial effect on the cell. Antimicrobial compounds that act on the membrane can cause depolarization or increased permeability through various mechanisms. For example, some antimicrobial peptides form pores (Cotter et al., 2005; Fantner et al., 2010) while other compounds, such as certain essential oil constituents, have a fluidifying effect on the membrane (Trombetta et al., 2005; Cristani et al., 2007). Membrane properties like lipid packing can be investigated in membrane vesicles by LAURDAN staining combined with spectrofluorometry (Nielsen and Otzen, 2010), and membrane fluidity can be investigated directly in bacteria by differential scanning calorimetry (Trombetta et al., 2005) or fluorescence anisotropy measurements of DPH using a spectrofluorometer (Liao et al., 2010). AFM imaging has also in recent years allowed highresolution visualization of native membranes on a solid support. Structural changes resulting from integration of an antimicrobial compound into the membrane can thus be visualized directly (Brasseur et al., 2008), and the effect on membrane rigidity can be quantified by AFM force spectroscopy (Sullan et al., 2010). Functionalizing the AFM tip with the antimicrobial compound of interest furthermore allows investigation of interaction forces between the compound and its target. This approach wasfor example used to map binding events of vancomycin on the surface of bacteria and confirmed that binding occurred at the site of cell wall synthesis in dividing cells (Gilbert et al., 2007).

6.5 Components of Essential Oils with Antimicrobial Activity

The major constituents of EOs can constitute up to 85%, whereas other components are present in trace amounts (Bakkali et al., 2008). α-phellandrene (36%) and limonene (31%) in *Anethum graveolens* leaf oil, d-limonene (over 80%) in citrus peel oils, α-phellandrene (36%) and limonene (31%) in *Anethum graveolens* leaf oil, carvacrol (30%) and thymol (27%) in *Origanum compactum* oil, α/β-thujone (57%) and camphor (24%) in *Artemisia* herba-alba oil, carvone (58%) and d-limonene (37%) in *Anethum graveolens* seed oil, and menthol (59%) and menthone (19%) in *Mentha*

piperita oil are among the constituents present at relatively higher concentrations in essential oils (Gallucci et al., 2009). Generally, the biological properties of the essential oils are determined by their major components including two groups of distinct bio-synthetical origin (Rivas, et al., 2010; Zhou et al., 2007). Terpenes and terpenoids comprise the main groups whereas aromatic and aliphatic constituents comprise the other group, all characterized by low molecular weight.

6.5.1 Terpenes and Terpenoids

Several isoprene units (C5H8) upon combination result in the production of hydrocarbons called terpenes. Occurring in the cytoplasm of plant cells, biosynthesis of terpenes proceeds via the mevalonic acid pathway starting from acetyl-CoA. Having a backbone of hydrocarbons, cyclases can rearrange terpenes into cyclic structures, thus forming monocyclic or bicyclic structures (Ultee et al., 2000). Terpene biosynthesis consists of synthesis of the isopentenyl diphosphate (IPP) precursor, IPPs being added repetitively to form the prenyldiphosphate precursor of the various classes of terpenes, terpene-specific synthetase modification of the allylic prenyldiphosphate to form the terpene skeleton, and finally, secondary enzymatic modification (redox reaction) of the skeleton to attribute functional properties to the different terpenes (Lambert et al., 2001). Monoterpenes (C10H16) and sesquiterpene (C15H24) are the main terpenes, but longer chains such as diterpenes (C20H32), triterpenes (C30H40), etc., also exist. p-cymene, limonene, menthol, eugenol, anethole, estragole, geraniol, thymol, γ-terpinene, and cinnamyl alcohol are among the examples of some constituents of essential oils with antimicrobial activity. Angelica, bergamot, lemongrass, mandarin, mint, caraway, celery, citronella, coriander, eucalyptus, geranium, petitgrain, pine, juniper, lavandin, lavander, lemon, orange, peppermint, rosemary, sage, and thyme are among the representatives of plants with some of these compounds (Lambert et al., 2001). Oxygenated monoterpene (β-fenchol) and oxygenated sesquiterpene (α-eudesmol) were identified as the two main bioactive constituents in the essential oil obtained from fresh leaves of *Eucalyptus teretecornis* with a minimum inhibitory amount (MIA) of 28 µg and 10 µg against *Alternaria alternata* (Ultee et al., 2000). Similarly, another study reported β-fenchol and linalool as the two antimicrobial components in essential oil obtained from the fresh leaves of Zanthoxylum alatum (Mulyaningsih et al.,2010). Biochemical modifications of terpenes via enzymes that add oxygen molecules and move or remove methyl groups result in the formation of terpenoids (Ultee et al., 2000). Terpenoids can be sub-divided into alcohols, phenols, esters, aldehydes, ethers, ketones, and epoxides. Thymol, carvacrol, linalool, linalyl acetate, citronellal, piperitone, menthol, and geraniol are the examples of terpenoids. In one study, α-cedrol was reported as the bioactive constituent of the essential oil from fresh leaves of *Thuja orientalis* with

a minimum inhibitory amount (MIA) of 30.5 µg against *A. alternate* (Van Vuuren et al., 2007). Monoterpenoid phenols present in the essential oil of *Origanum vulgare*, thyme, pepperwort, and wild bergamot are carvacrol or cymophenol. Diarrheal toxin production by *Bacillus cereus* and growth of vegetative bacteria were inhibited by carvacrol. The precursor of carvacrol is p-cymene which is a monoterpene with a benzene ring without any functional groups on its side chains. When used alone, p-cymene is not an efficient antimicrobial compound (Tserennadmid et al., 2011; de Azeredo et al., 2011), but the activity of compounds like carvacrol is potentiated by p-cymene (Goñi et al., 2009) and polymyxin B nona peptide (Gutierrez et al., 2009). It has been shown that p-cymene is hydrophobic in nature and causes swelling of the cytoplasmic membrane to a greater extent (Bassolé et al., 2011). Also, p-cymene influenced the synthesis of protein in *E. coli* cells. It is expected that the antimicrobial action of phenolic compounds such as thymol and carvacrol is attributed to structural and functional damages in the cytoplasmic membrane (Mackay et al., 2000). The primary mode of antibacterial action of thymol is not completely understood but is believed to involve disruption of outer and inner membrane and interaction with membrane proteins and intracellular targets. Thymol (or 2-isopropyl-5-methylphenol), a natural monoterpene phenol derivative of cymene, is isomeric with carvacrol present in thyme essential oil and is extracted from *Thymus vulgaris* (common thyme) and various other plants (White et al., 1996). In a study by Di Pasqua et al. (2006) interaction of thymol with membrane proteins was further supported by exposing *Salmonella enterica* to sub-lethal concentrations of thymol, and accumulation of outer membrane proteins in misfolded pattern and upregulation of genes involved in synthesis of outer membrane proteins was also observed (Shin et al., 2003). The citrate metabolic pathway was also impaired by thymol and many enzymes involved directly or indirectly in ATP synthesis. Intracellular action of thymol indicates that it affects important energy-generating processes, which lower the ability of a cell to recover after exposure to thymol. Studies pertaining to investigation of the mode of action of thymol against yeast and fungi point towards the interaction of thymol with the cell envelope and intracellular targets. It has been shown that thymol disrupted vesicles and cell membranes, and impaired biosynthesis of ergosterol in *Candida* strains, which consequently affected the integrity of cell membrane because membrane fluidity and asymmetry is regulated by ergosterol similarly to cholesterol in animal cells (Shin et al., 2003). Rao et al. (2010) proposed that specific signaling pathways are activated by thymol in yeast, rather than causing non-specific lesion of membranes. This was based on the observation that cytosolic Ca2+ bursts caused by thymol and transcription responses similar to those in Ca2+ stress and nutrient starvation are activated (Ahmad et al., 2011). Moreover, an increase in the permeability of *P. aeruginosa* and *S. aureus* cells was observed in ethidium bromide (fluorescence nuclear stain), dissipated pH gradients irrespective of glucose

availability, and leakage of inorganic ions. These results were in accordance with a study that utilized a mixture of thymol and carvacrol (Lambertet et al.,2011). A major constituent of oregano is carvacrol (a phenolic monoterpenoid). Carvacrol is one of the most extensively studied essential oil constituents together with its closely related isomer thymol. EOs rich in carvacrol have been reported to possess remarkable antimicrobial activity (Magi et al., 2015). Although the outer membrane is affected by carvacrol, the cytoplasmic membrane is thought to be its site of action, causing passive transport of ions across the membrane. As an adaptation mechanism to maintain optimal membrane function and structure, it has been proposed that cells exposed to carvacrol change the fatty acid composition of the membrane because of the effect of carvacrol on fluidity (Di Pasqua et al., 2006; Di Pasqua et al., 2007). It has been demonstrated that carvacrol affects the outer membrane of Gram-negative bacteria (La Storia et al., 2011). According to Friedman et al. (2004) based on the time they take to produce significant action, essential oils can be divided into the following two types: compounds that act slowly and compounds with fast action. Examples of some antimicrobials considered as fast acting compounds are carvacrol, cinnamaldehyde, and geraniol since they inactivate organisms like *E. coli* and *Salmonella* in a short time of five minutes. It was reported that a time duration of 30–60 min was required to show efficient antimicrobial activity for the compounds acting slowly (Friedman et al., 2004). Carvacrols' mechanism of antifungal activity is like thymol, showing H+ homeostasis and disruption of Ca2+, up- and down-regulation of gene transcription similar to that found in Ca2+ stress and nutrient starvation (Rao et al., 2010), disruption of membrane integrity, and impairment of biosynthesis of ergosterol in *Candida* strains (Ahmad et al., 2011). Silva-Angulo et al. (2015) showed that citral exhibited antilisterial activity against *L. innocua* and *L. monocytogenes* and can be applied in active packaging to control possible recontamination of foods or in combination with other preservation technologies (Klein et al., 2013). Similarly, Klein et al. (2013), determined the antimicrobial activity of six essential oil components against the potential food spoilage bacteria *Aeromonas hydrophila*, *Escherichia coli*, *Brochothrix thermosphacta*, and *Pseudomonas fragi* for single use and in combination with each other (Silva-Angulo et al., 2015). They further showed that, for single use, the most effective oil components were thymol (bacteriostatic effect starting from 40 ppm, bactericidal effect with 100 ppm) and carvacrol (50 ppm/100 ppm), followed by linalool (180 ppm/720 ppm), α-pinene (400 ppm/no bactericidal effect), 1,8-cineol (1400 ppm/2800 ppm), and α-terpineol (600 ppm/no bactericidal effect).

6.5.2 Phenylpropenes

In plants, synthesis of phenylpropenes occurs from the amino acid precursor phenylalanine, constituting a subfamily among the various groups of organic compounds called phenylpropanoids. A relatively small proportion of essential oils is composed of phenylpropenes, and the phenylpropenes that have been most thoroughly studied are safrole, eugenol, isoeugenol, vanillin, and cinnamaldehyde. Some representative bioactive compounds present in essential oils are reported in Figure 5.

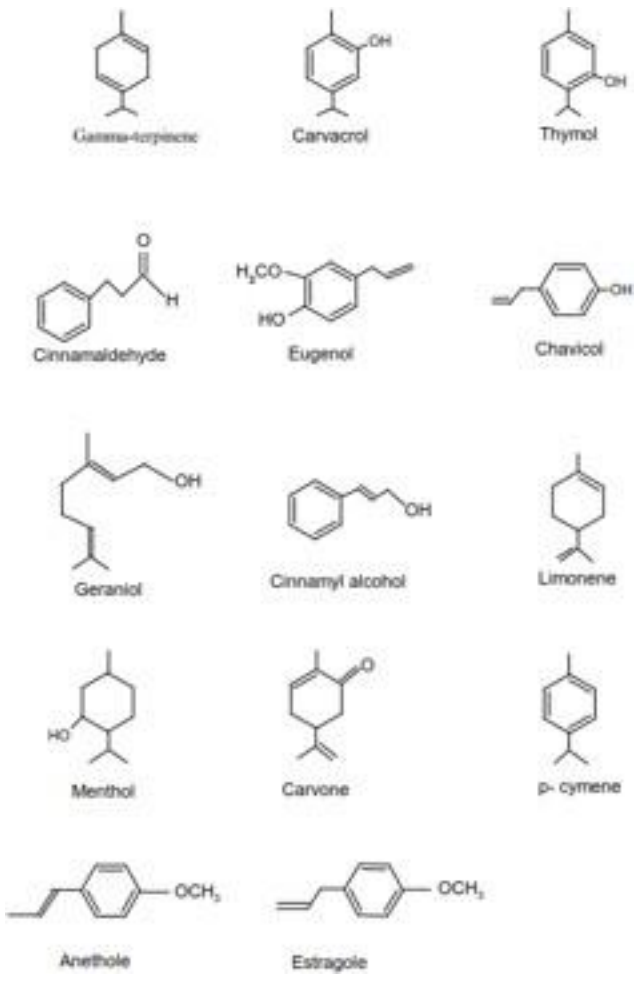

Figure 5: some representative bioactive compounds present in essential oils

Eugenol, which is a clear to pale yellow oily liquid is extracted from clove oil, nutmeg, cinnamon, basil, and bay leaves. A study reported eugenol as the antifungal bioactive molecule from *Cinnamomum tamala*, with a minimum inhibitory amount of 9.5 and 8.2 µg against *Alternaria alternata* and *Curvularia lunata*, respectively (Heer et al., 2017). Eugenol has also been shown to cause deterioration of the cell wall, lysis of cells, and prevention of enzyme action in *Enterobacter aerogenes* (Vergis et al., 2015). The antimicrobial activity of phenylpropenes is dependent on the selected microbial strains, the kind and number of substituents on the aromatic ring, and experimental parameters such as temperature and medium chosen for growth, etc. (Paul, 2011). Cinnamaldehyde is a flavor- and odor-giving organic compound. Being a pale-yellow viscous liquid, it occurs naturally in the bark of cinnamon trees and other species of the genus *Cinnamomum*. It is found as growth inhibitor of *Escherichia coli* and *Salmonella typhimurium* but does not disintegrate the outer membrane or deplete the intracellular ATP pool (Vergis et al., 2015).

6.6 Nano-Encapsulation of Essential Oils for Enhancing Their Antibacterial Effect

A process resulting in the formation of small capsules with many useful properties by surrounding droplets of the bioactive in nature with a coating or embedding them in a homogeneous or heterogeneous matrix is called encapsulation (Sagalowicz et al., 2010). Oil encapsulation may retard or even prevent thermo-oxidation reactions, leading to a widening of the intended range of enrichment purposes for food commodities (Soltani et al., 2015). Bioactive oils are commonly used for their pharmaceutical, cosmetic and nutritional properties. Generally, EOs are volatile substances sensitive to oxygen, light, moisture, and heat. These reported special characteristics could diminish their applicability in cosmetics, food and pharmaceutical industries. Thus, encapsulation is one of the most efficient methods for the formulation of bioactive oils and various studies have been developed in this aspect. The encapsulation system is selected in line with the intended usage of the final formulation, which can vary depending on the size, shape, or nature of selling components. The growing interest in the use of essential oils as natural antimicrobials and preservatives in the food industry has been driven in the last years by the growing consumer demand for natural products with improved microbial safety, and fresh-like organoleptic properties. Nano-emulsions efficiently contribute to support the use of EOs in foods by increasing their dispersibility in the food areas where microorganisms grow and proliferate, by reducing the impact on the quality attributes of the product, as well as by enhancing their antimicrobial activity (Donsì et al., 2016). Beyki et al. (2014) showed that MIC values of free as well as chitosan–cinnamic (CS–Ci) acid nanogel-encapsulated *Mentha*

piperita essential oils against *A. flavus* under sealed condition were 2100 and 500 ppm, respectively. Contrary to this, when tested under non-sealed conditions, the encapsulated oils performed better (800 ppm), while within the concentration range tested (up to 3000 ppm) the free oils failed to cause complete inhibition. As a carrier for essential oils to enhance their antimicrobial properties, these findings revealed the promising role of CS–Ci nanogel. A higher in vitro bactericidal action of nano-emulsions loaded with essential oils of lemongrass, clove, thyme or palmarosa against *Escherichia coli* has been reported as these nano-emulsions achieved log-reductions of 4.1, 3.6, 2.8 or 3.9, respectively, after a contact time of 30 min. In the case of nano-emulsions containing lemongrass or clove essential oils, faster and enhanced inactivation kinetics were also observed compared to their respective coarse emulsions (Salvia-Trujillo et al., 2015). Herculano et al. (2015) nano-encapsulated *Eucalyptus staigeriana* essential oil (EOs) using cashew gum (CG) as wall material with sizes of nano-emulsions ranging from 27.70 nm to 432.67 nm with negatively charged surfaces. The antimicrobial activity of nanoparticles against *Listeria monocytogenes* (Gram-positive) and *Salmonella Enteritidis* (Gram-negative) was evaluated by determining their minimum bactericidal concentration, The data from MBC showed greater antibacterial activity against Gram-positive bacteria, due to a likely synergistic effect between the EOs and CG. Thus, the data mentioned above suggest that the nanoparticles of EOs have potential to be used as natural food preservatives (Herculano et al., 2015). Another study revealed superior performance of encapsulated *Zataria multifloria* essential oil (ZEO) by chitosan nanoparticles (CSNPs) under both in vivo and in vitro conditions as compared to unmodified ZEO against *Botrytis cinerea*. The in vivo experiment also showed that at a 1500 ppm concentration, the encapsulated oils significantly decreased both disease severity and incidence of *Botrytis*-inoculated strawberries during 7 days of storage at 4 °C followed by 2–3 days at 20 °C. In another study, the role of CSNPs as a controlled release system for EOs has been suggested (Mohammadi et al., 2015). Tornuk et al. (2011) produced novel water-soluble and thermally stable chitosan nanoparticles loaded with different levels (1%, 1.2%, 1.4% and 1.5%) of summer savory (*Satureja hortensis* L.) essential oil using an ionic gelation method. NPs loaded with essential oils exhibited strong antibacterial activity against *Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli* depending on the concentration of EO encapsulated. It was concluded that the summer savory EO-loaded chitosan NPs were highly adapted to excessive environmental factors such as high temperature and acidic pH, possessing high bioactive properties convenient for future food processing and packaging applications (Feyzioglu et al., 2016). Also, Zhang et al. (2017) prepared blended cloves/cinnamon essential oil nano-emulsions using Tween 80 and ethanol as surfactant and co-surfactant, respectively. Even at far lower concentrations, the nano-emulsion showed higher antimicrobial activity against the four tested microorganisms *Escherichia*

coli, Bacillus subtilis, Salmonella typhimurium, and *Staphylococcus aureus*. This shows that blended cloves/cinnamon essential oil nano-emulsions have the potential to be a natural antimicrobial agent in the food industry (Zhang et al., 2016). Mohammadi et al. (2015), studied the performance of *Cinnamomum zeylanicum* essential oil (CEO) when encapsulated by CSNPs under both in vitro and in vivo conditions in comparison with unmodified CEO against *Phytophthora drechsleri*. The in vivo study showed that at a concentration of 1.5 g/L the encapsulated oils of *Cinnamomum zeylanicum* significantly decreased both disease severity as well as incidence of *Phytophthora* in inoculated cucumbers over 7 days of storage at 4 ◦C followed by 2–3 more days at 20 ◦C. Furthermore, the shelf life of cucumbers with CEO-CSN coating was extended up to 21 days at 10 ± 1 ◦C while uncoated fruit were unmarketable in less than 15 days. In addition to this, CEO-CSN coated fruits were firmer, maintained color, water content, had improved microbiological and physicochemical quality and showed lower microbial counts throughout storage. Thus, CEO-CSN coatings can be an effective method to extend cucumber shelf life. Similarly, Guerra-Rosas et al. (2017) assessed the antimicrobial activity of nano-emulsions containing oregano, thyme, lemongrass or mandarin essential oils and high methoxyl pectin during a long-term storage period (56 days) against *E. coli* and *Listeria innocua*. Regardless of the EO type, a higher antimicrobial activity was detected against *E. coli* as compared to *L. innocua*. Significant damage in the *E. coli* cells for both the cytoplasm and cytoplasmic membrane led to cell death which was revealed by the images of transmission electron microscopy (TEM). The antimicrobial activity of the nano-emulsions was found to be strongly related to the EO type rather than to their droplet size. The smallest droplet size (11 ± 1 nm) of the lemongrass-pectin nano-emulsion had a higher antimicrobial activity, reaching 5.9 log reductions of the *E. coli* population. Nevertheless, nano-emulsion of the freshly-made oregano, thyme and mandarin EO–pectin led to 2.2, 2.1 or 1.9 *E. coli* log-reductions, respectively. However, a significant decrease in the antimicrobial activity was observed during storage regardless of the EO type, which was related to the loss of volatile compounds over time (Guerra-Rosas et al., 2017). Besides EOs, there are numerous reports available on the antimicrobial efficacy of nanoparticles. Prabhu and Poulose reviewed various mechanisms of antimicrobial action of NPs, especially silver NPs (Prabhu et al., 2012), which are potential antimicrobial agents (Rai et al., 2014). Silver NPs create pits and thus anchor and penetrate the cell wall, causing release of free radicals followed by structural change in the cell membrane leading to a greater influx of the antibacterial agent through cytoplasmic membrane because of increased cell permeability, resulting in cell death. Another mechanism of the silver nanoparticles is the formation of free radicals, by which the cells die. It has been suggested by electron spin resonance spectroscopy studies that silver nanoparticles form free radicals, when in contact with bacteria, and these free radicals have the ability to make the cell membrane porous by damaging it

which can ultimately lead to cell death (Danilcauk et al., 2006). Moreover, silver nanoparticles attacking DNA bases can also inhibit signal transduction and cell wall formation, and interact with respiratory enzymes, liberating reactive oxygen species which is followed by cell death. Over the last decade, several research studies have focused on the synergy between EOs and various types of NPs for their superior antimicrobial efficacy. Cinnamaldehyde, a representative of EO, showed a strong synergistic activity with silver NPs against spore-forming *Bacillus cereus* and *Clostridium perfringens*. Bacterial kill curve analysis revealed rapid bactericidal action exerted by this combination of antimicrobial agents, while extensive damage to the cell envelope was evidenced by electron and atomic force microscopy (Ghosh et al., 2013). Release of silver ions by nanoparticles can inactivate many enzymes by interacting with the thiol groups (Feng et al., 2008; Matsumura et al., 2003). An enhancement in the antimicrobial potential of EOs can be attained by encapsulating with various nanomaterials e.g., solid lipid NPs, liposomes, polymeric NPs and nano-emulsions, where the inside core consists of EO while nanomaterial forms the outer nano-capsule. Essential oils upon nano-encapsulation exhibit physical stability, decreased volatility and protection from environmental interactions (e.g., light, oxygen, moisture, pH), enhanced bioactivity and reduced toxicity (Ravi Kumar et al., 2000). Small nano-emulsion droplets are able to bring the EOs close to the cell membrane surface, improving the accessibility to microbial cells and enabling the membranes of the cells to be disrupted, possibly by altering the integrity of phospholipid bilayer or by interfering with the embedded phospholipid bilayer active transport proteins (Moghimi et al., 2016). In order to modulate drug release i.e., burst release and/or controlled release, this represents a promising approach (Bilia et al., 2014). The in vitro release study of encapsulated oregano EO with chitosan NPs revealed an initial burst effect followed by slow release of drug (EO) (Hosseini et al., 2013; Abreu et al., 2012). Similarly, efficient biocidal activity against *Stegomyia aegypti larvae* was observed due to a slow and sustained release of EO by chitosan/ cashew gum nano-encapsulation. The effect of EO nano-emulsions on yeast cells has also been addressed in several studies among which *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae* are the most investigated. Yeast cells required longer incubation times with respect to bacterial inactivation, when exposed to carvacrol, cinnamaldehyde, and d-limonene nano-emulsions (Donsì et al., 2012), and exhibited lower minimum inhibitory concentration for encapsulated d-limonene (Zhang et al., 2014). In another study, an enhancement in the antimicrobial activity of carvacrol loaded in polylactic glycolic acid nano-capsules was reported due to significant transformation in the rheological characteristics of bacterial biofilm that potentially facilitated the activity of carvacrol (Iannitelli et al., 2011). EOs are protected from enzymatic degradation by the nano-carriers which transform them into powder and help to achieve the desired therapeutic levels for the required time duration to the target tissues with reduction

in number of doses and may also ensure an optimal pharmacokinetic profile (Bilia et al., 2014). Thus, the combination of various EOs with their inherent antimicrobial activity with other potent antimicrobial agents like NPs may greatly enhance their antimicrobial activity by complementing each other with the involvement of various mechanisms against different types of pathogens. Therefore, combinations of different antimicrobials appear to be the best strategy for controlling multidrug resistant microbes.

6.7 Synergies between Essential Oil components

The interaction between antimicrobials in a combination can have three different outcomes, synergistic, additive, or antagonistic. Synergy occurs when a blend of two antimicrobial compounds has an antimicrobial activity that is greater than the sum of the individual components. An additive effect is obtained when the combination of antimicrobials has a combined effect equal to the sum of the individual compounds. Antagonism occurs when a blend of antimicrobial compounds has a combined effect less than when applied separately (Davidson et al., 1989; Burt, 2004). The combined effect of a blend is analyzed by using measurements of the MIC to calculate the fractional inhibition concentration index (FICIndex) according to the formulas defined by (Davidson et al., 1989): FICA = MICA+B/MICA, FICB = MICB+A/MICB, FICIndex = FICA + FICB. The MICA+B value is the MIC of compound A in the presence of compound B, and vice versa for MICB+A. Calculating the FIC value for either substance A or B then requires determination of the MIC for the individual components. Theoretically, a FICIndex near 1 indicates additive interactions, while below 1 implicates synergy, and above 1 antagonism (Davidson et al., 1989). However, this definition has been replaced by a more general one where the FICIndex results are interpreted as synergistic if FICIndex < 0.5, additive if 0.5 < FICIndex < 4, or antagonistic if FICIndex > 4 (Odds, 2003). The antimicrobial activity of a given essential oil may depend on only one or two of the major constituents that make up the oil. However, increasing amounts of evidence indicate that the inherent activity of essential oils may not rely exclusively on the ratio in which the main active constituents are present, but also interactions between these and minor constituents in the oils. Various synergistic antimicrobial activities have been reported for constituents or fractions of essential oils when tested in binary or ternary combinations (Delaquis et al., 2002; Pei et al., 2009; García-García et al., 2011; Nguefack et al., 2012). For example, García-García et al. (2011) found the most synergistic binary combination against *L. innocua* to be carvacrol and thymol, and the most active ternary combination to be carvacrol, thymol, and eugenol. Reports on greater antimicrobial activity of crude essential oils compared to blends of their major individual components suggests that trace components in the crude

essential oils are critical to the activity and may have a synergistic effect (Marino et al., 2001; Delaquis et al., 2002; Burt, 2004; Koutsoudaki et al., 2005). In contrast to this, trace components may also cause antagonistic interactions, which were seen by comparing the antimicrobial effect of pure carvacrol to oregano oil where carvacrol is a major constituent. Pure carvacrol was 1500 times more effective than the crude essential oil (Rao et al., 2010). Among individual essential oil constituents, synergy has been observed for carvacrol and p-cymene on *B. cereus* (Ultee et al., 2002; Rattanachaikunsopon et al., 2010). It appears that p-cymene swells bacterial cell membranes, probably enabling easier entrance of carvacrol into the cell membrane where it exerts its action (Ultee et al., 2002). Furthermore, Bassolé et al. (2010) showed that if linalool or menthol was combined with eugenol it showed the highest synergy, suggesting that a monoterpenoid phenol combined with a monoterpenoid alcohol is an effective combination. Little is currently known about what governs synergy and antagonism among essential oil constituents. Four theoretical mechanisms of antimicrobial interactions produce synergy: (i) sequential inhibition several steps in a particular biochemical pathway, (ii) inhibition of enzymes that degrade of excrete antimicrobials, (iii) interaction of several antimicrobials with the cell wall, or (iv) interaction with the cell wall or membrane that leads to increased uptake of other antimicrobials (Davidson et al., 1989; Eliopoulos et al., 1996). Another possibility for synergistic effects could be that antimicrobials have different mode of actions, thereby attacking two different sites on or in the cell, which indirectly depend on each other. Even less is known about the cause antagonism, it is hypothesized to occur when: (i) combining bacteriostatic and bactericidal antimicrobials, (ii) antimicrobials have the same site of action, (iii) antimicrobials interact with each other (Davidson et al., 1989), Larson (1985) in Roller (2003). The hypothesized synergistic or antagonistic interactions are based on 15-year-old results, and with the emergence of new techniques this field is likely to see some significant advances in our understanding of how antimicrobial compounds affect each other when acting in concert. In practice, the knowledge needed to exploit synergistic combinations of essential oils in food products is (i) the site and mode of action of each essential oil constituent, and (ii) the mechanisms resulting in synergy or antagonism between several compounds, and (iii) how each compound interacts with food matrix components in a way that affects is antimicrobial properties. When the mechanistic details for synergistic interactions are better understood, it will be easier to exploit synergies using intelligent combinations of constituents to combat food spoilage microorganisms.

6.7.1 Synergism between Different Essential Oils

The antimicrobial properties of EOs have been reported in several studies (Bakkali et al., 2008; Burt, 2004; Bajpai et al., 2012). In many cases the activity results from the complex interaction between the different classes of compounds such as phenols, aldehydes, ketones, alcohols, esters, ethers or hydrocarbons found in EOs (Kim et al., 1995; Lambert et al., 2001). Though in some cases, the bioactivities of EOs are closely related with the activity of the main components of the oils (Juliani et al., 2002). Several studies have found that a number of these compounds exhibited significant antimicrobial properties when tested separately (Bassolé et al., 2010). It has been reported that EOs containing aldehydes or phenols, such as cinnamaldehyde, citral, carvacrol, eugenol or thymol as major components showed the highest antibacterial activity, followed by EOs containing terpene alcohols. Other EOs, containing ketones or esters, such as β-myrcene, α-thujone or geranyl acetate had much weaker activity. While volatile oils containing terpene, hydrocarbons were usually inactive (Ait-Ouazzou et al., 2011). High antimicrobial activity of *Thymus* and *Origanum* species has been attributed to their phenolic components such as thymol and carvacrol (Soković et al., 2009) and those of *Eugenia caryophillus* (Ait-Ouazzou et al., 2011), *Syzygium aromaticum* (Juliani et al., 2004), *Ocimum basilicum* (Bassolé et al., 2010) to eugenol. The antimicrobial activity of the EO of *Cinnamomum zeylanicum* has been related to its cinnamaldehyde content (Prabuseenivasan et al., 2006), though cinnamaldehyde-containing oils (non-phenolic) showed lower antimicrobial activities than eugenol oils (Hazzit et al., 2009). In basil, the strongest antimicrobial activity of sweet basil was attributed to eugenol (19%) and linalool (54%) content, and a synergistic effect was observed. The importance of the hydroxyl group (-OH) of phenols was demonstrated by the higher antimicrobial and antioxidant activities of eugenol in relation to methyl eugenol (-O-Me) (Juliani et al., 2009). Terpinen-4-ol is the principal active component *of Melaleuca alternifolia* (tea tree) oil (Southwell et al., 1993). Lis-Balchin and Deans (1997) showed that EOs containing large amounts of 1,8-cineole were better anti-listerial agents than EOs devoid of it. The weak antimicrobial activity of the EOs of *Chaerophyllum libanoticum* (Demirci et al., 2007), *Tanacetum argenteum* subsp. flabellifolium (Tabanca et al., 2007), *Cupressus arizonica* (Chéraif et al., 2007) has been attributed to their high hydrocarbon content. Different terpenoid components of EOs can interact to either reduce or increase antimicrobial efficacy (Delaquis et al., 2002). The interaction between EO compounds can produce four possible types of effects: indifferent, additive, antagonistic, or synergistic effects (Delgado et al., 2004). An additive effect is observed when the combined effect is equal to the sum of the individual effects. Antagonism is observed when the effect of one or both compounds is less when they are applied together than when individually applied. Synergism is observed when the effect of the

combined substances is greater than the sum of the individual effects (Burt, 2004) while the absence of interaction is defined as indifference. Interestingly, phenolic monoterpenes and phenylpropanoids (typically showing strong antimicrobial activities) in combination with other components were found to increase the bioactivities of these mixtures. Most of the studies have focused on the interaction of phenolic monoterpenes (thymol, carvacrol) and phenylpropanoids (eugenol) with other groups of components, particularly with other phenols, phenylpropanoids and monoterpenes alcohols, while monoterpenes and sesquiterpenes hydrocarbons were used to a lesser extent (Table 9). The combination of phenolics with monoterpenes alcohols produced synergistic effects on several microorganisms, in particular, the combination of phenolics (thymol with carvacrol, and both components with eugenol) were synergistically active against *E. coli* strains. Though other reports have observed additive (Lambert et al., 2001) and antagonism effects (Gallucci et al., 2009) (Table 9). Mixtures of cinnamaldehyde with carvacrol or thymol yielded in most cases synergistic effects against *E. coli* and *S. typhinurium*, though in one case an additive effect was observed (Table 9). Other monoterpenes have also been tested, particularly the oxide 1,8-cineole that in combination with sesquiterpene and monoterpene hydrocarbons (e.g., aromadendrene and limonene) were found to have additive and synergistic effects, respectively. Other combinations including a monoterpene hydrocarbon (α-pinene) with limonene or linalool also showed additive and synergistic effects (Table 9). Mixture of EOs have also been shown to interact with each other acting as additive, synergistic and in a few cases antagonistic agents (Table 9). The essential oil of oregano (*Origanum vulgare*) was the most used EO (rich in thymol and carvacrol) and combined with rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris*), basil (*Ocimum basilicum*), marjoram (*O. majorana*) and lemon balm (*Melissa officinalis*) (Table 9). In most cases only additive effects were observed, only the combination with rosemary oil yielded synergistic effects (Table 9). Most studies attributed additive and synergism effects to phenolic and alcohol compounds (Table 9). Generally, compounds with similar structures exhibit additive rather than synergistic effect. The occurrence of additive interaction of some essential oils has been related to their main phenolic compounds (carvacrol and thymol) (de Azeredo, et al., 2011). Antagonistic effect has been attributed to the interaction between non-oxygenated and oxygenated monoterpene hydrocarbons (Lambert et al., 2001; Goñi et al., 2009).

Pair combination	Organism	Method	Interaction	References
Thymol/carvacrol	*Staphylococcus Aureus, Pseudomonas. Aeruginosa*	Half dilution	Additive	Lambert *et al.* [23]
	Escherichia Coli	Checkerboard	Synergism	Pei *et al.* [54]
	S. aureus, Bacillus. cereus, E coli	Checkerboard	Antagonism	Gallucci *et al.* [55]
	S. aureus, P. aeruginosa	Mixture	Additive	Lambert *et al.* [23]
	E. coli	Checkerboard	Additive	Rivas *et al.* [56]
	Salmonella typhinurium	Mixture	Synergism	Zhou *et al.* [57]
Thymol/eugenol	*E. coli*	Checkerboard	Synergism	Pei *et al.* [54]
Carvacrol/eugenol	*E. coli*	Checkerboard	Synergism	Pei *et al.* [54]
	S. aureus, B. cereus,	Checkerboard	Antagonism	Gallucci *et al.* [55]
α-pinene/Linalool				
Linalool/ Terpinen-4-ol				
O. vulgare/Rosmarinus officinalis	*L. monocytogenes, Yersinia enterocolitica, Aeromonas hydrophilla, P. fluorescens*	Mixture	synergism	de Azeredo *et al.* [63]
O. vulgare/T. vulgaris	*P. fluorescens*	Mixture	Additive	
Lippia multiflora/ Mentha piperita	*E. coli, E. aerogenes, Enterococcus faecalis, L. monocytogenes,*	Checkerboard	Synergism, additive	Bassole *et al.* [30]
L. multiflora/ O. basilicum	*P. aeruginosa, Salmonella enterica, S. typhimurium, Shigella. dysenteriae, S. Aureus*			
M. piperita/ O. basilicum	*E. coli, E. aerogenes, E. faecalis, L. monocytogenes, P. aeruginosa, S. enterica, S. typhimurium, S. dysenteriae, S. aureus*			
S. aromaticum/ R. officinalis	*Staphylococcus. epidermidis, S aureus, B. subtilis, E. coli, Proteus vulgaris, P. aeruginosa*	Mixture	Additive	Fu *et al.* [42]
	Candida albicans		Synergism	
	Aspergillus niger		Antagonism	
C. zeylanicum/ S. aromaticum	*E. coli*	Mixture	Antagonism	Goni *et al.* [64]
	Y. enterocolitica, L. monocytogenes, B. Cereus	Mixture	Synergism	
O. vulgare/O. basilicum	*B. Cereus, E. Coli, P. Aeruginosa*	Checkerboard	Additive	Gutierrez *et al.* [20]
O. vulgare/ Melissa officinalis	*B. cereus*			
O. vulgare/O. majorana	*B. cereus, E. coli*			
O. vulgare/R. officinalis	*B. cereus*			
O. vulgare/T. vulgaris	*Enterobacter cloacae, P. fluorescens, Listeria Innocua*	Checkerboard	Additive	Gutierrez *et al.* [65]

O. vulgare/ Salvia triloba	*B. cereus*			
O. vulgare/T. vulgaris	*B. cereus, P. aeruginosa*			
O. vulgare/T. vulgaris	*Enterobacter cloacae, P. fluorescens, Listeria Innocua*	Checkerboard	Additive	Gutierrez *et al.* [65]
T. vulgaris/O. majorana	*E. cloacae*			
T. vulgaris/ M. officinalis	*L. innocua*			
Cymbopogon citratus/ C. giganteus	*E. coli, E. aerogenes, L. monocytogenes, S. typhimurium, S. dysenteriae, S. aureus*	Checkerboard	Synergism, additive	Bassole *et al.* [66]

Table 9: Combination of component and essential oils their antimicrobial interactions against several microorganisms.

6.8 Characteristics and antimicrobial properties of the Essential Oils active against *Listeria monocytogenes* used in the present study

6.8.1 Thyme

Thyme is an aromatic plant and is widely distributed over the Mediterranean area (Europe, Asia and North Africa). Taxonomically, thyme belongs to the family of the Labiatae (*Lamiaceae*), the genus *Thymus* (etymologically from the Latin «Thymún» and from the Greek «Thymon») and to the class of Dicotyledons, native to the countries of the western Mediterranean basin. Thyme is one of the medicinal aromatic plants found in the Iberian Peninsula, and its essential oil has become one of the most widely used in the food industry. Thyme has been used since ancient times for its health properties, which are associated with its essential oils and chemical components. Its economic importance is associated with its essential oils. The generic name comes from the Greek verb Thym, which translates to perfume, in allusion to the intense and pleasant aroma of the plant. It is an aromatic, vivacious, woody, very polymorphic plant that is 10–40 cm high, with numerous branches that are woody, erect, compact, and brownish or velvety-white. The linear, oblong leaves are 3–8 mm, with the petiole or its margins ciliated and whitish on its underside. The flowers are axillary and grouped at the tip of the branches, forming a kind of terminal node. The fruit is a tetraquenium and brown in color. It blooms from March onwards. It is a highly variable species, both in its phenology and in the chemical composition of its essential oil, in which seven chemotypes have already been detected. For this reason, there is often taxonomic confusion in this genus, the varieties, or ecotypes of which have been identified as different species. Its habitat is in the countries of the western Mediterranean Basin in dry and sunny soils. It is abundant in the east, center and south of the Iberian Peninsula. It develops in limy, clayey and, less frequently, siliceous soils. It grows at an altitude of 0–1800 m. The climate is warm-temperate and mountainous. It is resistant to frost and drought, but not waterlogging or excess environmental humidity. The number of species currently cataloged exceeds 500, although there are perhaps many more that exist because of, among other things, the

great ease in which this aromatic plant produces hybridizations and mutations. Among the best-known species in Spain that experience greater propagation and exploitation are *Thymus rumidicus hispánicos*, *Thymus zygis*, *Thymus vulgaris*, *Thymus hyemalis*, *Thymus mastichina*, *Thymus citríodotus*, *Thymus corydothymus*, *Thymus loscossi*, *Thymus pipirella*, *Thymus communits*, etc. In all thyme species and varieties, the main part used commercially is its leaves, with purposes that vary from seasoning to herbalism. Another important use that mainly involves the species *Thymus zygis*, *Thymus mastichina*, *Thymus corydothymus* and some others is the extraction of essential oils through the distillation process. The essence of thyme has multiple applications, both in medicine and perfumery. From the essence of thyme, balsamic, vermicidal and bactericidal substances are extracted for very diverse uses.

6.8.1.1 Antimicrobial Activity of Thyme

Essential oils that are rich in phenolic compounds appear to be the most effective compounds against infections caused by microorganisms. As reported above, thyme essential oil is a "natural" preservative with the ability to control microorganisms (Meeran., 2012). It has been proven that there is a synergistic effect between different compounds of the EO, as occurs, for example, between carvacrol and its precursor p-cymene (Kalemba et al., 2003). In addition, it is also necessary to consider other constituents of these essential oils for their possible antagonistic or synergistic effects, especially thymol and carvacrol (Vardar-Ünlü et al.,2003). The physical conditions that improve the performance of these oils are low temperature, low oxygen levels and low pH (Burt, 2004). Anaerobic environments favor the action of both thyme EO and thymol against microorganisms such as *Salmonella thyphimurium* or *Staphylococcus aureus* (Burt, 2004). On the other hand, Gram-positive bacteria appear to be slightly more sensitive to the action of EO than Gram-negative bacteria (Burt, 2004). Due to the interaction between the compounds of the EO and the constituents of food, the antimicrobial efficacy of these substances is reduced when they are used in food products, so a higher concentration of essential oil is necessary to achieve results similar to those obtained in vitro (Juven et al.,1994). In a study conducted by Shapiro and Guggenheim (Shapiro et al.,1995), with bacteria that affect the oral cavity, it was observed that thymol produces a perforation in the plasma membrane of the bacterial cell, which causes a rapid outflow of intracellular constituents. This compound induces a decrease in intracellular ATP as a direct consequence of infiltration, and, in some bacteria, it also inhibits the synthesis pathways of this biomolecule. The effects of thymol on the potential of membranes are probably the result of the infiltration of substances caused by this compound. Evans and Martín (Evans et al., 2000) also proved the effectiveness of thymol as a growth inhibitor of

ruminal microorganisms, such as *Streptococcus bovis* or *Selenomonas ruminantium*. In fact, thyme has also demonstrated its potential antibacterial activity in vitro against food pathogens, such as *Salmonella, Staphylococcus, E. coli, Klebsiella, Pseudomonas and Enterococcus*, at concentrations from 5 to 20 µL EO (Boruga et al., 2014; Manconi et al., 2018). Indeed, the antifungal, antibacterial, antiparasitic and antiviral activities of thyme plants can be related to their expectorant, anti-inflammatory, antitussive, analgesic, sedative and antibroncholitic properties. On the other hand, carvacrol and thymol are isomeric phenolic compounds that can act against the pathogen *Bacillus cereus* (Ultee et al., 2002). Carvacrol shows a marked hydrophobic character, so it accumulates in the plasma membrane of the bacterial cell, which, as with thymol, affects its integrity and causes a fall in the membrane potential. In their study, Ultee et al. (Ultee et al., 2002) reported that carvacrol acted to reduce the pH across the plasma membrane, acting as a proton exchanger. This compound, which has a hydroxyl radical in the ortho position, diffuses through the membrane into the cytoplasm of the cell, where it releases its proton. Subsequently, it returns to the cellular membrane to carry a potassium ion from the cytoplasm. The cation is released, and carvacrol captures a new proton, repeating the cycle. The result is the depletion of ATP deposits in the cell, which leads to a deterioration of vital processes and ultimately to the death of the bacteria. Therefore, thymol (with the hydroxyl radical located in the meta position) and carvacrol have strong antibacterial activity. However, ρ-cimeno, the biological precursor of these two constituents in the essential oil of thyme, lacks a hydroxyl group and shows less activity, which suggests that this radical is related to toxicity against microorganisms. This work also showed a synergistic effect between carvacrol and ρ-cimeno, which may be due to the fact that this precursor contributes to the destabilization of the bacterial plasma membrane, which favors the entry of carvacrol into the cell. Thymol and carvacrol are also active against bacteria such as *P. aeruginosa* or *S. aureus* (Di Pasqua et al., 2006). These components show an additive effect that causes the inhibition of the growth of these microorganisms by damaging the integrity of the plasma membrane, affecting the pH and the balance of inorganic ions. Di Pasqua et al. (2006) investigated the bactericidal and bacteriostatic activity of essential oils obtained from various plants on bacteria such as *E. coli, S. typhimurium, L. monocytogenes* and *lactic acid bacteria*, and thyme oil was found to be the most effective spice against the greatest number of microorganisms tested. Di Pasqua et al. (2006) showed the lipophilic nature of thymol and carvacrol, as well as other constituents that can be found in thyme oil, such as limonene or eugenol, and reported that these molecules interact with bacterial membranes, altering their structure and making them more permeable. Other authors have shown the effectiveness of thyme on *Escherichia coli*. Both the EO obtained from *Thymus vulgaris* and that originating from Origanum vulgare exert a strong action on this microorganism, which is observed in a wide range of temperatures (Lambert et al., 2001). For

example, Burt et al. (2003) analyzed the effect of EOs of thyme and oregano and their four major components (thymol, carvacrol, ρ-cimeno and γ-terpinene) on *E. coli*, and they proved that thymol and carvacrol have clear bactericidal activity, reporting that they were similarly effective against *E. coli*. The antimicrobial activity of the EO depends on these two components, which have an additive effect and are not influenced, in this case, by the other major constituents of oil, namely, the precursors ρ-cimeno and γ-terpinene, which apparently do not act against this bacterium. The absence of synergism between carvacrol and ρ-cimeno contrasts with that expressed by other authors (Ultee et al., 2000). This could be due to the physiological differences between the bacteria used in each of the studies since the structure of the cell wall of *Escherichia coli* and other Gram-negative bacteria can inhibit the action of ρ-cimeno. As in previous studies, it was also demonstrated that thymol destroys the integrity and affects the electrical potential of the plasma membrane of the bacterial cell of *E. coli*, which finally leads to cell lysis (Vasala et al., 1999). Investigating the activity of various antimicrobial agents obtained from plants, Dorman and Deans (Dorman et al., 2000) determined that the EO of thyme has a greater spectrum of action, and, by studying these components separately, these authors confirmed the greater effectiveness of the phenolic compounds present in these oils, especially thymol and carvacrol, with ρ-cimeno being the least active constituent. This test also shows the influence of the hydroxyl group in the phenolic structure since a great difference can be seen between the antibacterial activity of carvacrol and that of its methyl ester, which is a relatively inefficient component; the authors also noted the importance of the position of this group in the benzene ring, contrary to what was previously reflected in this section. They agreed, however, with what was stated by Domingo et al. (2004), who affermed that the presence of hydroxyl groups in phenolics is related to the toxicity of these compounds, and the position of these radicals influences the effectiveness of phenols against bacteria. In addition, *Thymus albicans* and *Thymus mastichina*, with 1,8-cineole as the main constituent, also have positive inhibitory effects on bacteria, such as *L. monocytogenes, S. aureus or Salmonella sp*. (Faleiro et al., 1999). Similarly, the terminal alcohol linalool is active against bacteria of the genus *Leishmania* (Rosa et al.,2003) and other microorganisms such as *Lactobacillus plantarum, Citrobacter freundii* or *Clostridium sporogenes* (Dorman et al., 2000). Alcohols have more bactericidal than bacteriostatic activity, as they denature the proteins of microorganisms (Rota et al., 2004). In summary, essential oils are effective against a wide variety of microorganisms and can be used as food preservatives when added in small amounts; they can delay microbiological contamination and food deterioration without affecting its organoleptic properties (Dorman et al., 2000). Moreover, baicalein, another flavone identified in the *Thymus vulgaris* extract, potentiates the antimicrobial effect of tetracycline on *S. aureus* (Fujita et al., 2005). Other studies have reported the antibacterial potential of different plant extracts (thyme, fennel, sage, tea and mint)

and determined that thyme is the most effective against common pathogenic bacteria and lactic acid bacteria, so these concentrates are considered natural foods or food additives that could have a positive effect on the digestive system of humans and animals (Sagdic et al., 2005). The aqueous extract obtained from *Thymus vulgaris* was screened and found to be one of the most effective against bacteria, such as *H. pylori* (Tabak et al., 1996). The ability of compounds to act against different microorganisms from the extracts of other thyme species, such as *Thymus serpyllum* or *Thymus spathulifolius* (Tabak et al., 1996), has also been successfully tested. In the latter article, the authors compared the effects of the EO of the plant, containing thymol (36.5%) and carvacrol (29.8%) as the major constituents, with those of the extract. The result showed the strong antimicrobial activity of the EO, both against bacteria and against most of the fungal species tested by these authors, while the extract acted moderately against bacteria and was not effective against fungi. In other work, the antioxidant capacity of these two substances was evaluated, with positive results for both (Rota et al., 2008).

6.8.1.2 Sensory Implications

The main objective of the use of thyme in food is to extend the shelf life; however, the main limiting aspect for the use of the essential oil and plant extract of thyme is the development of negative organoleptic characteristics in foods, contributing to an unpleasant odor and taste. To avoid these sensory limitations, several strategies can be employed, such as the use of low concentrations and other conservation methods or the inclusion of natural compounds encapsulated with nanocarriers or added to bioactive films. On the one hand, the essential oil can be encapsulated with nanocarriers, such as nanoemulsions, nanofibers, cyclodextrins or amylose (Rezaei et al., 2019), which mask flavor and contribute to their controlled release while also protecting against oxidative degradation (Liu et al., 2020). This strategy increases the bioactivity of compounds present in the EO and plant extract; for example, encapsulation increases the stability of volatile components in the EO and increases cellular uptake, improving antimicrobial activity (Donsi et al., 2010). Another strategy to decrease the EO concentration is to apply the thyme EO in combination with other antimicrobial and antioxidant compounds to provide synergistic effects without the negative organoleptic aspect. (Nguefack et al., 2012). On the other hand, natural compounds can be included in bioactive films in order to increase the sensory acceptability while also allowing the gradual release of the compound and avoiding the negative organoleptic effects (Lv et al., 2011). In spite of the demonstrated potential of thyme and its constituents in vitro, its use as a food preservative has been limited by the organoleptic problem. In many food products, hydrophobic EO compounds are impaired by several

interactions with the components of the food matrix, such as fat (Cava-Roda et al., 2010; Rattanachaikunsopon et al., 2010), starch (Gutierrez et al., 2009) and proteins (Cerrutti et al., 1996). Moreover, the antimicrobial properties of EO compounds also depend on pH (Juven et al., 1994), temperature and microbial contamination (Somolinos et al., 2009). Given these interactions between natural extracts and food components, it is useful to study how the constituents of the EO can interact with the food matrix. This interaction can be studied by measuring the microorganism's growth in a culture medium containing different concentrations of starch, protein and fat.

6.8.1.3 Incorporation of Thyme in Fish and Seafood

The strategy of using natural extracts as fish and seafood preservative agents is a response to the consumer's concern about synthetic additives and environmental impacts (Román et al., 2017). In general, for this type of application, both thyme essential oil and extracts are used for immersion (for 30 min, followed by drainage of the product) or applied to films or added to the surface of the fish (on both sides). Different studies on the use of thyme in fish and seafood have applied concentrations ranging from 0.1 to 3%. Another alternative is the inclusion of the EO in coatings and packaging films on seafood to contribute to their preservative effects. To explore the effects of polymer films containing thyme essential oil, Jouki et al. (2014) studied the inclusion of 2% thyme oil in films of quince seed mucilage integrated into refrigerated rainbow trout fillets. These authors reported that thyme inhibited the development of lactic acid bacteria, psychrophilic bacteria, *Pseudomonas* spp. and *Enterobacteriaceae*. Moreover, Gómez-Estaca et al. (2010) studied the effects of different EOs (including thyme) against 18 bacterial strains in fish muscle extract and concluded that clove oil was the best antimicrobial; the second best was rosemary, followed by thyme and lavender oils. These essential oils were able to inhibit *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*; however, in accord with these results, Huang et al. (2018) showed that thyme EO was not able to inhibit *Pseudomonas spp.* Another food-borne pathogen related to the intake of lightly cooked or raw seafood is *Vibrio parahaemolyticus*. For example, Yano et al. (2006) reported that thyme exhibited antibacterial activity at 30 °C; however, this activity against *Vibrio parahaemolyticus* was weak at low temperatures. To investigate sensory acceptability, Navarro-Segura et al. (2019) studied the inclusion of nanoencapsulated essential oils into cyclodextrin films, and these authors concluded that the use of both strategies (nanoencapsulation and the film) improved the sensory acceptability and decreased microbiological counts and trimethylamine, so the shelf life was extended by 4 days. These authors reported an improvement in the sensory acceptability of seabream stored on ice and packaged with films (containing nanoencapsulated essential oils with cyclodextrin). The lower microbiological counts (*Enterobacteriaceae*, mesophilic bacteria and psychrophilic bacteria) and trimethylamine

allowed the shelf life of the fish to be extended by 4 days. In addition, several examples of the use of thyme in fish have been reported, such as the study of Mahmoud et al. (2004) who showed that submerging fish fillets (carp) in a 1% thymol/carvacrol solution reduced the initial mesophilic counts, and the shelf life was extended from 4 days to 12 days for fillets stored at 5 ◦C refrigeration. Similarly, Harpaz et al. (2003) observed an increase in the shelf life of perch during its storage for 33 days at 2 ◦C in comparison with the 12 days of shelf life of the control when the fillets were treated with a mixture of EOs of oregano and thyme at 0.05%. In addition, Ouattara et al. showed that the shelf life of prawns was extended using a combination of thyme oil, γ-irradiation and trans-cinnamaldehyde.

6.8.1.4 Public Health and Dietary Implications Concerning the Use of Thyme in Foods

The European Commission has accepted different EO components as flavoring in food, such as thymol, eugenol, carvacrol, citral, vanillin, limonene, linalool, carvone and cinnamaldehyde, because they do not present a risk to consumer health. The FDA (United States Food and Drug Administration) classifies these substances as GRAS (generally recognized as safe). Included in this category (essential oils classified as GRAS by FDA) are thyme, clove, cinnamon, oregano, mustard, nutmeg and basil. In addition, in the USA, before an EO can be added to foods, there are regulatory limitations on the accepted daily intake; therefore, before adding an EO, a daily intake survey should be available for evaluation by FDA. In general, recommendations for the intake of food for healthy eating do not yet include suggested amounts of spices and herbs. Therefore, the recommended intake of spices and herbs should be considered for incorporation into guides for healthy eating in different countries. In addition to the health effects, the use of this plant in foods can be used to totally or partially replace other less desirable ingredients, such as synthetic additives, sugar or salt. The addition of these herbs and spices may be a strategy to stabilize stir-fry dishes, dressings and marinades, casseroles, curries, soups and Mediterranean-style cooking. Before the application of thyme as a natural extract, several factors must be taken into account, such as the nature of the extracts, fruiting stage, mode of extraction, the concentration of active extract components and the possible synergistic effect between thyme and other components. Thyme extract can be applied as a complementary food preservative in food systems such as milk products, dressings, meat and oils and for the enhancement of functional foods. In addition, other considerations regarding thyme are important and warrant its inclusion in the food industry, such as its non-toxicity, availability and low cost.

6.8.2 Salvia officinalis

Salvia officinalis L. (Sage) is a perennial round shrub in the family of *Labiatae*/*Lamiaceae* (Bisset et al., 2001). Salvia is the largest genus of this family and includes near 900 species. Plants of this genus grow all over the world and the specie of *S. officinalis* is native to Middle East and Mediterranean areas. Today's, it has been naturalized throughout the world particularly in Europe and North America. The aerial parts of *S. officinalis* shrub has a long history of use in cookery and traditional medicine (Miura et al., 2001). Because of its flavoring and seasoning properties, this plant has been widely used in preparation of many foods. In folk medicine of Asia and Latin America, it has been used for the treatment of different kinds of disorders including seizure, ulcers, gout, rheumatism, inflammation, dizziness, tremor, paralysis, diarrhea, and hyperglycemia. In traditional medicine of Europe, *S. officinalis* has been used to treat mild dyspepsia (such as heartburn and bloating), excessive sweating, age-related cognitive disorders, and inflammations in the throat and skin (Zargari, 1990; Garcia et al., 2016). German Commission E has accepted the use of *S. officinalis* for a number of medical applications included inflammation and dyspepsia. In recent years, many research studies have been conducted to document the traditional uses of *S. officinalis* and to find new biological effects for this plant (Perry et al.,1999; Adams et al.,2007; European Medicines Agency 2009; Badiee et al., 2012). These studies have revealed a wide range of pharmacological activities including anticancer, anti-inflammatory, anti-nociceptive, antioxidant, antimicrobial, antimutagenic, antidementia, hypoglycemic, and hypolipidemic, effects.

6.8.2.1 **Bioactive compounds in *Salvia officinalis***

The major phytochemicals in flowers, leaves, and stem of S. officinalis are well identified. A wide range of constituents include alkaloids, carbohydrate, fatty acids, glycosidic derivatives (e.g., cardiac glycosides, flavonoid glycosides, saponins), phenolic compounds (e.g., coumarins, flavonoids, tannins), poly acetylenes, steroids, terpenes/terpenoids (e.g., monoterpenoids, diterpenoids, triterpenoids, sesquiterpenoids), and waxes are found in *S. officinalis*. Most of the phytochemicals which are reported from *S. officinalis* have been isolated from its essential oil, alcoholic extract, aqueous extract, butanol fraction, and infusion preparation. More than 120 components have been characterized in the essential oil prepared from aerial parts of S. officinalis (Capek et al., 2004). The main components of the oil include borneol, camphor, caryophyllene, cineole, elemene, humulene, ledene, pinene, and thujone. Alcoholic and aqueous extracts of S. officinalis are rich in flavonoids

particularly rosmarinic acid and luteolin-7-glucoside. Also, the phenolic acids such as caffeic acid and 3-Caffeoylquinic acid have been found in methanolic extract of *S. officinalis* (El Hadri, 2010). Several flavonoids like chlorogenic acid, ellagic acid, epicatecin, epigallocatechin gallate, quercetin, rosmarinic acid, rutin, and luteolin-7-glucoside, as well as several volatile components such as borneol, cineole, camphor, and thujone have been identified in infusion prepared from *S. officinalis* (Hayouni et al., 2008). Rosmarinic acid and ellagic acid are the most abundant flavonoids in *S. officinalis* infusion extract, followed by rutin, chlorogenic acid, and quercetin. The most abounding carbohydrates described in this plant are arabinose, galactose, glucose, mannose, xylose, uronic acids and rhamnose (Capek et al., 2004). Comparing the phytochemicals in flowers, leaves, and stem of *S. officinalis*; linalool is the most present phytochemical in the stem; the flowers have the highest level of α-pinene and cineole; and bornyl acetate, camphene, camphor, humulene, limonene, and thujone are the most present phytochemicals in the leaves. However, it should be considered that, like other herbs, the chemical composition of *S. officinalis* would be varied depending on the environmental conditions such as climate, water availability, and altitude (Hayouni et al., 2008).

6.8.2.2 Antibacterial effects

Several lines of evidence support antimicrobial effects of *S. officinalis*. The essential oil and ethanolic extract of *S. officinalis* show strong bactericidal and bacteriostatic effects against both Gram-positive and Gram-negative bacteria. Among Gram-positive pathogens, *Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Enterococcus faecalis, Listeria monocytogenes*, and *Staphylococcus epidermidis* show high sensitivity to *S. officinalis* (Hayouni et al., 2008; Mitic-Culafic et al., 2005). Effects of *S. officinalis* on Gram-negative bacteria depend on the type of extract used. While essential oil of *S. officinalis* has significant inhibitory effect on the growth of *Aeromonashydrophila, Aeromonassobria, E. coli, Klebsiella oxytoca, Klebsiella pneumonia, Pseudomonas morgani, Salmonella anatum, Salmonella enteritidis, Salmonella typhi,* and *Shigellasonei,* effect of ethanolic extract on *E. coli, Pseudomonas aeruginosa,* and *S. enteritidis* is weak. In addition to antibacterial action, *S. officinalis* has been reported to induce antifungal, antiviral, and anti-malarial effects (Russo et al., 2013; Seidel, 2006). The antifungal activity has been reported against *Botrytis cinerea, Candida glabrata, Candida albicans, Candida krusei*, and *Candida parapsilosis*. Antimicrobial effects of *S. officinalis* are attributed to terpens and terpenoids compounds found in this plant. It has been shown that camphor, thujone, and 1,8-cineole have antibacterial effects against *Aeromonas hydrophila, Aeromonas sobria, B. megatherium, B. subtilis, B. cereus*, and *Klebsiella oxytoca*. Also, oleanolic acid and ursolic acid, two triterpenoids of *S. officinalis*, have inhibitory action on growth of multidrug-resistant bacteria

such as vancomycin-resistant *enterococci*, penicillin-resistant *Streptococcus pneumonia*, and methicillin-resistant *Staphylococcus aureus* (Veličković et al., 2003; Bozin et al., 2007). The effect of ursolic acid on *Enterococcus faecium* and multidrug-resistant bacteria is stronger than that of ampicillin. Carnosol, a diterpenoid, and its related compound carnosic acid are two other antibacterial compounds obtained from *S. officinalis*. These compounds potentiate the effects of aminoglycosides on methicillin-resistant *S. aureus*. The antiviral activity of *S. officinalis* is most probably mediated by safficinolide and sage one, two diterpenoids which are found in its aerial parts (Akkawi et al.,2012).

6.8.2.3 Toxicological studies

Several clinical trials have reported that consumption of *S. officinalis* does not induce severe side effects. However, in the case of prolonged use or following overdose of ethanolic extract and volatile oil of *S. officinalis* (corresponding to more than 15 g of the leaves) some unwanted effects such as vomiting, salivation, tachycardia, vertigo, hot flushes, allergic reactions, tongue swallowing, cyanosis, and even convulsion may occur (Tada et al., 1994). The proconvulsant action of *S. officinalis* oil is due to its direct effect (at doses more than 0.5 g/kg) on nervous system. Camphor, thujone, and terpene ketones are considered as the most toxic compounds in *S. officinalis*. These compounds may induce toxic effects on the fetus and newborn (Carta et al., 1996; Horiuchi et al., 2007). Therefore, consumption of *S. officinalis* is not recommended in pregnancy and lactation. Results from animal studies have demonstrated that the LD50 of *S. officinalis* oil (when consumed orally) and the methanolic extract (when injected intraperitoneally) is 2.6 g/kg and 4 g/kg, respectively. It has been reported that *S. officinalis* tea enhances CCl4-induced hepatotoxicity in mice. However, in clinical studies no hepatotoxic effects were reported (Horiuchi et al., 2007).

6.8.3 Mentha piperita

The genus Mentha is part of the family of *Lamiaceae* and includes about 30 species grown in temperate climate zones around the world, especially in Europe, North America, North Africa (Syria, Ethiopia), and northern parts of Iran. Due to the high variability of the species and great ease of crossing species, the chemical composition of the EOs obtained from them is very diverse (Stringaro et al., 2018). One of the peppermint species is a natural hybrid of two species: *Mentha spicata* L. and *Mentha aquatic* L. Peppermint is a perennial with a height of 30–90 cm with numerous underground and aboveground runners and a purple stem. The leaves are oblong and dark green. Purple flowers are collected in capitate inflorescences. This plant requires fertile and permeable soil. EO from

peppermint is obtained after distillation of dried leaves with water vapor. As a result of this process, a light yellow or greenish liquid with an intense mint aroma is obtained (Lis-Balchin et al., 1997; Orchard et al., 2017).

6.8.3.1 Chemical Composition of Peppermint EO

Approximately 300 compounds have been identified in the EO. The main components are menthol (30–55%) and mentone (14–32%). Menthol occurs mainly as the isomer with the (1R, 3R, 4S) configuration (20–60%), while the main isomer of mentone is (1R, 4S) (5–35%). The Polish Pharmacopoeia VIII defines the content of other ingredients as follows: cineol (3.5–14%), menthyl acetate (2.8–10%), isomenton (1.5–10%), menthofuran (1.0–9.0%), limonene (1.0–5.0%), pulegone (<4.0%), and carvone (<1.0%). The quantitative composition of EO depends on many factors, such as the growing conditions, and the date of harvesting. Therefore, the date of the congregation should be chosen to ensure that it contains as much menthol as possible (Astani et al., 2010; Orchard et al., 2017).

6.8.3.2 Antimicrobial Properties of Peppermint EO

The broad spectrum of biological activity of plants of the genus Mentha was discussed recently in a review article (Stringaro et al., 2018). Peppermint essential oil exhibited high levels of virucidal activity against HSV-1 and HSV-2 in viral suspension tests. Both kinds of viruses were significantly inhibited when Herpes simplex virus was pre-treated with the EO prior to adsorption. Peppermint EO affected the virus before adsorption, but not after penetration into the host cell (Spirling et al., 2001). Peppermint EO has a weak antibacterial activity, hence it is usually included in complex preparations. Its widespread use is due more to a pleasant mint flavor and the feeling of coolness than to its antimicrobial properties. However, it is believed that the higher menthol content in peppermint EO has more antimicrobial activity (Orchard et al., 2017). In the diffusion test, peppermint EO (20 µL) inhibited the growth of bacterial strains, such as *E. coli* WDCM 00013, *L. monocytogenes* WDCM 00020, *P. aeruginosa* WDCM 00024, *S. enterica* WDCM 00030, and *S. aureus* WDCM 00032. The inhibition zone was from 12 mm for *P. aeruginosa* up to 37.66 mm for. *S. aureus* (Marjanovi´c-Balaban et al., 2018). Recently, the effect of peppermint EO on the development of yeast has been investigated. These microorganisms may be responsible for the spoiling of cashew, guava, mango,

and pineapple juices. In preliminary studies, the MIC was 1.875 L/mL against *C. albicans, C. tropicalis, Pichia anomala,* and *S. cerevisiae*. However, when used in mango and pineapple juices, even at higher concentrations of EO (7.5 L/mL), no significant reduction in yeast was observed. In the case of *S. cerevisiae*, the addition of 1.875 L/mL of mint EO to the cashew and guava juices strongly weakened the membrane permeability, membrane potential, and activity of the efflux pump in the yeast cells. It is true that mint EO did not affect the appearance, smell, and viscosity of fruit juices, but negatively influenced their taste (Rathod et al., 2017). In turn, Benzaid et al. (2019) determined that mint EO in the volatile form inhibits the development of *C. albicans* comparable to amphotericin B, influencing the expression of various genes, such as secreted aspartyl proteinases (SAP 1, 2, 3, 9, 10), and being associated with the process of adhesion of hyphal wall protein 1 (HWP1). It is worth noting that although the mint EO alone has a weak antibacterial activity, it may have a synergistic effect with other EOs or substances. For example, it increased the activity of *Pongamia pinnata* EO and additionally increased over 30 times the sensitivity of bacteria to gentamicin, *E. coli* pMG309 harboring and plasmid encoding lactamase, KPC-3 on meropenem, and caused a strong anticandidal effect with azole antibiotics, such as Fluconazole and Ketoconazole, and a weaker synergistic effect with Clotrimazole and Itraconazole. The research was carried out using *Candida* strains isolated from patients affected by skin diseases and urine samples (Rathod et al., 2017). One of the popular preparations used to treat headaches, colds, coughs, mild spinal gastrointestinal complaints, and to relieve local muscle pain is Olbas® Tropfen (Olbas). It contains EOs such as peppermint EO (5.3g), eucalyptus (2.1g), cajuput, and a smaller amount of juniper EO (0.3g). Olbas® has shown antibacterial activity against many strains, including methilicin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus*. Hence, Olbas® could be used to treat uncomplicated skin and respiratory infections (Hamoud et al., 2012). This activity may result from the high content of monoterpenes, especially menthol, which due to their hydrophobicity, affect the fluidity and permeability of the cell membrane. Monoterpenes also affect the conformation of proteins embedded in the membrane, thus inhibiting the process of cellular respiration and disrupting the transport of ions through cell membranes, which can lead to cell death (Trombetta et al., 2005; Kamatou et al., 2013). The possibility of using peppermint EO introduced into a chitosan nanogel was investigated in the protection of plaque against *S. mutans* causing caries. The maximum release of peppermint EO from the nanogel was about 50% after 360 h in an aqueous-alcoholic solvent at ambient temperature. The adhesion of bacterial cells was highly sensitive to nanoformulation of mint EO, as compared to the unloaded chitosan nanogel. Inhibition of biosynthesis of S. mutans occurred at a concentration of 50 g/mL, compared to 400 g/mL for a nanogel without EO. In addition, it was found that chitosan nanogel containing mint EO inhibited the activity of glycosyltransferase genes

(gtf B, gtf C, and gtfD) involved in the formation of extracellular polymers (Ashrafi et al., 2019). Moreover, peppermint EO also inhibited the growth of fungi strains, such as *Alternaria alternata, Aspergillus flavus, Aspergillus niger, Colletotrichum gloeosporioides, Fusarium solani,* and *Macrophomina phaseol* (França et al., 2018).

6.8.4 Citrus lemon

Citrus are the most important crops in the world in terms of production according to the Food and Agricultural Organisation (FAO), with 240,780 million metric tons produced in 2013 (FAO Statistics, 2016). Citrus plants are grown in many countries all over the world and among the major African citrus-producing countries is Tunisia. Thus, *Citrus* would be considered as one of the most economically important crops in Tunisia. The genus *Citrus* belongs to the *Rutaceae* family that comprises of about 140 genera and 1300 species and, for instance, *Citrus limon* (Lemon) is among important species of genus *Citrus* (Kamal et al., 2011). Essential oils were composed of many valuable natural products that may be described as mixtures of hydrocarbons, oxygenated compounds and nonvolatile residues. They include terpenes, sesquiterpenes, aldehydes, alcohols, esters and sterols (Darjazi, 2013). Citrus plants constitute one of the main sources of essential oil, which are extensively studied for their potential uses in the food industry (Mustafa, 2015). Foods contaminated with *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli O157:H7* and *Salmonella* has been reported as the causal agents of foodborne diseases (Ben Hsouna et al., 2011; Rahman et al., 2009). One of the most important psychrotrophic food pathogens related to anaerobically packed cooked meat products and shelf-life failures of conserved foods is *Listeria monocytogenes*. This organism is the causal agent of listeriosis, a disease that can be serious and is often fatal in susceptible individuals, caused by eating contaminated food (Cornu et al., 2006). Thus, to prevent contamination during the production, sale, and distribution and to extend the shelf lifetime of raw and/or processed foods, synthetic additives should be used. However, there is a strong debate about the safety aspects of these chemical preservatives since they are considered responsible for many carcinogenic and teratogenic attributes as well as residual toxicity (Skandamis et al., 2001). Thus, a growing attention is being paid to plants and herbs naturally derived compounds as a new alternative to prevent the proliferation of microorganism and protect food from oxidation. Generally, little information exists on the in vivo antimicrobial efficacy of plant essential oils against food-borne pathogens in meat. To the best of our knowledge, the antimicrobial activity of *Citrus limon* essential oil (ClEO) against a wide range of food-associated microorganisms (bacteria, moulds, and yeasts) has not been studied. The purposes of the present work were (i) to evaluate the chemical composition of Tunisian lemon

EO (ClEO) by GC-MS, (ii) to study in vitro the antioxidant and antimicrobial activities of ClEO, (iii) to assess the effect of ClEO on physicochemical of raw minced beef meat stored at 4 °C, and (iv) to determine the efficacy of ClEO in inhibiting *L. monocytogenes* growth in raw minced beef meat during refrigerated storage (Zheljazkov et al., 2012).

6.8.2.4 antibacterial activity of *Citrus Limon*

Lemon (*Citrus limon* Osbeck) EO is a natural stress reliever. Inhaling lemon EO causes anti-stress effects through modulating the 5-HT and dopamine (DA) activities in mice (Komiya et al., 2006; Ogeturk et al., 2010). Lemon EO showed cytotoxic effects against human prostate, lung, and breast cancer cells (Zu et al., 2010). It also induced apoptosis in HL-60 cells due to the presence of citral, decanal, and octanal (Massadeh et al., 2013). Oral administration of lemon EO inhibited 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced neoplasia of the lungs and forestomach of female mice (Wattenberg et al., 1991). Lemon EO causes activation of the sympathetic nerve activity innervating the white adipose tissue (WAT), which increases lipolysis and results in the suppression of body weight gain (Niijima et al., 2003). Lemon EO significantly reduces lipid peroxidation levels and nitrile content, but increases reduced glutathione (GSH) levels, as well as superoxide dismutase, catalase, and glutathione peroxidase activities in mouse hippocampus (Campêlo et al., 2011). The neuroprotective effect of lemon EO is attributed to its remarkable radical-scavenging activity (Choi et al., 2000; De Freitas et al., 2011). Prolonged exposure (for 2 weeks) to lemon EO induces significant changes in neuronal circuits involved in anxiety and pain in rats (Ceccarelli, et al., 2004). Lemon EO improves creativity and mood and is thought to affect heart rhythm (Ceccarelli, et al., 2002). The analgesic effect of lemon EO is induced by dopamine-related activation of anterior cingulate cortex (ACC) and the descending pain inhibitory system (Ikeda et al., 2014). Inhalation of lemon EO reduces the intensity of nausea and vomiting of pregnancy (NVP) by 33% (Yavari et al., 2014). It also showed anti-spasmodic activity (Ogeturk et al., 2010). Lemon EO significantly enhanced attention level, concentration, cognitive performance, mood, and memory of students during the learning process (Akpinar et al.,2005). Rats exposed to lemon EO were able to find a target point faster than a control group (Ogeturk et al., 2010). Lemon EO is a safe and effective penetration enhancer for topical administration of lipid- and water-soluble vitamins which are critical issues for the protection of anti-ageing formulations. It significantly enhances the trans-epidermal release of α-tocopherol (vitamin E), retinyl acetate (vitamin A), pyridoxine (vitamin B6), and ascorbic acid (vitamin C) from topical emulsions in reconstructed human epidermis (Valgimigli et al., 2012). In addition, lemon EO is a potent antibacterial against *Bacillus cereus, Mycobacterium smegmatis,*

Listeria monocytogenes, Lactobacillus curvatus, L. sakei, Micrococcus luteus, Escherichia coli, Klebsiella pneumoniae, Pseudococcus aeruginosa, Proteus vulgaris, Enterobacter gergoviae, E. ammnigenus, Staphylococcus aureus, S. carnosus, and *S. xylosus* (Viuda-Martos et al., 2008; Viuda-Martos et al., 2011), and a strong antifungal against *Aspergillus niger, A. flavus, Penicillium verrucosum, P. chrysogenum, Kluyveromyces fragilis, Rhodotorula rubra, Candida albicans, Hanseniaspora guilliermondii, and Debaryomyces hansenii*. Lemon EO has insect repellent effects against the malaria vector, *Anopheles stephensi* (Oshaghi et al., 2003). It also showed remarkable miticidal activity against *Sarcoptes scabiei var. cuniculi*, both in vitro and in vivo. When lemon EO was tested at 20% and applied topically on the infected parts of rabbits once a week for four successive weeks, the infected rabbits completely recovered after the second week of treatment (Aboelhadid et al., 2016).

6.8.2.5 Safety of Citrus Oils

Generally, *Citrus* EOs are non-toxic, non-mutagenic, and non-carcinogenic (Skandamis et al., 2011). They are not hazardous in pregnancy and do not alter the maternal reproductive outcome (Skandamis et al., 2001; Volpato et al., 2015). Sweet orange, bitter orange, neroli, petitgrain, lemon, lime (both distilled and expressed), bergamot, and grapefruit oils have GRAS status (Skandamis et al., 2001). However, there is a possible skin sensitization issue if old or oxidized oil is used. The distilled oils are not phototoxic, while the expressed oils carry a low to moderate risk of phototoxicity (Opdyke, 1974) due to the presence of furanocoumarins (Naganuma et al., 1985). In case of applying expressed EOs to the skin in a dose higher than the maximum dermal use level, it is recommended to avoid exposure to sunlight for at least 12h (Skandamis et al., 2001). Neroli and yuzu oils are neither irritating nor sensitizing. Expressed sweet orange oil was neither irritating nor sensitizing to 25 volunteers when tested at 8 and 100% (Opdyke, 1974), whereas it caused sensitivity to 0.13% of total dermatitis patients when tested at 2% (Rudzki et al., 1976). Bitter orange EO was neither irritating nor sensitizing to 25 volunteers when tested at 10% (Opdyke, 1974), while it caused sensitivity to 1.5% of total dermatitis patients when tested at 2% (Rudzki et al., 1976). Lemon oil was neither irritating nor sensitizing to volunteers when tested at 10% (Opdyke, 1974), and similar results were observed for distilled lime oil when tested at 15 and 100% (Opdyke, 1974). No irritation or sensitization data were found for the expressed lime oil. The high citral content of lime EO causes potential toxic and myelotoxic effects. Grapefruit oil was neither irritating nor sensitizing to volunteers when tested at 10 and 100% (Opdyke, 1974). Mandarin EO was neither irritating nor sensitizing to 25 volunteers when tested at 5 and 8% (Opdyke, 1974). The expressed bergamot oil was neither irritating nor

sensitizing to 25 volunteers when tested at 10% (Opdyke, 1974). It caused no irritation when tested at 2% on 1200 dermatitis patients, with only two (0.17%) patients showing sensitivity reaction (Santucci et al., 1987), whereas when tested at 10% in 590 eczema patients, 0.5% of the patients had reactions (Menenghini et al., 1971). Expressed bergamot oil caused severe phototoxic effects in hairless mice and pigs using simulated sunlight, and in humans using natural sunlight and may be photocarcinogenic (Opdyke, 1974). When applied to mice, then irradiated with UV light, bergamot oil showed a carcinogenic action due to the presence of bergapten (Young et al., 1990). Chronic skin pigmentation (also known as berloque dermatitis, bergapten dermatitis, or photophytodermatitis) can also develop. Increased exposure to UV light can lead to serious burns. In the absence of UV light, bergamot oil is not carcinogenic and even low concentration sunscreens can completely inhibit bergapten-enhanced phototumorigenesis (Young et al., 1990). No hazards found for the furanocoumarin-free (FCF) or rectified bergamot oil. The rectified oil was not sensitizing when tested at 30% on 25 volunteers (Opdyke et al., 1973). To avoid oxidation of d-limonene, Citrus oils should be stored in a dark air-tight container and placed at 4 °C (Skandamis et al., 2001). The use of old or oxidized oils should be avoided. To avoid any possible adverse skin reactions, it is recommended to dilute *Citrus* oils with a carrier oil before topical use (Opdyke et al., 1973). Also, adding an antioxidant to preparations containing *Citrus* oils is recommended (Skandamis et al., 2001).

6.9 Essential Oils in food preservation

Food-borne diseases are a growing public health problem worldwide. It is estimated that each year in the United States,31 species of pathogens cause 9.4 million cases of food-borne illnesses (Scallan et al., 2011). Successful control of food-borne pathogens requires the use of multiple preservation techniques in the manufacturing and storage of food products. A recent consumer trend toward preference for products with lower salt and sugar content presents an increased need for efficient food preservatives, as lowering the salt and sugar content would otherwise compromise the product's shelf-life (Zink, 1997). A wide range of preservatives are used to extend the shelf-life of a product by inhibiting microbial growth. However, an increasingly negative consumer perception of synthetic food additives has spurred an interest in finding natural alternatives to the traditional solutions (Zink, 1997). Although originally added to change or improve taste, the antimicrobial activity of essential oils makes them an attractive choice for substituting synthetic preservatives.

6.9 Perspectives and limitations in application of Essential Oils in food

A range of essential oil components have been accepted by the European Commission for their intended use as flavorings in food products. The registered flavorings are, e.g., linalool, thymol, eugenol, carvone, cinnamaldehyde, vanillin, carvacrol, citral, and limonene, all of which are considered to present no risk to the health of the consumer. The United States Food and Drug Administration (FDA) also classifies these substances as generally recognized as safe (GRAS). The crude essential oils classified as GRAS by FDA include amongst others clove, oregano, thyme, nutmeg, basil, mustard, and cinnamon. There are regulatory limitations on the accepted daily intake of essential oils or essential oil components, so before they can be used in food products, a daily intake survey should be available for evaluation by FDA. Despite the demonstrated potential of essential oils and their constituentsin vitro, their use as preservatives infood has been limited because high concentrations are needed to achieve sufficient antimicrobial activity. In many food products, the hydrophobic essential oil constituents are impaired by interactions with food matrix components, such as fat (Cava-Roda et al., 2010; Rattanachaikunsopon et al., 2010), starch (Gutierrez et al., 2008), and proteins (Cerrutti et al., 1996; Kyung, 2011). Furthermore, the antimicrobial potency of essential oil constituents also depends on pH (Juven et al., 1994), temperature (Rattanachaikunsopon et al., 2010), and the level of microbial contamination (Somolinos et al., 2010). Extrapolation of results from in vitro tests to food products is thus difficult at best, and a lower performance of the antimicrobial compound must be expected. For example, Cilantro oil had significant antibacterial activity at 0.018% in vitro, but when applied to a ham model, even 6% cilantro oil had no antimicrobial activity (Gill et al., 2002). Before being added to food products, it is therefore useful to investigate how essential oils, or their constituents interact with food components in vitro. Food matrix interactions with the essential oils or their constituents can be investigated by measuring the growth of microorganisms in culture medium containing a range of concentrations of fat, protein, or starch as well as the antimicrobial compound of interest. Such experiments have been performed using a so-called food model media (Gutierrez et al., 2009), and can be used to provide quick answers to which kind of food products the compound in question can be used in. The intense aroma of essential oils, even low concentrations, can cause negative organoleptic effects exceeding the threshold acceptable to consumers (Lv et al., 2011). Having to increase the concentration of essential oils to compensate for their interactions with food matrix components is therefore highly unfortunate and limits their application to spicy foods where the acceptable sensory threshold is relatively high. Different strategies can be used to circumvent this problem. One option is to use essential oils in active packaging rather than as an ingredient in the product itself. Essential oils can be encapsulated in polymers of edible and biodegradable coatings or sachets that provide a slow release to the food surface or to the headspace of packages of, e.g., fruit, meat, and fish (Pelissari et al., 2009; Sánchez-

González et al., 2011). Sachets that release volatile essential oils into the headspace environment are simply placed within an enclosed food package (Ahvenainen, 2003). The advantage of incorporating volatile components of essential oils in films or edible coatings is that the diffusion rate of the agents away from the food product can be reduced, thereby maintaining the active compounds in the headspace or on the product surface for extended periods of time (Phillips et al., 2011; Sánchez-González et al., 2011). A way to minimize organoleptic effects of essential oils added to the matrix of a food product is to encapsulate essential oils into nanoemulsions. This approach increases the stability of volatile components, protecting them from interacting with the food matrix, and increases the antimicrobial activity due to increased passive cellular uptake (Donsí et al., 2011). Lowering the concentration of essential oils without compromising their antimicrobial activity can also be obtained by applying them in combination with other antimicrobial compounds that provide a synergistic effect (Nguefack et al., 2012). Synergies are known to occur for essential oil combinations, and it is therefore a field with countless opportunities to find potent antimicrobial blends, which may be the key to implementing essential oils in food preservation without simultaneous organoleptic effects.

7. Edible coating

7.1 Background

Since ancient times, humans have tried to find optimal solutions for packing their food products in the simplest and most productive way. Along with technological advancements, society found better and more resistant materials to improve food packing. The research for new, unconventional materials that can be used for packaging food in a more sustainable and eco-friendly way has seen a constant increase in the last few decades. Packaging has many duties such as: to preserve substances against contamination and perishability, to move easily and keep goods, and to give a constant measure of the contents. A package has three important tasks: to protect the contents, to give good marketing to a product, and to deliver useful information to the customer. A fourth purpose is related to advertisement because easy to use packaging increases market opportunities. Thus, four significant functions of packaging have been identified: security, containment, communication and accessibility. All these attributes are interconnected, and all must be evaluated and taken into consideration within the process of packaging development (Kaewprachu et al., 2016).

The recent advances regarding the applicability of animal and vegetal derived proteins in developing food (edible) films, coatings, and innovative packaging materials are reported below. Protein

characteristics, suitability, protein-based functionality, and economic aspects are also discussed.

7.2 Materials Used for Protein-Based Packaging and Edible Packaging

Over the years, humans started developing different kinds of films and coatings meant to protect food against spoilage. For example, in the 12th century fruits were coated in a wax layer to slow down the water loss process and during, the 15th century, a Japanese scientist named Yuba discovered the first edible film from soymilk (Brandenburg et al., 1993). Starting in the 16th century in Europe, enough people figured out how to use different substances in order to collect products and to prevent alteration, by reducing the moisture and covering the meat with oil. In the 19th century, they started coating food in oil and gelatin. In the same period, foods like hazelnuts, nuts and almonds were coated with sucrose, to prevent rancidness and oxidation (Erginkaya et al., 2014). In order to have a waste disposal value close to zero, there is edible packaging made from ingredients that can be eaten alongside the contained food or beverage, the edible packaging and the food forming together a constant and cohesive system (Biris-Dorhoi et al.,2020). An edible package is defined as a thin layer that covers the food surface. If it is not consumed, the edible film degrades very fast and in this way is reduces the demands of landfills, in comparison with plastic and biodegradable products (Jeya Jeevahan et al., 2020). The quality of the food might be improved by using edible films and coatings, and the composition of films or coatings has a great role in this process. According to Pavli et al. (2018) flavorings, antioxidants, antimicrobials, probiotics and nanomaterials are known to be beneficial in enhancing the quality of food (Pavli et al., 2018). For the development of an effective edible package, the selective permeability and gas barrier are specific conditions (Umaraw et al., 2020). These thin layers of edible substances are created between food parts or on the surface. They have different properties, from controlling oxygen, carbon dioxide, taste and aroma between other food parts or the surrounding atmosphere to the capability of carrying a various array of food additives as preservatives, antimicrobial agents and antioxidants. They could offer all these functional properties as a packaging material if they are prepared in the correct way (Erginkaya et al., 2014). Regarding the edible food packaging, polysaccharides are a great option with respect to edible packaging material. To reduce conventional plastic packaging, starch, xanthan gum, carrageenan, pectin and alginate (polysaccharides) were used to produce edible films and coatings, because they are natural products, with low toxicity and selective permeability for oxygen and carbon dioxide. All these attributes of the coatings and edible films made from polysaccharides can prolong fruit shelf life (Mohamed et al., 2020). Brown algae (*Phaeophyceae*) contains a polysaccharide named alginate. This polysaccharide consists of α-L-guluronate (G) and R-D-mannuronate (M) bonds in the (1-4)

chain (Mohamed et al., 2020; Pop et al., 2012). Another polysaccharide with microbial properties is pullulan, composed of maltotriose and α (1,6) glycosidic units, produced by *Aureobasidium pullulans* from starch (Qian et al., 2013). Pullulan is water-soluble, it has no color, smell and taste, and is also an oil permeable and heat sealable edible film (Mohamed et al., 2020). A very important question to ask is how can we obtain almost unlimited amounts of prime ingredients? The answer in the case of polysaccharides is by looking back at what nature has to offer. Cellulose is the earth's most abundant organic compound, and scientists rapidly found ways to procure plant origin polysaccharides, marine origin polysaccharides and even microbial polysaccharides (Von Schantz et al., 2014). An excellent cellulose compound with thermal gelatinization and water-soluble characteristics film-forming is the (carboxymethyl cellulose (CMC) (Mohamed et al., 2020). Researchers started to study and develop nanostructured antimicrobial edible films in order to further protect and contain the food matrix (Alexandre et al., 2016). Overtime, edible films have become widely used for a variety of different products and different food categories such as meat products, vegetables or dairy products. According to Farhan et al. (2020), the edible film of semi-refined κ-carrageenan can be developed by a water extract from the process of fenugreek seed germination. For fresh chicken breast this edible film can be used as an alternative to conventional plastic films that are used in the packaging of chicken meat (Farhan et al., 2020; Pop et al., 2020). Furcellaran, a genus of red algae, is one of the most important sources of carrageenan. Jamróz et al. (2019) used furcellaean with nanofillers, nanoparticles of maghemite, and graphene oxide with good antimicrobial activity (for the nanofillers film), but not excellent mechanical properties (Jamróz, et al., 2019). Three types of coating mixture using carboxymethyl cellulose, sodium alginate and carrageenan were used for the effective protection of cherry tomatoes. These coverings of the tomatoes, in combination with preservation in a controlled atmosphere, were used to validate the preservation system. The carrageenan edible film was proven protect the cherry tomatoes, from where it can be used for other vegetables and fruits as preservation packaging (Zhou et al., 2016). According to Cruz-Diaz et al. (2019), protein-based films treated with ultrasound have lower water-vapor permeability than the films treated with heat. Only the color of the protein-based film was affected by addition of microbial transglutaminase into the solutions treated with ultrasound, while the properties of the film were not affected. Another study with whey protein films has favorable results for cheese slices and more studies will be continued on this subject (Cruz-Diaz et al., 2019). Furthermore, protein-based packages may be an active package. The interaction of the package (or of one of the ingredients) with the packaged food or the nearby environment makes it active. The most common ingredients used in order to obtain an active film or coating are antioxidant and antibacterial compounds. Delaying the oxidation (by binding pro-oxidation compounds or by releasing antioxidant ones) and stopping pathogen development (organic

acids, negatively charged phosphate groups, essential oils, anthocyanins, chitosan) are, in the stated cases, the main objectives of the active packages (Adilah et al., 2018; Mousavi Khaneghah et al., 2018). Chemical, biochemical or biological changes on the surface of the product turn on the release of the active compounds and ensure a prolonged freshness and shelf life. Food packaging is a broad area where innovation has no limit. An important step was made in the food sector when intelligent and smart packages were applied. Intelligent packages equipped with sensors, indicators (pH, temperature), or tagged with radio frequency inform the consumer based on the ability of the package to feel, notice, or record outer or inner changes in the product. These systems are advanced, using computer applications, nanotechnologies and microelectronics (Kalpana et al., 2019; Ghoshal et al., 2018). In Figure 6 multiple valences that a protein-based food package can have can be seen.

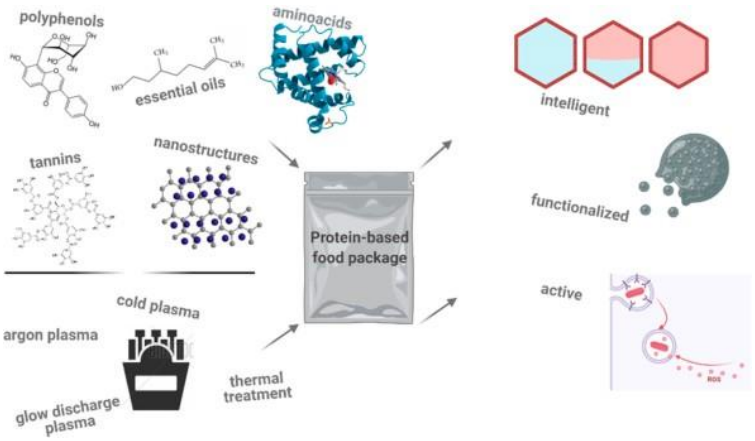

Figure 6. Compounds and technologies that add values to protein-based food packages.

7.3 Proteins Used for Food Films or Coatings

Many researchers have dedicated their work to the insertion of biopolymers in active packaging. Proteins, units formed by a covalent peptide bond, are among these biopolymers (Hanani et al., 2014). Many important protein sources are found in different vegetable or animal sources. Because of the widespread of resources within these basic products, researchers started to extract polypeptides from a large variety of vegetable and animal products or by-products (Parimi et al., 2015; Soto-Sierra et al., 2018). For example, *Moringa oleifera* seeds are an important source of proteins (40%) with antioxidant activity (Liang et al., 2019); insects are also an explored source together with fish by-products (Gasco et al.,2020) or plant-based by-products (Gençda et al., 2020). There are different types of proteins such as the plant-derived proteins from corn (Cho, S.Y et al., 2010), wheat and soy (Cho, S.Y et al., 2010), etc., and animal-derived proteins such as collagen (Wang, et al., 2015), keratin

(Pardo-Ibáñezet et al., 2014), casein (Moreira et al., 2011) and gelatin (Ramos et al., 2016; Liu et al., 2018).

7.4 Vegetable Protein-Based Packages

Vegetable proteins used in food packaging are suitable for vegan diets and bring several advantages such as active ingredients, large diversity, and economic sustainability (Li et al., 2020). When adding plant extracts or plant byproducts into protein-based food packages, an increased bacterial protection can be registered due to the presence of phenolic compounds (Umaraw et al., 2020). The main protein found in corn is zein, which has hydrophobic, antioxidant, and antibacterial properties, and forms an adhesive film (Mohamed et al., 2020). By adding sugar plasticizers into zein films, the hydrophobicity can be enhanced (Hanani et al., 2019). One method for obtaining soy protein film is by boiling soy milk in a thin pot until the film is formed, while another method is based on baking soy protein isolates on pans for 1 h at a temperature of 100 °C (Mohamed et al., 2020). In a study, canola and sorghum proteins were added to soy proteins to improve adhesion (Hanani et al., 2019). In another study, a coating based on Plantago major seed mucilage and enriched with *Anethum graveolens* essential oil inhibited bacterial (*E. coli, S. aureus, S. pyogenes, B. subtilis, B. cereus, L. monocytogenes*) and fungi (*Candida albicans, Aspergillus fumigatus*) growth (Fărca et al., 2015). In a review describing the food preservation of active films and coatings, the authors reported significant inhibition on the growth of spoilage bacteria (Umaraw et al., 2020). The authors obtained significant reductions in the growth of spoilage microorganisms and achieved 18 days of storage by using the active coating, whereas the shelf life of control samples was 6 days (Umaraw et al., 2020). Another agro-food by-product with promising reuse potential due to the significant content of proteins, fibers and phenolic compounds is the malt spent grain biomass generated in the brewing process (Behbahani et al., 2017). Due to its low cost and high availability, brewers' spent grains proteins could be considered as a cheap alternative for the preparation of biodegradable film with antioxidant activity. In this sense, Proaño et al. (2020) investigated the potential of developing brewers' spent grain protein films (casting protein dispersion) with active packaging properties. Of all the tested parameters (different pH and plasticizers levels), the films prepared at pH 2 and plasticized by polyethylene glycol (PEG) exhibited enhanced mechanical properties. In a similar study, Lee et al. (2015) demonstrated that the incorporation of chitosan into the brewers' spent grain protein resulted in a composite film with good antioxidant and antimicrobial activities. Edible coatings can be formed by treating the grass pea flour suspension with microbial transglutaminase (mTGase) using a small quantity of glycerol (8%) as a plasticizer. The enzyme treatment has a small influence on the typical protein agglomerate size, by

decreasing the particle dimension; meanwhile, the treatment with mTGase does not influence the zeta-potential and the polydispersity index of the resulting film forming solution. While the microbial transglutaminase is present, the film opacity is eight times smaller than the non-transparent polypropylen and bigger by seven times than the one produced by the transparent cellulose triacetate, the grass pea flour being slightly transparent, which is shown by the optical analysis. The SEM analyses of the coating surface and the crosssection, proven by morphology research, shows that mTGase offers a constant and soft structure. The presence of the microbial transglutaminase created bioplastics with an increased extensibility, which were less hard and more resistant, in terms of the mechanical properties (Giosafatto et al., 2018). According to Takma et al. (2019) an active packaging film was made with coatings of alginate, antimicrobial chitosan and incorporated black cumin essential oil. The chicken breast was stored at 4 °C over 5 days, time in which the film demonstrated a lower color change, lower microbial growth, and fewer pH changes. To give fresh and safe meat products, black cumin essential oil can be used in active packaging due to its antimicrobial activity against *E. coli*, a Gram-negative bacterium which is responsible for foodborne disease; it is bacteria especially found in the raw meat foods (Takma et al., 2019). In another study of Badr et al. (2014), the antimicrobial efficiency of edible films made from whey protein, and incorporated with 1–2.5% thyme, cinnamon and cumin essential oils on fresh beef was evaluated. After the storage of this meat at 5 °C for 12 days, it was observed that the sliced meat containg thyme essential oil had a stronger inhibition on the bacteria because of a higher antimicrobial activity under these parameters. The results showed that whey protein edible film which contains 2.5% w/w of cumin, thyme and cinnamon essential oil can double the shelf life of fresh beef meat when stored under refrigeration (Badr et al., 2014). Another type of edible film was prepared from a composition of alginate-clay by adding some essential oils (cumin, marjoram, coriander, cinnamon, clove and caraway) and its antimicrobial activity was evaluated on rainbow trout slices. The maximum inhibitory effect was demonstrated by marjoram essential oil, followed by the clove and cinnamon oil. Furthermore, the results showed that the film which contained alginate-clay with 1% incorporated marjoram essential oil delayed the development of *L. monocytogenes* during 15 days under refrigerated storage with a total of 6.23 log CFU/g (colony forming units), while in control samples a 7.38 log CFU/g ($p < 0.05$) was reached. As a conclusion, the intensity of the antimicrobial activity has the following order: marjoram > clove > cinnamon > coriander > caraway > cumin essential oil (Alboofetileh et al., 2016). It is known that some types of quinoa proteins are used to create edible films and presented remarkable consequences on their physical properties (water vapor permeability, water sorption, roughness and solubility). Being cross-linked with transglutaminase helped with the improvement of the edible films' properties. In addition, the quinoa protein variety combined with its proteins profile is directly

connected to the interactions between proteins and transglutaminase (Caro et al., 2016). The association of the transglutaminase with the lysine of wild quinoa and quinoa Pasankalla is shown in a study presented by Escamilla-García et al. (2019). The lowest solubility (14.02 ± 2.17%, w/w) was shown by the mixture of chitosan: wild quinoa (1:5, w/w). The water vapor permeability was different and varied because of the composition of the mixture. The water vapor permeability of the chitosan: quinoa protein varied from 2.85 to 9.95 × 10−11 g cm Pa−1 cm−2 s −1, in the absence of transglutaminase. When transglutaminase was added to the mixture the range was reduced to 2.42–4.69 × 10−11 g cm Pa−1 cm−2s−1. The film surface roughness was reduced from 8.0 ± 0.5 nm to 4.4 ± 0.3 nm by adding transglutamminase to the chitosan: quinoa Pasankalla composition. Regarding the sorption isotherm, the added transglutaminase enhanced the stability of the chitosan: quinoa wild films (monolayer (Xm) = 0.13 ± 0.02%). Consequently, the enumerated physical properties showed a much higher improvement when the edible films had a higher quantity of cross-linking. The interactions between the proteins that were caused by the amount of transglutaminase depended on profile and the protein source (Escamilla-García et al., 2019). In the research presented by Porta et al. (2015), it was proven that the surface of films is smoother and more compact in the presence of transglutaminase than when prepared without this enzyme. Studies about surface roughness show significant difference between films' preparation with (Rq 1441.1 ± 1.2 nm) or without (Rq 1484.4 ± 1.5 nm) transglutaminase. In addition, the control films prepared with transglutaminase enzyme are much more homogeneous, resistant, firm, and permeable (oxygen 700-fold and carbon dioxide 50-fold), than the control films prepared without the enzyme which have irregular zones in the films' cross-sections (Porta et al., 2015). Another study of the same topic included bitter vetch protein films but with spermidine, without or with a low quantity of glycerol and it was demonstrated that, by increasing the plasticizer amount, the tensile strength was gradually reduced. It was shown that the film extensibility and flexibility were improved by the spermidine, by helping the reduction in glycerol-dependence of the intermolecular forces beside the chain proteins, and also by acting as a plasticizer by ionic interaction with proteins. In this way, spermidine can be considered a second plasticizer because of its capacity to improve glycerol plasticizing action. The films which contain spermidine were analyzed under a microscope and it was confirmed that the matrices are more uniform, cohesive and compact (Porta et al., 2017). Figure 7 shows the most utilized proteins for food films and/or coatings.

Figure 7. Most utilized materials for protein-based (edible) films coatings.

7.5 Protein-Based Films and Coating Functionalization

In the agro-food industry, proteins, polysaccharides, and lipids represent the highest amount of macroelements present in crops and waste streams. In plant and animal tissues, proteins can be found in a large variety of structures and accomplish various functions. Among these functions, probably the most known are related to their involvement in biochemical reactions and the building of tissues. The excellent and wide range of functional properties of proteins will fill the need for high-performance renewable materials. Chemical, enzymatic or physical modifications and treatment of protein films as well as the preparation together with other hydrophobic polymers can have a positive influence on the mechanical strength of protein films and on their poor water vapor resistance. For the preparation of functionalized (edible) films and coatings, the most utilized proteins are casein, gelatin, wheat gluten, soy protein or zein (Coltelli et al., 2016). In the processing technique, coatings are applied to food products by liquid methods and films are obtained as solid laminates and then applied to food products (Falguera et al., 2011). Regarding the biodegradability, protein-based food packages are among the most feasible ones (Moosavi et al., 2020). Low price and sustainability are the most important aspects from the industrial point of view (Li et al., 2019). The inclusion of different components like natural antioxidants improves the antioxidant properties or strengthens the protein networks. The insertion of functional groups in the amino acid side chain of proteins allows covalent and non-covalent crosslinking. Chitosan derivatives acts as non-covalent crosslinking agents based on hydrogen bonding with plant proteins like whey (Braber et al., 2021). The crosslinking leads to an increase in the film's insolubility and elongation and an increase in the surface hydrophobicity with

a contact angle larger than 90° These effects on the film properties are possibly caused due to conformational change of the proteins after the crosslinking. An important functionalization of the protein-based films is described by Cano et al. (2020). They analyzed the antioxidant activity of tannins incorporated in protein-based packages. Furthermore, tannins from different sources (white peel grape, red peel grape, from oak bark, guava leaves etc.) have also proved to have antimicrobial activity. The addition of tannin makes the package less soluble. An emerging technology—cold plasma—is used for the protein film and coating properties modeling, namely by improving the adhesion properties (Moosavi et al., 2020). The influence of different plasma treatments as a method for modification is investigated and described by Romani et al. (2020). Thermal treatment of food proteins, e.g., from yellow peas, causes physical and chemical changes to their structures due to the fact that proteins rearrange which cause their secondary and tertiary structures to unfold due to the breaking of hydrogen bonds. This effect leads to an increase in surface hydrophobicity (Acquah et al., 2020). Incorporation of montmorillonite clay together with citric acid increased the barrier properties and leads to a sequential decrease in the physicochemical quality loss in processed apples (Azevedo et al., 2018). The addition of cellulose nanocrystals (CNCs) can lower the moisture amount of the protein-based package by disintegrating the hydrogen bonds between the proteins' amino groups and the molecules of water. Additionally, the filling effect of the incorporated CNCs make the film more rigid (Yu et al., 2018). The incorporation of natural antioxidants into protein films influences the antioxidant activity. The incorporation of mango kernel extracts (MKE) in soy protein isolate (SPI) and fish gelatin (FG) films maximizes the antioxidant activity. The FG films showed improved thickness, higher tensile strength, and ensured more transparency, meanwhile, SPI film showed higher antioxidant activity and improved water barrier properties (Adilah et al., 2018). Into a whey protein-based active film, rosemary and thyme extracts were incorporated and the antioxidant activity of the extracts was evaluated (Andrade et al., 2018).

7.6 Antioxidant, Antimicrobial/Antifungal Activity of Protein-Based Films

Oxidation and microbiological contamination are the main processes blamed for food spoilage and food-borne illnesses. There are plenty of studies (Table 10) on protein-based films and coatings that sustain bioactive compounds (with antioxidant and antimicrobial
activity) incorporation into the package to obtain an extended shelf life of the food product. The conveniences of this practice are given by the fact that the biomolecules incorporated into the film or coating, (i) do not influence the product teste, (ii) are released in a controlled manner and (iii) may ensure less additives are inserted (antioxidants, preservatives) into the product. Moreover,

biodegradable and inexpensive make characteristics them even more suitable. Bioactive compounds may have various sources and thus various modes of action.

Film/Coating	Formulation	Antioxidant Capacity	Antimicrobial/Antifungal Activity Against	Reference
Edible coating	whey protein isolate whey protein concentrate hydroxypropyl methylcellulose beeswax or carnauba wax	decrease enzimatic browning (just for the whey protein-based coating)		[85]
Film	Polyvinyalcohol with lysozyme	-	*Micrococcus lysodeikticus*	[86]
Film	mung bean protein pomegranate peel (0, 2.5, 12.5, and 25% w/w)	13.88 mg GAE/g (gallic acid equivalents) (25% pomegranate peel)	*Escherichia coli O157:H7* *Listeria monocytogenes*	[87]
Film	soy protein isolate with cortex *phellodendron* extract (0, 10, 12.5, 15, 17.5, 20, 22.5% w/w)	14.87 mg GAE/g (22.5% *phellodendron* extract)	*Staphylococcus aureus* ↓ *Escherichia coli*	[88]
Film	soy protein isolate fish gelatin mango kernel extracts	3.77 μg GAE/g film	-	[18]
Film	soy protein isolate licorice residue extract (10, 30, 50, 70 g/kg)	20% higher than in the control	-	[89]
Film	distiller dried grains with soluble (protein) green, black and oolong tea extract (0.1, 0.3, 0.5%)	all 0.3% samples had over 50% higher antioxidant activity than control	-	[90]
Film	soy protein isolate chestnut (*Castanea mollissima*) bur extracts (20, 50, 80, and 100 g/kg)	at least 20% higher than the control	-	[91]
Film	fish myofibrillar protein catechin–Kradon extract	at least 40% higher than the control	-	[92]
Coating	Whey protein TiO_2 nanotubes	over 50% higher than the control	*Listeria monocytogenes* *Staphylococcus aureus* *Salmonella enteritidis* *Escherichia coli*	[93]
Film	cassava starch and whey protein rambutan peel extract cinnamon oil	over 30% higher than the control	*Bacillus cereus* *Staphylococcus aureus* *Escherichia coli*	[94]
			Aspergilus niger CGMCC	[95]
Film	soy protein isolate plant-sourced cinnamaldehyde zinc oxide nanosheets	-		
Film	gelatin nano-chitin	-	*Aspergillus niger*	[96]
Film	gelatin mannoprotein (extracted from *Saccharomyces cerevisiae* cell wall)	-	*Aspergillus flavus* binding aflatoxin B1	[97]

Table 10. Antioxidant, antimicrobial and antifungal activity of protein-based films and coatings (Mihalca et al., 2021).

7.7 Safety Issues

There are many ways in which food quality can be altered causing spoilage of the products, the most common reasons being oxidation and microbial growth. To make food packages more reliable, scientists began using the packaging system not only to provide critical information about the product, but also to be a line of defense against microorganisms and oxidation. Active packaging begins to be used on a larger scale simply because it helps improve the products' shelf-life and quality. A common procedure to overcome the simple barrier of (protein-based) films and coatings and bring more value to the safety aspect of the package, is to incorporate active materials. These procedures develop a new class of packages—active packages (Cuibus et al., 2015). The safety issues are controlled by the active protein-based films and coatings using mechanisms such as decreasing the microbial development, delaying oxidation through antioxidant compounds and decreasing moisture migration. By implementing these active components, the food quality, shelf-life, and freshness are also improved (Kalpana et al., 2019). Intelligent packaging can overcome safety concerns, as monitoring systems can give information about the status and quality of a product directly from the package, and therefore helps to reduce food waste and spoilage (Müller et al., 2019). The most common indicator used in this kind of packages is the change in color, and among the quality change the microbial activity is the most monitored one (Pavelková, 2013). Various researchers discuss whey protein-based films' property of having a relatively low oxygen permeability (Sabato et al., 2001). This fact makes them potentially useful for coatings or other film materials used for oxygen-sensitive food products (Schmid et al., 2019). Minimizing the growth rate of foodborne pathogens by using antimicrobial agents in packaging material could extend the shelf life of packaged foods. In the last decade, food additives, preservatives, were the main option for food shelf-life extension. Consumers and healthcare institutions' concerns about additives levels led to the development of innovative antimicrobial films and coatings application methods. An antimicrobial protein or non-protein packaging system is developed by the incorporation of antimicrobial fillers directly into the films, by coating the packaging films with antimicrobial compounds, or by using polymers that have natural antimicrobial features. Afterwards, the antibacterial agent is slowly released on top of the food surfaces. The slow-release system helps retain a sufficient concentration of the antibacterial agent, ensuring antimicrobial protection during the product shelf life (Jafarzadeh et al., 2020). Frequently utilized antimicrobial agents are herbs and nanoparticles (Mesaros et al., 2019; Cuibus et al.,2015). The typical herbs such as thyme, oregano and tarragon contain caffeic acid (Pop, O.L. & Vodnar, D.C., 2016), which has a strong effect against pathogenic bacteria, viruses, and fungi. Flavones have phenolic structures with one carbonyl group. These Polymers 2021, 13, 769 15 of 23 kinds of compounds are synthetized by

the plants to protect them against microbial attack. Due to this fact, their action is efficient against a broad range of pathogens (Jafarzadeh et al., 2020; Mustapha et al., 2017). Nanoparticles and nanostructures of silver or gold inhibit the growth of foodborne pathogens due to their broad-spectrum antimicrobial activities. Moreover, nano-silver and nano-gold could catalyze the absorption and decomposition of ethylene emitted from fruit metabolism, which is blocking the ethylene and thus ensure prolonged shelf life (Blanke et al., 2012). An increase activity of the nano-functionalized package can be explained by the fact that, for example, ZnO nanoparticles directly interact with the food, significantly increasing the shelf life (Sharma et al., 2020; Baldea et al., 2020). Nanotechnology has is gaining field not only in medicine but also in cosmetics and food sectors (Diaconeasa et al., 2015). Due to their nanosized structure, their exact behavior in the human body (i.e., edible packages containing nanoparticles) is under safety concerns. Recent analyses regarding the toxicity, behavior, and long-term use of nanoparticles in food systems underline the urgent need for more studies. Nanoparticles, in any food system, are only allowed to be used, in Europe (EU), if they are stated to be safe in Annex I of the 10/2011 EU regulation (Das et al., 2009). Safety concerns rise especially in the cases of edible packages, intelligent and active packages where the nanoparticles are released and come into contact with the food on purpose. Authorities and consumers' concerns are related to nanoparticles' toxicity and accumulation in the body. Brugè et al. in their cytotoxicity study, found that all investigated nanostructured lipid carriers are biocompatible with skin cells, but some of them are sensitive to UV irradiation (Brugè et al., 2015). Furthermore, in any new formulation, the components (new protein sources, extracts) must be verified as being accepted for contact with food products or for ingestion, in the case of edible packages. Anyway, an important issue regarding food safety is protein-based edible films and coatings made from edible ingredients. In this sense, it is imperative that edible films and coatings ingested together with the food product be safe for consumption, with no health risk involved (Mohamed et al., 2020).

8. Biofilm and biofilm control

Biofilm formation is a cyclical and dynamic process that comprises different steps, starting from a reversible attachment of planktonic cells to a surface, followed by an irreversible attachment with the production of extracellular polymeric substance (EPS) that include proteins, polysaccharides, lipids and DNA. Biofilms are usually made up of a single or multiple species, forming the so-called multi-species biofilms, which show also higher resistance to antimicrobial and sanitizing agents and could involve spoiling and pathogenic bacteria (Srey et al., 2013; Giaouris et al., 2014; Galié et al.,2018). The ability of pathogens to adhere and to form biofilm are relevant concerns for the food industry, since these characteristics influence their persistence in the environment and on the equipment of the

food processing facilities, compromising food safety and quality (Abdallah et al., 2014). *Listeria monocytogenes* is a foodborne pathogen well known for its resistance to harsh environmental stresses including antimicrobial agents, sanitizers, and disinfectants. *L. monocytogenes* persists in the food processing environment by forming biofilms, leading to the cross-contamination of associated food products, resulting in economic losses due to recalls of contaminated foods (Ferreira et al., 2014). Chemical-based methods for the control of biofilms have been widely used; however, they are often insufficient to completely remove sessile bacterial cells. In addition, current sanitization approaches have some well-known draw backs, such as the possible toxicity and even carcinogenicity due to possible food contamination by residues, and the continuous exposition to these agents has been contributing to the acquisition of resistance by the pathogenic bacteria. So, the study of new agents able to inhibit/prevent biofilm formation and eradicate/remove already established biofilms is of particular importance for the food industry. Furthermore, in response to consumer demands for natural products, alternatives to the use of chemicals are required. Among the environmental-friendly strategies, the use of Lactic Acid Bacteria (LAB), bacteriocins produced by LAB and essential oils (EOs) have great potential for food industry application against biofilms formed by foodborne pathogens, such as *L. monocytogenes*. LAB have been used in food due to their limited health risk to humans and their metabolic characteristics. Currently, research is focused on the application of LAB to colonize different types of surfaces (Gómez et al., 2016; Winkelströter et al., 2014). Their colonization on these surfaces counteracts the formation of biofilm by pathogenic bacteria based on the competitive exclusion as well as the effect of several potent metabolites such as lactic acid, hydrogen peroxide, antimicrobial peptides, and bacteriocin produced by the LAB (Hibbing et al., 2010). Apart from the bactericidal activity (killing of the cells) of the above metabolites, bacteriostatic effects (cell growth prevention) by some of the LAB and their derivatives have also been observed towards pathogenic microorganisms (Mao et al., 2020; Prabhurajeshwar et al., 2017; Niederhäusern et al., 2020). These derivates contribute in the prevention of bacterial infection by inhibiting the adhesion, colonization, biofilm formation, attenuating the QS signaling system, and eradicating the mature biofilm of spoilage and food-borne bacteria (Kiymaci et al., 2018; Melo et al., 2016; Iseppi et al., 2020).

In recent times, the interest in the anti-biofilm activity of essential oils (EOs) has been increasing, because their different chemical components can be exploited as antimicrobials not only against planktonic cells but also against the sessile cells. In fact, EOs can interfere with the mechanisms involved in biofilm formation, thus enabling to impair and control this process.

The literature suggests a promising use of EOs in sanitizing formulations to control microbial biofilms in food industries, and EOs based solutions applied to sanitize surfaces appear to be easily removed

with washing procedures without residual odor problems (de Oliveira et al., 2010). EOs rich in monoterpenes or phenylpropanoids demonstrated high efficacy in bacterial biofilm prevention. Indeed, during the whole evolution of the biofilm formation process, EOs exert antimicrobial activity on planktonic and sessile cells; most of the studies on the mechanisms of action of EOs (Serio et al., 2010; Zhang et al., 2016) reported that they can increase membrane permeability, disturbe cell membrane integrity, then inhibiting microbial growth. Although the interest in biofilm is growing, the reports describing the anti-biofilm mechanisms of action of EOs are limited and the phenomenon is not completely understood. Overall, the potential controlling mechanism of EOs is mainly due to the action on the multiple stages of biofilm formation. In fact, during the life cycle of biofilms (adhesion, microcolonies formation, and maturation) the anti-biofilm effects are principally related to the inhibition of EPS matrix, the suppression of cell adhesion and the QS system alteration.

9. Aim of the study

Foodborne infections due to bacterial pathogens like *L. monocytogenes*, responsible for over 90% of all cases of food poisoning with a lethality of 20-30%., remain a serious clinical problem, and the employment of chemical additives is less and less accepted by the consumers and limited by restrictive laws. Given the wide spread of this foodborne pathogen endowed with psychrotrophic feature, the severity of the pathologies sustained (nervous-meningeal, abortigenic or septicemic forms), the occurrence of antibiotic resistance in *L. monocytogenes* strains isolated from various RTE food products and the expansion of the categories at risk, preservative interventions aimed to prevent food contamination by this microbial agent are necessary. Chemical additives are frequently used to limit microbial growth on foods; however, their use is less and less accepted by consumers, who prefer natural antimicrobial substances as food preservatives. Moreover, the growing demand for high quality and safe foods is leading to an increase in the study of new preservation methods to be combined with refrigeration for the control of *L. monocytogenes.* Edible coatings or films based on the incorporation of natural substances like EOs and bacteriocins may be a new green preservation method to reduce the use of synthetic polymers. EOs have been extensively studied as natural compounds to provide benefits in food and human health. EOs or their components can be added directly to foods or incorporated into films or coatings made of non-renewable materials or biomaterials to be released during transport and storage. Bacteriocins are endowed with bactericidal (causing cell death) or bacteriostatic (causing a slowdown in growth) activity and, for these reasons offer potential applications in food preservation. Moreover, their use in the food industry can help to reduce the addition of chemical preservatives as well as the intensity of heat treatments, resulting in naturally preserved foods that have maintained their organoleptic and nutritional properties. Microbial contamination of RTE products remains a challenge as a large proportion of products originates in developing countries. Shrimp, for example, is one of the most important aquatic products and the psychrotropic pathogen *L. monocytogenes* is frequently associated with this slightly preserved seafood product, showing a great ability to attach to the cooked shrimp carapace. For these reasons, the food industry is in general interested in the use of natural substances like EOs or bacteriocins and the incorporation of the same in packaging materials for both the pathogens control and maintenance or extension of product shelf life could be a stepping stone to future solutions.

Therefore, the aim of the study was:

(i) to evaluate the anti-*Listeria* and anti-*L.m* biofilm activity of two types of natural compounds: bacteriocin LP17, produced by *Enterococcus mundtii* LP17 (isolated from red mullet) and EOs

derived from *S. officinalis* and *T. vulgaris*, condiment plants commonly used in the Mediterranean area, by themselves or in combination. **Part 1**

(ii) to evaluate in artificially contaminated shrimps the anti-*Listeria* activity of four EOs (*Salvia officinalis, Citrus limon, Mentha piperita and Thymus vulgaris*) and bacteriocin bacLP17, previously isolated and characterized, used alone or in combination and/or added to edible coating. **Part 2 (A and B)**

10. Characteristics of bacteriocin bac LP17 produced by *Enterococcus mundtii* strain isolated from seafood and used in the present study

The new bacteriocin bacLP17 used in the present study was produced by the bacteriocinogenic LAB *Enterococcus mundtii*, isolated in a previous investigation (Iseppi et al., 2019) in the laboratory of Applied Microbiology (Department of Life Sciences – University of Modena and Reggio Emilia). This natural antibacterial substance was studied and characterized for its potential use in food preservation.

Bacteriocin bacLP17 presents a large inhibition zone against some *Listeria* species and notably towards *Listeria monocytogenes* NCTC 10888 (Figure 8) and is sensitive to proteolytic enzymes (proteinase K and trypsin), indicating their proteinaceous nature, but activity was not altered by pepsin (Figure 9).

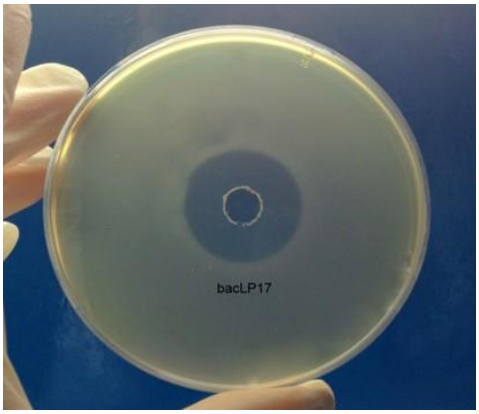

Figure 8: Agar well diffusion method used to show inhibition of *L. monocytogenes* NCTC 10888 by bacteriocin bacLP17.

(a) (b)

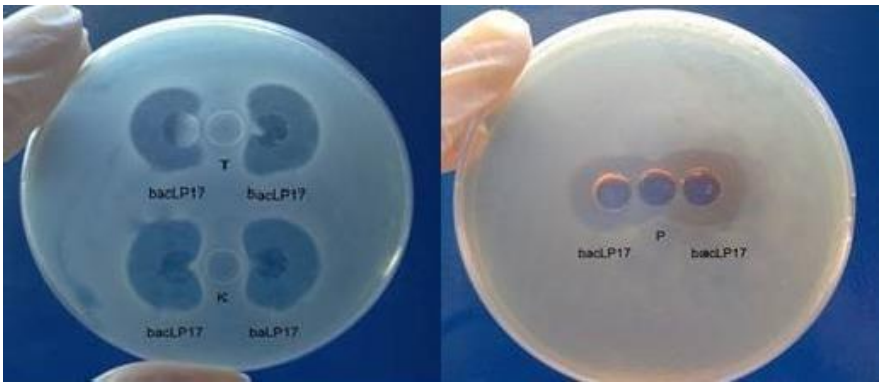

Figure 9: (a) Effect of trypsin (T), proteinase K (K) and (b) pepsin (P) on bacteriocins bacLP17.

BacLP17 is heat tolerant and remained active after treatment at 100 °C for 1 h (Figure 10a) and after autoclaving temperature. In addition, the exposure to different pH values for 1 h at room temperature showed its anti-listerial activity in the pH range of 2.0-12.0 (Figure 10b). Its storage 4 °C and -20 °C for six months did not affect the antibacterial activity. Furthermore, it remained stable when treated with 1% (by mass) of NaCl, sodium dodecyl sulfate (SDS), urea and 1% (by volume) of Triton-X100, Tween 20 and Tween 80 (Figure 11).

(a) (b)

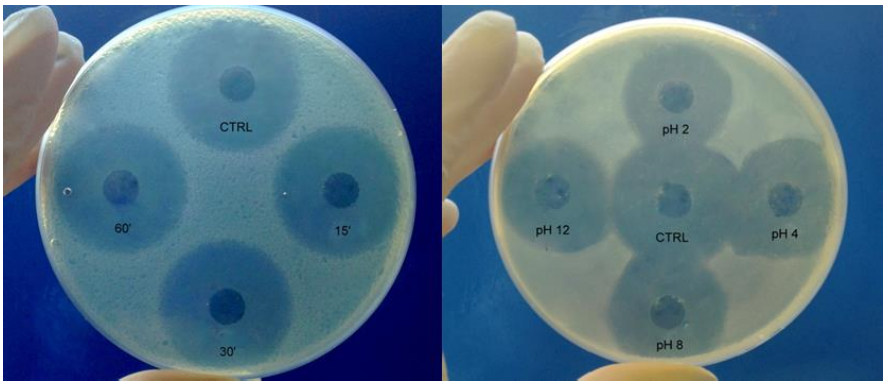

Figure 10: (a) Effect of temperatures (100°C for 15', 30' and 60') and (b) pH (2.0, 4.0, 8.0, and 12.0) on antimicrobial activity of bacteriocins bacLP17. CTR, refers to CFSF untreated.

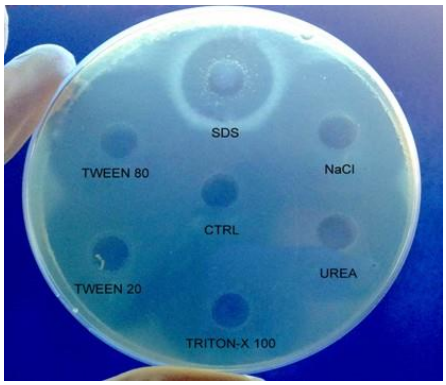

Figure 11: Effect of detergents and salts on antimicrobial activity of bacteriocins bacLP17. CTR, refers to CFSF untreated.

The antibacterial activity against the pathogen, translated as arbitrary units of inhibition (AU/ml), revealing for bacLP17 a strong antimicrobial titre of 1280 AU/ml (Figure 12). Optimal production of bacteriocin bacLP17 (1280 AU/ml) was recorded in DeMan, Rogosa and Sharpe (MRS) broth, adjusted to pH 6.0, 7.0, 8.0 and 9.0, during 24 h of incubation at 30°C.

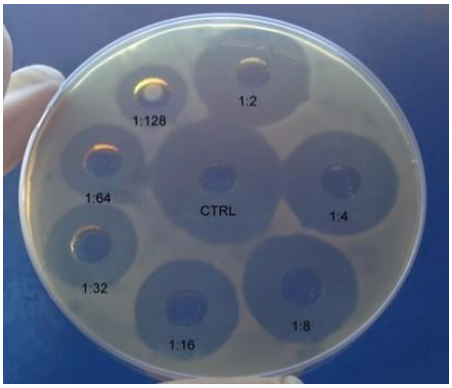

Figure 12: Antibacterial activity titration expressed as arbitrary units of inhibition. CTR, refers to CFSF untreated.

The production of bacteriocin bacLP17 reached 1280 AU/ml after 8 h of incubation at 30°C. During 24 h of growth, the cell density increased from 0.01 to 0.7 (OD595) and the pH decreased from 6 to 5.5 (Figure 13).

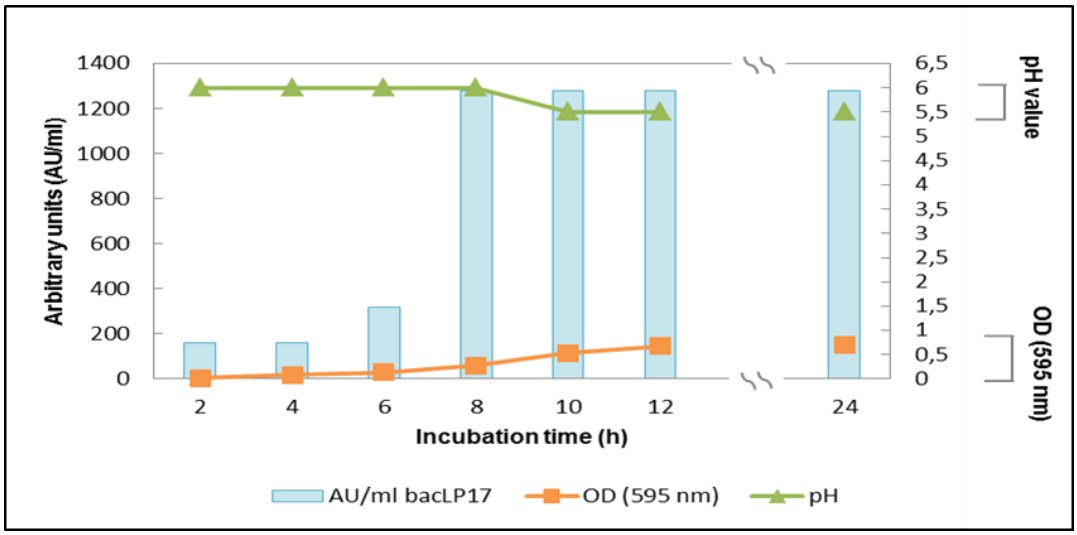

Figure 13: Growth of *Enterococcus mundtii* strain and bacteriocin bacLP17 production in MRS broth.

The adsorption of bacteriocins bacLP17 to *L. monocytogenes* NCTC 10888 cells were of 75%. This level of adsorption (75%) was observed at 20°C, 30°C and 37°C, while a reduction to 50% of adsorption to pathogen cells resulted at temperature of 4°C. Optimal adsorption of bacLP17 (87.5%) to *L. monocytogenes* NCTC 10888 was observed at pH=8.0 and pH=10.0, whereas lower levels of pH (2.0-4.0) resulted in reduction to 75% of the adsorption (Table 11).

	bacLP17
Control	75*
Temperature	
4°C	50
20°C	75
30°C	75
37°C	75
pH	
2.0	75
4.0	75
8.0	87.5
10.0	87.5

Chemicals 1%	
NaCl	75
Triton-X100	50
Tween 20	0
Tween 80	0

* results presenting the % of adsorption of bacteriocins bacLP17 to *L. monocytogenes* NCTC 10888

Table 11: Effect of temperature, pH and chemicals on the adsorption (%) of bacteriocins bacLP17 to *L. monocytogenes* NCTC 10888.

To study the mode of action of bacteriocin bacLP17, it was addictioned (1280 AU/ml) to an early-log culture of *L. monocytogenes* NCTC 10888 (3 h-old, OD595 ≈ 0.03) repressed pathogen growth for 6 h and 8 h, respectively. So, the bacteriocin demonstrated a bacteriostatic mode of action toward *L. monocytogenes* tested strain since no cell lysis was observed by OD measurement. In the untreated (control) samples, no repression or inhibition growth was observed. Finally, the molecular weight of bacteriocin bacLP17 was analyzed by SDS-PAGE. After incubation, an inhibition area around a band appeared (Figure 14), suggesting that its molecular weight was approximately 6.0 kDa in size.

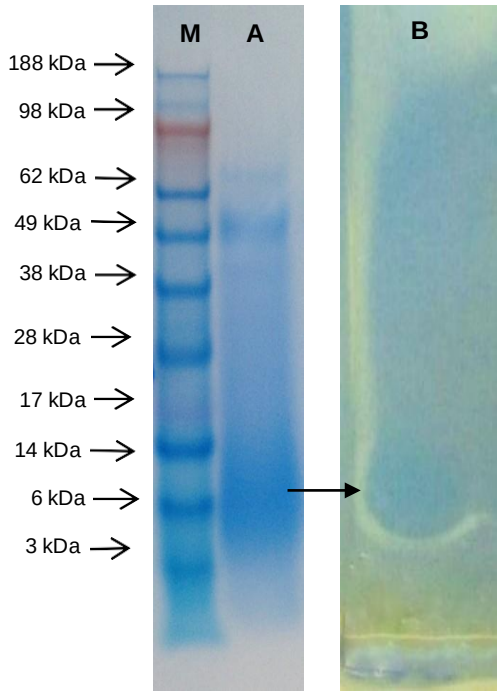

Figure 14: Separation of bacteriocins bacLP17 by SDS-page. Lane M: molecular mass marker; Lane A: peptide band of strains LP17 stained with Coomassie Brilliant Blue R; Lane B: zone of growth inhibition of bacteriocin bacLP17.

11. Experimental Section

11.1 Part 1. Study of the antimicrobial activity of bacteriocin bacLP17 and essential oils (EOs) by themselves or in combination against *Listeria monocytogenes* both in planktonic and in sessile forms

The first objective of the study was to confirm the antilisterial activity of bacLP17, produced by *Enterococcus mundtii* LP17, a strain previously isolated from red mullet and endowed with a strong activity toward the pathogen (Iseppi et al., 2019), and to evaluate the antibacterial activity of two EOs derived from *S. officinalis* and *T. vulgaris*, condiment plants commonly used in the Mediterranean area. Afterwards, these natural compounds have been studied against the pathogen, both in planktonic and in sessile forms (mature biofilm), by themselves or in combination.

Materials and methods

Bacterial strains, media and culture conditions

The EOs, obtained by hydro-distillation of dried spices were bought from a local herbalist shop. To assess the antibacterial activity of *T. vulgaris* and *S. officinalis* EOs and bacLP17, twelve bacterial strains, 2 classified *L. monocytogenes* (NCTC and ATCC) and 10 *L. monocytogenes* wild type, were used (Table 12). Both *E. mundtii* LP17 producer strain and all *L. monocytogenes* used as indicators were grown in Tryptic Soy Broth or Agar (TSB or TSA, Oxoid S.p.A, Milan, Italy). All strains were maintained in the same media containing 20% (w/v) glycerol at -80 °C until use.

Anti-Listeria activity determination

The preliminary determination of the antibacterial activity of *S. officinalis* and *T. vulgaris* against all *L. monocytogenes* strains, was carried out by using the agar disk diffusion assay, according to the standard procedure of the Clinical and Laboratory Standards Institute (CLSI 2012). Sterile disks of 6 mm in diameter, containing 10 mL of each EO, were placed on Mueller Hinton Agar (MHA, Oxoid S.p.A, Milan, Italy) plates, previously seeded with 100 ml of 107e108 cfu/ml of bacterial suspensions. A sterile disk added with sterile distilled water was used as negative control. After incubation at 37 °C for 24 h, the antagonistic activity of the EOs was quantified by a clear zone of inhibition in the

indicator lawn around the disks and the diameters in millimeters of these zones were measured (Klancnik et al., 2010). The antibacterial activity of bacLP17 was determined by the agar well diffusion assay (Rogers et al., 1991) against the same 11 strains of *Listeria* as indicators. To extract the bacteriocin released in liquid medium, the crude filtrate supernatant fluid (CFSF) of *E. mundtii* LP17 was prepared from a culture in TSB broth grown at 37 °C for 24 h. Cultures were centrifuged at 12,000xg for 30 min at 4 °C and supernatant fluid was collected, dialysed against 30 mmol/l sodium acetate buffer (pH 5.3) and filter sterilized (0.45 mm-poresize filter; Millipore Corp., Bedfort, Mass.). To eliminate the anti-microbial effect of organic acids, the pH of the supernatants was adjusted to 6.0 with sterile 1 M NaOH. The obtained crude bacteriocin bacLP17 was used at the concentration of 1280 arbitrary units for milliliter (AU/ml), previously determined as the reciprocal of the highest dilution of CFSF producing a distinct inhibition of the indicator lawn (Mayr-Harting et al., 1972). 50 ml of CFSF containing bacLP17 (1280 AU/ml) was brought to 100 ml volume with phosphate buffer (pH 6) and dispensed into 8 mm diameter wells previously performed in Tryptic Soy agar plates (TSA, Oxoid S.p.A, Milan, Italy). 100 ml of phosphate buffer was used as negative control. After diffusion of the solutions, plates were slowly seeded with 5 ml of warm TSA (0.7%) containing 107 cfu/ml from over-night cultures of the same indicators listed in Table 1 and incubated at 30 °C for 24 h. The presence of the antagonistic activity was determined by a clear zone of inhibition in the indicator lawn around the wells.

Minimal inhibitory concentration (MIC)

The MIC values of bacLP17, *S. officinalis* and *T. vulgaris* EOs were determined against *L. monocytogenes* by using the broth microdilution method in 96-well microplates, according to the Clinical Laboratory Standards Institute (CLSI) guidelines 2012. The test was performed in sterile 96-well microplates by dispensing into each well 95 ml of Tryptic Soy Broth (Oxoid S.p.A, Milan, Italy) and 5 ml of bacterial suspensions, to a final inoculum concentration of 106 CFU mL^{-1}. Then, 100 ml of bacLP17 and EOs serial dilutions were added to obtain concentrations ranging from 512 to 0.125 ml/ml (Afonso et al., 2019, Sahin et al., 2004). Negative control wells consisted of bacteria in TSB without bacteriocin and EOs. The plates were incubated at 37 C for 24 h, mixed on a plate shaker at 300 rpm for 20 s, and the MIC was defined as the lowest concentration of bacteriocin and EOs that inhibited visible growth of the tested microorganisms after the optical density (OD) measure at 570 nm using a microtiter-plate reader.

Combination of essential oils and bacteriocin

The combined effects of *T. vulgaris*/*S. officinalis* and EOs/bacLP17 were calculated in terms of fractional inhibitor concentration index (FIC-Index). The FIC-Index is calculated by comparing the value of the MIC of each agent alone with the combination-derived MIC. Antimicrobial combinations that result in a fold reduction in the MIC compared with the MICs of agents alone are synergistic (FIC 0.5), whereas FICs in the 0.5 to 1.0 range are non-synergistic or additive. FIC-Index values from 1 to 4 are defined as indifferent, while those with a value greater than 4 are antagonistic. For the determination of the FIC-Index we used the chessboard method with a 96-well microplate. The FIC-Index was calculated as follows: MIC of the combination of essential oils/MIC of the essential oil only. Essential oils have been combined with MIC + MIC; MIC + 1/2 MIC; MIC + 1/4 MIC; MIC + 1/8 MIC; 1/2 MIC + 1/2 MIC; 1/2 MIC + 1/4 MIC; 1/2 MIC + 1/8 MIC; 1/4 MIC + 1/4 MIC; 1/4 MIC + 1/8 MIC; and 1/8 MIC + 1/8 MIC. The same calculation was used for the evaluation of FIC-Index among essential oils and bacteriocins.

Anti-biofilm activity determination

The effects of both EOs, bacLP17 and the combination between bacLP17/EOs were tested on '3 days old' pre-formed biofilm, obtained using 96-well polystyrene microtiter plates, as previously described (Condò et al., 2020). Polystyrene microtiter plates were inoculated with 200 ml of 18-h-old bacterial culture containing cell count of approximately 10^6 cfu/ml. The medium was refreshed every 24 h. After biofilm formation, the medium was gently aspirated, and plates were washed three times with a sterile phosphate-buffered saline solution (PBS, pH 7.2) to remove planktonic bacteria and the natural compounds were added at MIC concentration. Following an additional incubation for 24 h at 37 C, the biofilm biomass was determined by the crystal violet staining method (Stepanovic et al., 2007). Briefly, the supernatant was removed, and the wells were washed three times with PBS. For fixation of the biofilm's biomass, 150 ml of methanol for 15 min was added, and the supernatant was removed again. Then, 150 ml of crystal violet (CV) solution at 0.1% was added to each well and, 15 min later, the excess dye was removed by washing the plates three times with sterile PBS. The bound of crystal violet was released by adding 200 ml of 33% acetic acid followed by incubation for 10 min at room temperature. The optical density (O.D.) was measured at 570 nm using a microplate reader (Sunrise Tecan, Austria). Both 50% ethanol solution and TSB with bacterial culture only were used as negative and positive controls, respectively.

Statistical analysis

All the experiments were carried out in triplicate and the bacterial count was performed on three plates. The arithmetic means of the three determinations, expressed as log bacterial count, was plotted against the control. The results were analysed statistically with the student's t-test and differences were considered significant when $p < 0.05$.

Results

Anti-Listeria activity determination

Both EOs were active against all *L. monocytogenes* strains, as demonstrated by using the agar disk diffusion assay, even if *T. vulgaris* resulted more effective than *S. officinalis* (Table 12). Difference in *T. vulgaris* and *S. officinalis* activity was observed, with the inhibition zone greater than 3 and 10 mm (8 out 11 indicators and 3 out 11 indicators, respectively). Figure 15 shows an example of EOs anti-*Listeria* activity. The results obtained by the Agar Well Diffusion assay demonstrated the good anti-*Listeria* activity of bacLP17, with 9 out 11 showing inhibition zone greater than 5 mm, thus confirming the good anti-Listeria activity of the compound, already emerged in our previous investigation (Iseppi et al., 2019).

Table 12: Antilisterial activity detected with agar well diffusion assay deferred antagonism (bacLP17) and agar disk diffusion assays (*T. vulgaris* and *S. officinalis*).

Indicator strains	*Bac*LP17	*Thymus vulgaris*	*Salvia officinalis*
L. monocytogenes NCTC 10888	+++	+++	++
L. monocytogenes ATCC 13932	+++	+++	++
L. monocytogenes 53 A	+++	+++	++
L. monocytogenes 25 C	+++	+++	++
L. monocytogenes 40 A	+++	+++	+
L. monocytogenes 33	+++	+++	+
L. monocytogenes 722	++	++	+
L. monocytogenes 30 C2	++	++	+
L. monocytogenes 37	+++	++	+
L. monocytogenes 66	+++	+++	+
L. monocytogenes 692	+++	++	+
L. monocytogenes 4 C	+++	+++	+

(−) = no inhibition zone; (+) = 1–3 mm inhibition zone; (++) = 3–5 mm inhibition zone; (+++) = >5 mm inhibition zone.

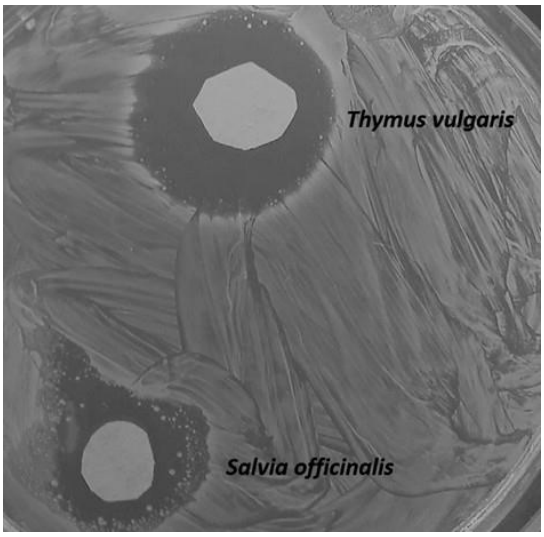

Figure 15: Agar well diffusion assay: *Thymus vulgaris* and *Salvia officinalis* EOs against *Listeria monocytogenes* NCTC 10888.

Minimal inhibitory concentration (MIC)

The MIC against all *L. monocytogenes* strains of EOs and bacLP17 confirmed the results of both the disk diffusion test and the deferred antagonism method. No strain showed resistance to both the bacLP17 and the EOs. A good antimicrobial activity was observed for bacLP17 and *T. vulgaris* EO (Table 13), with values ranging from 0.5 ml/ml to 4 ml/ml against all tested strains. *S. officinalis* EO, as already observed with the agar disk diffusion assay, resulted less active against all the indicators, with values ranging from 2 ml/ml to 16 ml/ml, and with the highest MIC value (16 ml/ml) observed for 4 out 11 indicator strains.

Combination of essential oils and bacteriocin

In general, the FIC-Index showed a good synergy between all the natural substances tested, EO/EO and EOs/bacLP17, with values equal to or less than 0.5 (Table 13). In particular, the combination EOs/bacLP17 revealed an excellent synergistic effect. The best synergy (FIC-Index < 0.5) was observed for the combination *T. vulgaris* EO/bacLP17, with values ranging from 0.195 to 0.484. A less evident synergy emerged when *S. officinalis* EO and bacLP17 were used together, with values ranging from 0.285 to 0.484. *T. vulgaris*/*S. officinalis* EOs also showed a synergistic effect (ranging from 0.312 to 0.5) against six strains out of twelve (50%) and with a FICIndex value of 0.5.

Indicators strains	Essential oils MIC		FIC-Index of essential oils/SD	MIC of BacLP17	FIC-Index of BacLP17 and *S. officinalis*/SD	FIC-Index of BacLP17 and *T. vulgaris*/SD
	Salvia officinalis	*Thymus vulgaris*				
L. monocytogenes NCTC 10888	16	0.5	0.5 (S) ± 0.00	0.25	0.285 (S) ± 0.15	0.328 (S) ± 0.12
L. monocytogenes ATCC 13932	16	4	0.5 (S) ± 0.00	0.5	0.320 (S) ± 0.08	0.484 (S) ± 0.03
L. monocytogenes 4 C	2	2	0.312 (S) ± 0.12	2	0.328 (S) ± 0.1	0.289 (S) ± 0.1
L. monocytogenes 25 C	4	2	0.5 (S) ± 0.02	0.5	0.367 (S) ± 0.02	0.406 (S) ± 0.02
L. monocytogenes 30 C2	2	1	0.375 (S) ± 0.11	0.5	0.367 (S) ± 0.07	0.195 (S) ± 0.15
L. monocytogenes 33	4	2	0.5 (S) ± 0.03	1	0.367 (S) ± 0.00	0.464 (S) ± 0.03
L. monocytogenes 37	16	0.5	0.5 (S) ± 0.01	2	0.328 (S) ± 0.1	0.390 (S) ± 0.05
L. monocytogenes 40 A	8	0.5	0.375 (S) ± 0.08	2	0.484 (S) ± 0.01	0.445 (S) ± 0.05
L. monocytogenes 53 A	4	2	0.5 (S) ± 0.02	1	0.328 (S) ± 0.06	0.242 (S) ± 0.1
L. monocytogenes 66	16	2	0.36 (S) ± 0.13	4	0.375 (S) ± 0.02	0.5 (S) ± 0.00
L. monocytogenes 692	16	2	0.375 (S) ± 0.05	4	0.484(S) ± 0.00	0.406 (S) ± 0.04
L. monocytogenes 722	2	1	0.375 (S) ± 0.07	1	0.367 (S) ± 0.12	0.343 (S) ± 0.04

Minimal inhibitory concentration (MIC): data are expressed as μl/mL.
FIC-Index, fractional inhibitory concentration index: ≤0.5 represents synergy (S); >0.5 to ≤4.0 represents indifference (I); >4.0 represents antagonism (A).
SD, Standard deviation.

Table 13: MICs of EOs and bacLP17 and synergistic interaction between EOs and for single EO in combinations with bacLP17 against *L. monocytogenes* strains.

Anti-biofilm activity determination

All *L. monocytogenes* strains employed in the study proved to be good biofilm producers, nevertheless both the EOs and bacLP17 were effective against all their mature biofilm's biomass (Figure 16). The anti-biofilm activity of both single used EOs and bacLP17 was similar, with a significant difference to the controls (range of p-value from 0.0388 to 0.000014), whereas the association between EO/EO and EO/bacLP17 was synergic in reducing the mature biofilm's biomass. In particular, the synergic activity of combined EOs led to a significant reduction both with respect to the control (range of p-value from 0.018 to 0.00011) and when compared to the single EOs (range of p-value from 0.042 to 0.0028 for *S. officinalis* and from 0.046 to 0.00115 for *T. vulgaris*). The best anti-biofilm activity was observed with the combination bacLP17/*S. officinalis* and bacLP17/ *T. vulgaris* both with respect to the control (p-value from 0.00089 to0.0000009) and when compared to the single EOs (p-value from 0.062 to 0.0016 for *S. officinalis* and from 0.034 to 0.00023 for *T. vulgaris*).

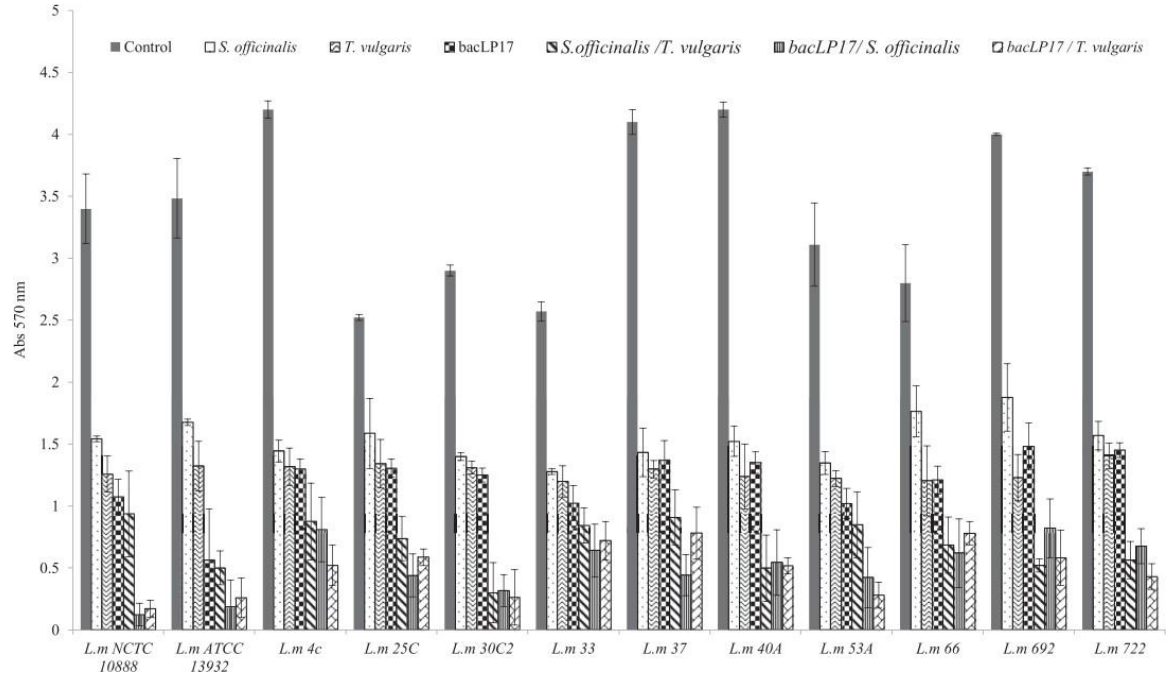

Figure 16: Anti- *L. monocytogenes* NCTC 10888 Biofilm activity of *T. vulgaris*, *S. officinalis* EOs and bacLP17 used both alone and in combination. Error bars represent standard deviation.

Discussion

The consumption of minimally processed ready-to-eat (RTE) or raw foods has affected the incidence of diseases caused by psychotropic bacteria, such as *L. monocytogenes*. A major safety risk is associated to psychotropic pathogens in substrate where they can reach high viable counts during storage at refrigeration temperatures. In these products, and mainly in foods eaten without thermal treatments like ready to eat seafood, the employ of natural products as biopreservatives might be of great interest for producers and consumers (Mate et al., 2015). Natural substances as bacteriocins and essential oils have already shown an important role in the control of pathogens, with feasible application in various foods. Their use in the food industry can help to reduce both the addition of chemical preservatives and the intensity of heat treatments, resulting in a more natural fresh food. Several bacteriocins already offer potential applications in food preservation, but to date, only few studies have described the antilisterial characteristic of a compound obtained from a bacteriocinogenic Lactic Acid Bacteria (LAB), a strain isolated from fish and therefore well adapted to growth in this organic matrix and therefore capable to compete with pathogens better than LAB from other sources. On the other hand, an increased focus on new preservation methods based on the use of natural substances like EOs has also been observed. Essential oils, which use in vegetable

products have already been approved by the Food and Drug Administration (2001) show their activity against both food-borne pathogens and spoilage bacteria (Singh et al., 2002; Marrelli et al., 2016). It seems that the EOs are more effective in vegetables because they have a low-fat content (Singh et al., 2002). *T. vulgaris* EO confirmed its good antilisterial effect observed in our previous investigation, where this essential oil led to a significant decrease in *L. monocytogenes* NCTC 10888 cell viability after 4 days of exposure and to a final reduction of 3 log cfu/g in artificially contaminated RTE vegetable, stored at refrigeration temperatures. Another study (Ghorbani et al., 2017) has shown a lot of pharmacological properties of *S. officinalis*, with its biological activity attributed to terpenes and terpenoids, compounds extensively found in the plant. The authors refer a strong bactericidal effect on *L. monocytogenes*, whereas in the present investigation *S. officinalis* was less effective than *T. vulgaris* against all *L. monocytogenes* strains tested. Regarding the sensitivity of microorganisms to these natural antimicrobial substances, no resistance mechanisms developed by bacteria towards essential oils have been reported, most likely due to the complexity and to the variety of mechanisms of action of their active compounds. Moreover, the essential oil components may act synergistically with antibiotics, for their capability to affect multiple targets, to perform physicochemical interactions and to inhibit antibacterial-resistance mechanisms. Reports on bacteriocins resistance developed by some Gram-positive bacteria are referred for the most used bacteriocin nisin, compound already approved as food preservative. Many studies have however shown that the bacteriocin's resistance can be overcome by using the combination of different bacteriocins or by the association with other natural antimicrobial compounds like essential oils (Langeveld et al., 2014; Kumariya et al., 2019). The combined use of the natural compounds against *L. monocytogenes* proposed in the present investigation has produced encouraging results, consistent with other studies. Turgis et al. 2012 refer that the combination of nisin with *Origanum vulgare* EO induces a synergistic effect against *L. monocytogenes*. Addition of oregano or savory essential oil exhibited a synergistic effect with CAB (cell-adsorbed bacteriocin) to control *L. monocytogenes* in pork meat during storage at 4 °C, and anti-*Listeria* activity of AS-48 (30 mg/g) in ready-to-eat food was strongly enhanced by essential oils (Ghalfi et al., 2007; Cobo Molinos et al., 2009). The natural substances employed in the present study are Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) (2001) but have some limits: bacteriocins do not have a broad host range, and the use of EOs is often limited for undesirable organoleptic impact. The synergistic effect observed in the present investigation consists of a more enhanced antilisterial activity of bacteriocin (used at its lower MIC) and a contextual significant reduction of the amount of EOs to use toward the pathogen. For this last reason, a low MIC permits the use of EOs as preservative without affecting the sensorial quality of foods. The combination EO/bacteriocin allows to overcome this limitation and could be an alternative to the

traditional chemical preservatives. The combined use of EOs and bacteriocins could also help to overcome the problem of bacteriocin resistance in Gram-positive bacteria, as reported for nisin (0-200 IU/ml) and garlic extract (0-6 mg/ml) (Singh et al., 2001). Lastly, the present study also showed a significant antilisterial biofilm activity of the single EOs and bacLP17. The synergistic association between EO/EO and EO/bacLP17 led to a significant reduction of the mature biofilm and the association between EOs and bacteriocin was the most active. Our results demonstrate the antibacterial potential of *T. vulgaris, S. officinalis* and bacLP17, alone and in combination, both to control *L. monocytogenes*, and to impair the biofilm produced by the same. Although the natural antimicrobial compounds are becoming popular in the food industry for the control of foodborne pathogens, there are some difficulties with their effective use. Both natural antimicrobials used in the present investigation present limits like the reduced sensitivity of Gram-negative bacteria to LAB bacteriocins, and the strong smell of EOs. Our results show that the synergism emerged with the combined use of EOs and bacteriocin is a promising natural way to overcome both the narrow range of activity and the unpleasant sensory impact. The use of EOs and bacteriocins together opens new promising opportunities for the development of novel preservative agents effective in controlling *L. monocytogenes* growth in seafood and other minimally processed RTE foods.

11.2 Part 2 (A and B) Study of the anti-*L. monocytogenes* activity of edible coatings added with a mixture of natural products carried out on shrimp samples

Food packaging is an area of interest not just for food producers or food marketing, but also for consumers who are more and more aware that food packaging has a great impact on food product quality and on the environment. The most used materials for the packaging of food are plastic, glass, metal, and paper. However, over time edible films have become widely used for a variety of different products and different food categories such as fresh fish, meat products, vegetables, or dairy products. For example, proteins are excellent materials used for obtaining edible or non-edible coatings and films. Consumers usually prefer natural additive substances to synthetic ones (Pizzal et al., 2002). Herbs and herbal extracts which contain antioxidants and aromatic substances with antimicrobial effects are commonly used in foods. Since the chemical components and active ingredients of these herbs and spices are different, their relative impacts are different from each other as well (Singhal et al., 2001). Many EOs are used in the industry as flavoring agents, but same of these possess a broad range of antimicrobial properties for food preservation (Lambert et al., 2001). Although many studies

have been carried out concerning the use of EOs as antimicrobial agent in edible films (Ojagh et al., 2010), very few have thoroughly discussed its effect on nutritional properties. In food systems, different concentrations of EOs are needed to exert similar antibacterial effects as those obtained in in vitro assays. The use of combinations of EOs and edible coating are thus new approaches to increase the efficacy of anti-listeria activities in foods, taking advantage of their synergistic and additive effects. With regard the use of bacteriocins in the active packaging field, several lactic acid bacteria (LAB) isolates from the *Lactobacillus* genera have been applied in food preservation, partly due to their antimicrobial properties. Their application in the control of human pathogens holds promise if appropriate strains are scientifically chosen and a suitable mode of delivery is utilized. LAB have the generally recognized as safe (GRAS) status and can produce antimicrobial compounds such as organic acids (lactic, acetic, or propionic acid), diacetyl, bacteriocins as well as other metabolites (Solarte et al., 2017). Their formation is strain and species dependent, but it is also related to the characteristics of the fermented substrates. Natural products represent a promising source of bioactive molecules, and edible coating, bacteriocins and essential oils have attracted much attention due to their myriad of biological properties, including anti-listeria activities.

Materials and strain

The EOs, obtained by hydrodistillation, were purchased from a local herbalist's shop in Modena, Italy, bacteriocin bacLP17 derived from a strain of *Enterococcus mundtii* isolated from fresh red muletts, while the edible coating obtained from pea proteins coming from processing residues was provided by SSICA research foundation (Parma, Italy). The shrimp samples were purchased from a supermarket in Modena and brought to the laboratory using dry ice (4 °C). *L. monocytogenes* NCTC 10888 was used as microorganism test.

Minimal inhibitory concentration (MIC) and the Fractional Inhibitory Concentration Index (FICI) determination

The MIC values of *S. officinalis, M. piperita, C. limon* and *T. vulgaris* EOs were determined against *L. monocytogenes* NCTC 10888 by using the broth microdilution method in 96-well microplates, according to the Clinical Laboratory Standards Institute (CLSI) guidelines 2012. The test was performed in sterile 96-well microplates by dispensing into each well 95 ml of Tryptic Soy Broth (Oxoid S.p.A, Milan, Italy) and 5 ml of bacterial suspensions, to a final inoculum concentration of 10^6 CFU/ mL. Then, 100 µL of EOs serial dilutions were added to obtain concentrations ranging from

512 to 0.125 µL/mL (**Part 2A**). 100 µL of bacteriocin bacLP17 serial dilutions were also added to obtain concentrations ranging from 512 to 0.125 µL/mL (in the **Part 2B** only). Negative control wells consisted of bacteria in TSB without EOs and bacLP17. The plates were incubated at 37°C for 24 h, mixed on a plate shaker at 300 rpm for 20 s, and the MIC was defined by the growth of the bacteria on the TSA plate. The checkerboard methods were carried out in TSB by using the microdiluition method to obtain the FICI for the following combinations: (i) EOs mixtures of *S. officinalis* and *M. piperita, S. officinalis* and *T. vulgaris, S. officinalis* and *C. limon, M. piperita and T. vulgaris, M. piperita* and *C. limon, T. vulgaris* and *C. lemon*. (ii) EOs and bacLP17 mixture of *S. officinalis* and *M. piperita* and bac LP17, *S. officinalis* and *T. vulgaris* and bacLP17, *S. officinalis* and *C. limon* and bacLP17, *M. piperita* and *T. vulgaris* and bacLP17, *M. piperita* and *C. lemon* and bacLP17, *T. vulgaris* and *C. limon* and bacLP17 (Hemaiswaryaa et al., 2008).

Edible coating preparation

Pea proteins obtained from industrial processing residues of peas were used as major coatings-forming component. These by-products, principally constituted by non compliant seeds of peas, empty pods and a mixture of leaves and stems, have been subjected to an extraction process of proteins, developed and set up by SSICA in a specific Italian patent (SSICA Industrial Invention Patent di n° Conc.1.399.500). The protein content was 79.6 ± 0.4 %. The determination of the protein content is based on the Kjeldhal method. (Bradstreet R.B., 1965; Meloan, E. Clifton and Y. Pomeranz, 1978; Benton J.J., 1991; AOAC International, 1995). The protein coating has been prepared first by dissolving 6.25% (w/v) pea protein and 3.12% (w/v) glycerol in double deionized water. The pH was measured and check to 7.2 and solubilization phase of 1 hour and the phase of denaturation at 70°C for 20 minutes has been performed. After the denaturation and once the solution was cooled down, X-Gum was finally added as thickener, in a quantity equal to 0.5% (w/w) (control). To obtain the active coating EOs and bacLP17 alone and in combinations were added to the solution in a quantity equal to 3% (w/w) of the final solution, after the check of pH, the solubilization and the denaturation, when the temperature of the solution was about room temperature and before adding the thickener.

Contamination of shrimps and packaging with the active coatings

Samples (44) were contaminated in sterile condition by inoculating each shrimp with a Hamilton syringe with approximately 10^6 CFU/mL. Suspension of an overnight *L. monocytogenes* NCTC 10888 cultures diluted in sterile saline solution (NaCl 0.85%) resulted in a final absorption of about 10^6 CFU/ g. of *L. monocytogenes* in food samples. Edible coating added with different concentrations

of EOs, alone and in combination (Part A) or EOs/bacLP17 (PartB) was applied to all samples by dipping (Table 14-16), including the controls inoculated with *Listeria monocytogenes* NCTC 10888 but coated without essential oils. Samples of doped and undoped coating stored at 4°C were analyzed at regular intervals: 0h, 24h, 72h, 4 days and 7 days.

Bacterial strain	***S. officinalis***	***M. piperita***	***T. vulgaris***	***C. lemon***	bacLP17
Listeria monocytogenes NCTC 10888	128 µL/mL	32 µL/mL	8 µL/mL	32 µL/mL	16 µL/mL

Table 14: MICs of EOs and bacLP17 used alone against *L. monocytogenes* NCTC 10888

Bacterial strain	***S. officinalis* + *M. piperita***	***S. officinalis* + *T. vulgaris***	***S. officinalis* + *C. limon***	***M. piperita* + *T. vulgaris***	***M. piperita* + *C. limon***	***T. vulgaris* + *C. limon***
Listeria monocytogenes NCTC 10888	6µL/mL +1,5µL/mL	6µL/mL +0,8µL/mL	6µL/mL +1,5µL/mL	3µL/mL +0,8µL/mL	3µL/mL +3µL/mL	0,8µL/mL +3µL/mL

Table 15: MICs of EOs used in combination against *L. monocytogenes* NCTC 10888

Bacterial strain	***S. officinalis* + *M. piperita*** + bacLP17	***S. officinalis* + *T. vulgaris*** + bacLP17	***S. officinalis* + *C. lemon*** + bacLP17	***M. piperita* + *T. vulgaris*** + bacLP17	***M. piperita* + *C. lemon*** + bacLP17	***T. vulgaris* + *C. lemon*** + bacLP17
Listeria monocytogenes NCTC 10888	3,1µL/mL +3µL/mL +0,8µL /mL	6µL/mL +0,4µL/mL +1,5µL/mL	3µL/mL +3µL/mL +1,5µL/mL	3µL/mL +1,5µL/mL +0,4µL/mL	1,5µL/mL +3µL/mL +0,8µL/mL	0,8µL/mL +3µL/mL +0,8µL/mL

Table 16: MICs of EOs and bacLP17 used in combination against *L. monocytogenes* NCTC 10888

Anti-*Listeria* activity determination

Portions of samples (25 g) were placed in sterile plastic bags, added with 225 ml of buffered peptone water (Oxoid, Milan, Italy) and homogenised for 1 min in Stomacher (Lab Blender, Seward, London, UK) (El-Shenawy and M. A. El-Shenawy, 1995). Serial tenfold dilutions of the obtained homogenates were spread in triplicate on Palcam agar added with selective supplement (Oxoid) and plates were incubated aerobically at 37 °C for 24 hours. All bacterial counts were recorded as CFU/g.

Statistical Analysis

Each experiment was carried out in triplicate. The statistical significance was determinedby the t-test and ANOVA test using statistical program GraphPad Prism 9.2.0. (SanDiego, CA, USA). The p-values were declared significant at ≤ 0.05.

Part A Edible antimicrobial coating with a mixture of Essential Oils against *Listeria monocytogenes* on shrimp samples

Results

The anti-*Listeria* properties of all the chosen EOs were still confirmed even when singularly added to the coatings. In this study, the synergistic effects of mixtures of essential oils on *L. monocytogenes* were also investigated. The findings demonstrated that when the EO/EO were used in combination, the FIC-index showed a good synergy between the natural compounds inside the coating, showing the antilisterial activity at values much lower than when used alone (Tables 14 and 15).

Figures 17-26 report the mean values of the *L. monocytogenes* NCTC 10888 viable counts (log CFU/g) detected in the contaminated samples packaged either with doped (EOs alone or in combination) or undoped (control) coatings, after storage at 4°C. The results showed a good anti-listerial activity for the coating added with the four EOs, with significant differences on *L. monocytogenes* NCTC10888 viable counts compared to the control. At 24 h of experimentation, *M. piperita* showed the best activity against *L. monocytogenes*, with a significant difference compared to the control ($p<0.001$) (Figure 18). After 3 days all the EOs displayed a good anti-listerial activity, in particular *C. limon* ($p<0.001$) (Figure 20). After 4 days the activity of all the compounds on *L. monocytogenes* turns out to be excellent, with no significant differences between them (Figures 17-20). At the end of the study (7 days), *S. officinalis* and *T. vulgaris* showed the best activity towards the viable cells of *L. monocytogenes* ($p=0.0001$ and $p<0.0001$, respectively) (Figures 17 and 19).

A good anti-listerial activity also emerged when the mixtures of EOs were used, demonstrating an evident synergy between the chosen compounds. After 24 h only the combination *M. piperita* / *T. vulgaris* displayed a significant difference compared to the control ($p<0.01$) (Figure 24). This synergy was very active on the 3rd day of experimentation, together with the combination *M. piperita* / *C. limon* ($p<0.01$) (Figure 25) and *T. vulgaris* / *C. limon* ($p<0.01$) (Figure 26). The mixtures *M. piperita* / *T. vulgaris* (Figure 24) and *T. vulgaris* / *C. limon* (Figure 26) yielded to the best activity against viable *Listeria* cells ($p<0.001$) already on the 4thday, and their good synergistic effect was maintained

until the end of the study (7 days). Lastly, *S. officinalis* EO exerted synergistic effect with *M. piperita* showing the best activity towards *L. monocytogenes* on the last day ($p<0.001$) (Figure 21).

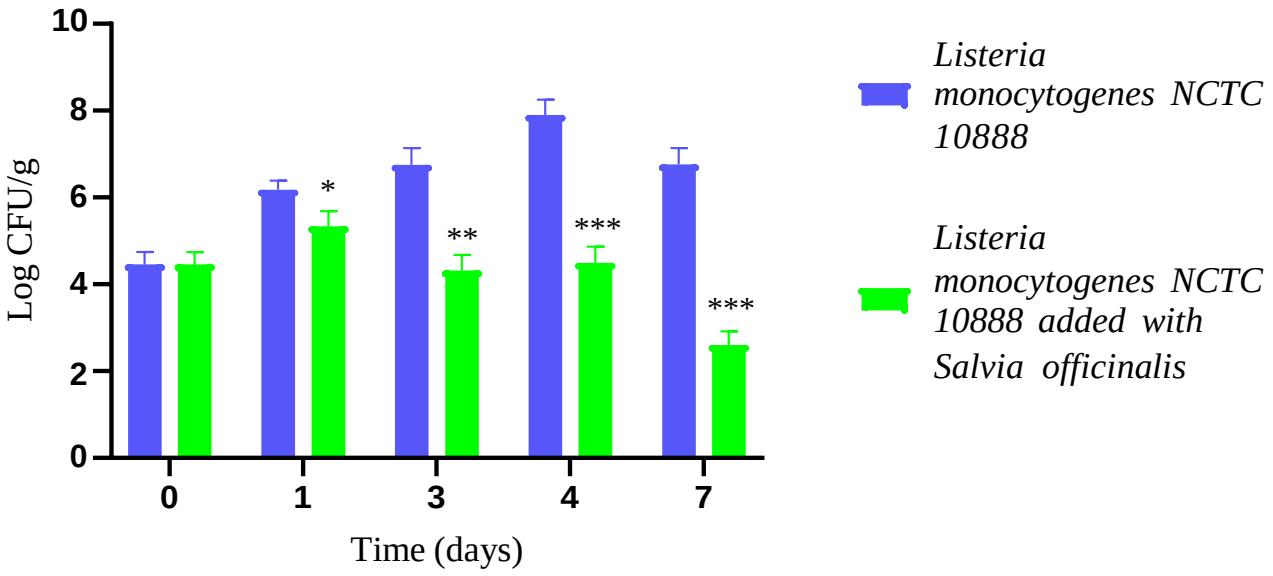

Figure 17: Antibacterial activity of *Salvia officinalis* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered significant by t-test and ANOVA.

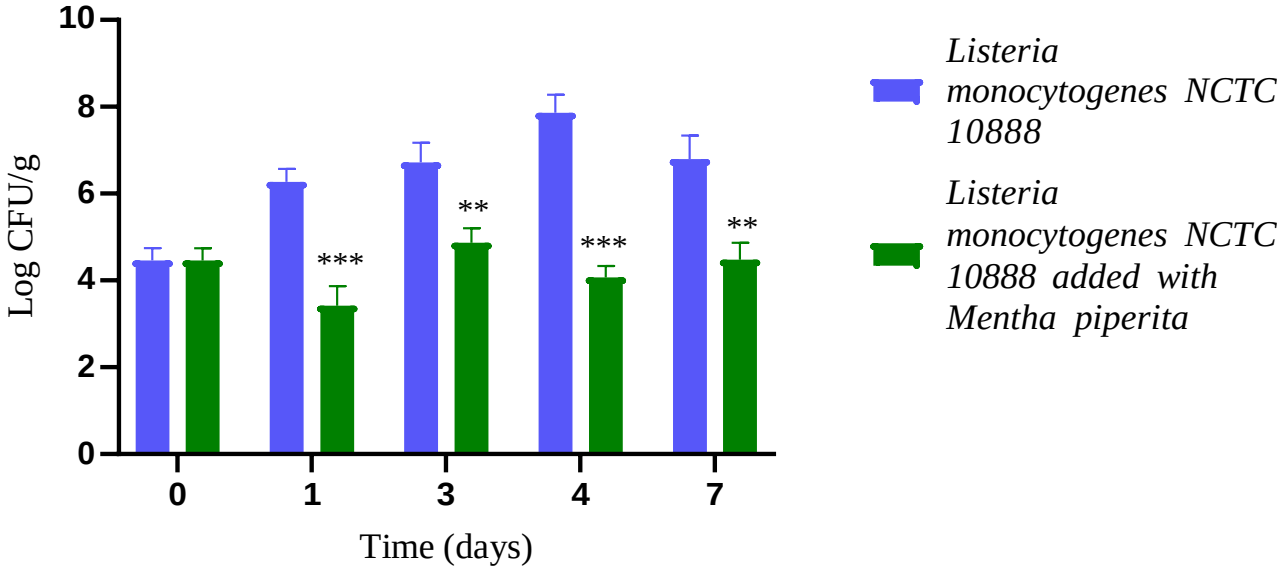

Figure 18: Antibacterial activity of *Mentha piperita* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered significant by t-test and ANOVA.

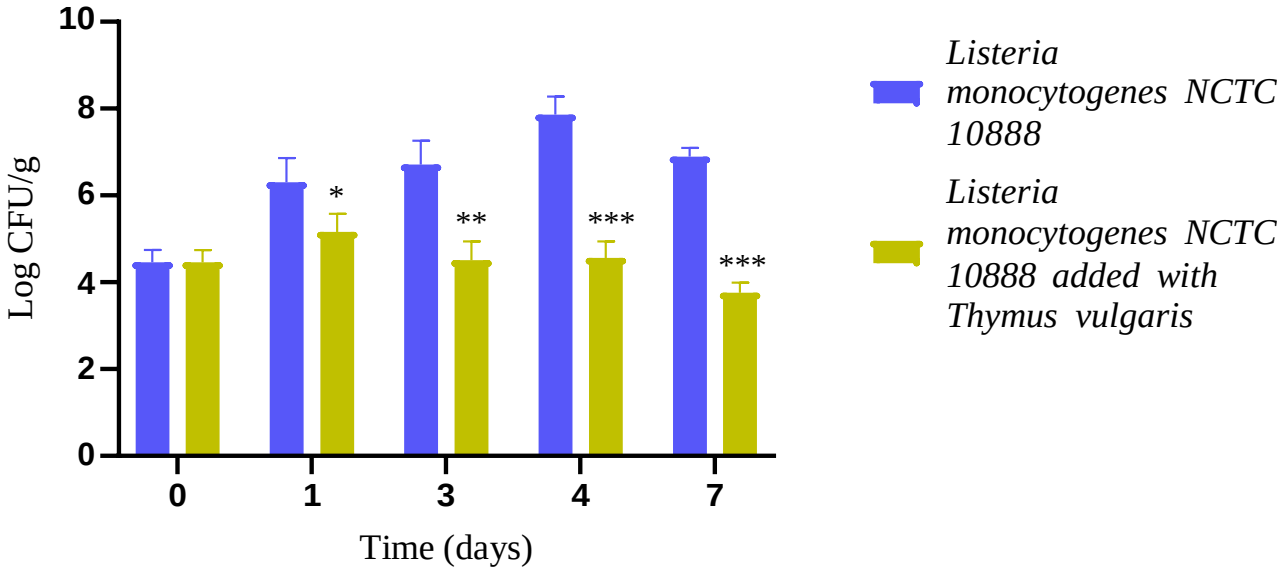

Figure 19: Antibacterial activity of *Thymus vulgaris* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered significant by t-test and ANOVA.

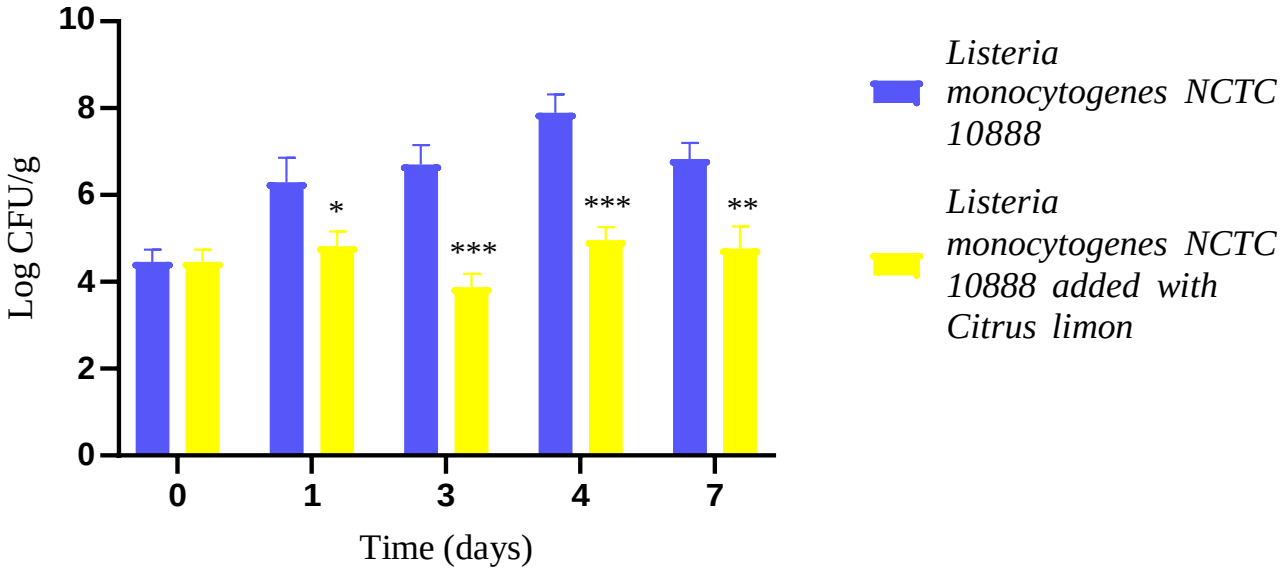

Figure 20: Antibacterial activity of *Citrus limon* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered significant by t-test and ANOVA.

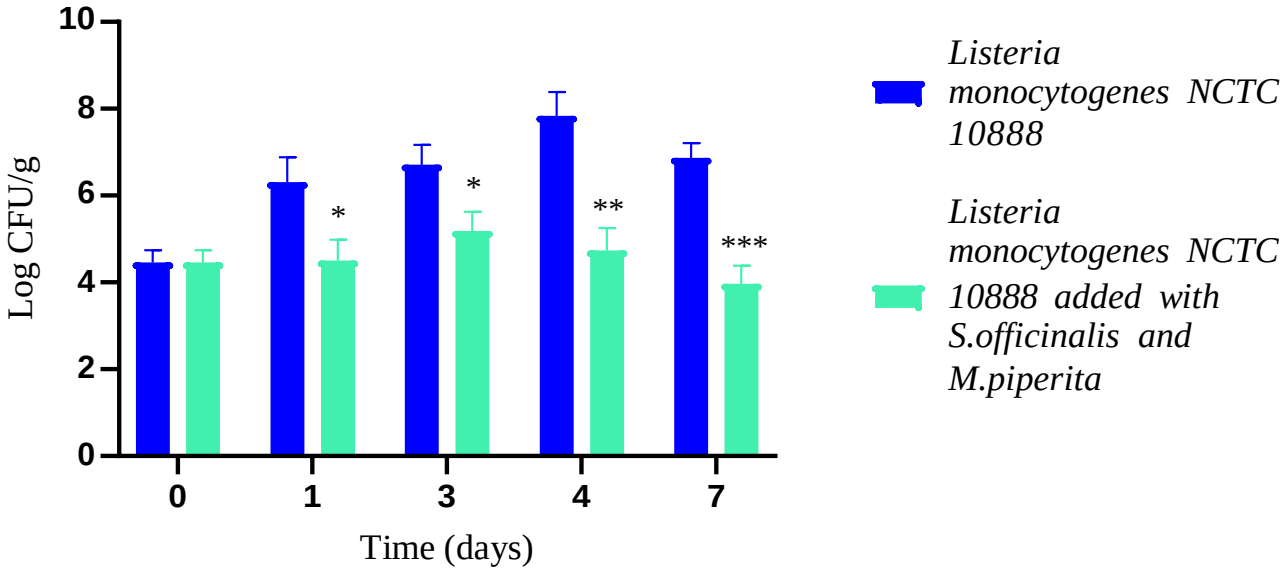

Figure 21: Antibacterial activity of *Salvia officinalis* and *Mentha piperita* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered significant by t-test and ANOVA.

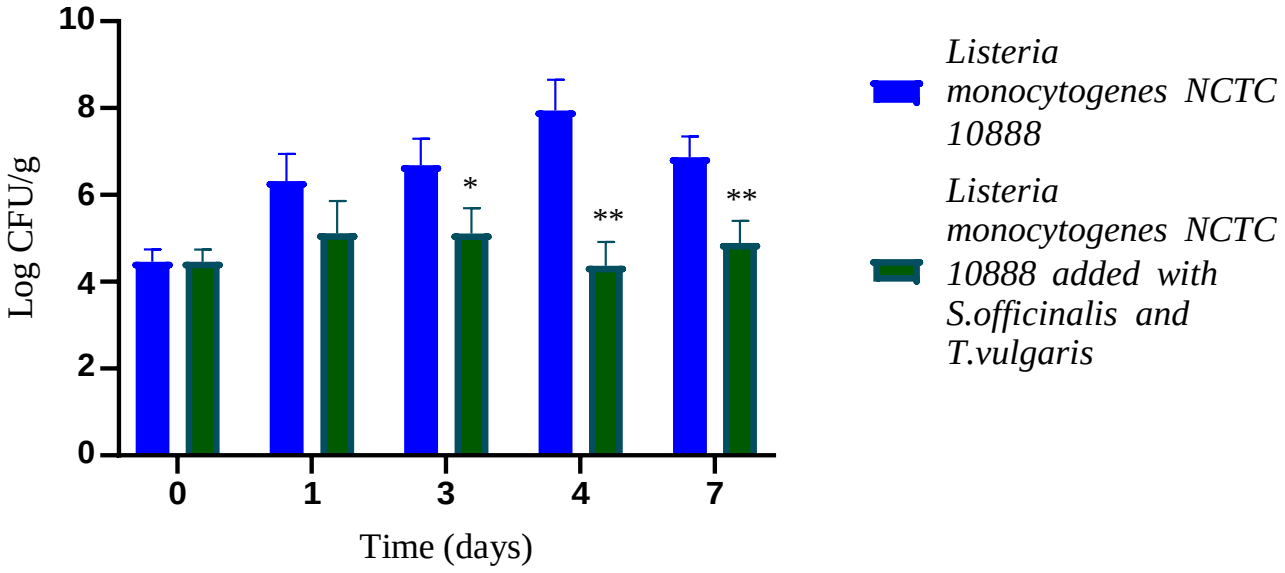

Figure 22: Antibacterial activity of *Salvia officinalis* and *Thymus vulgaris* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*) and p < 0.01 (**) were considered significant by t-test and ANOVA.

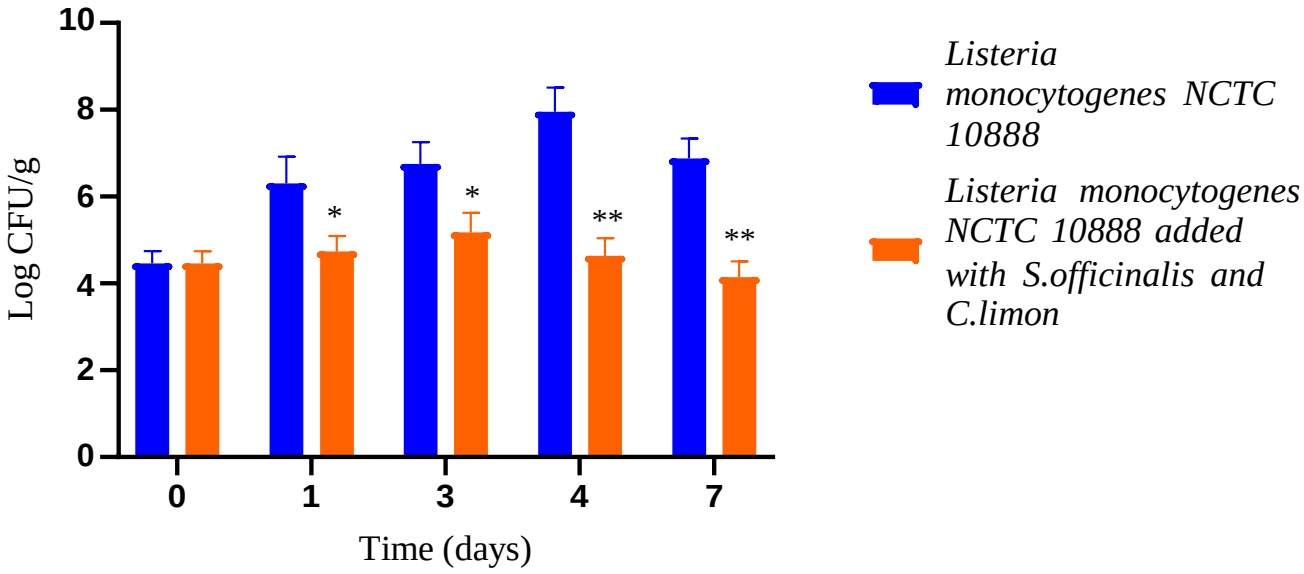

Figure 23: Antibacterial activity *of Salvia officinalis* and *Citrus limon* against *Listeria monocytogenes* NCTC 10888.

p-values of < 0.05 (*) and p < 0.01 (**) were considered significant by t-test and ANOVA.

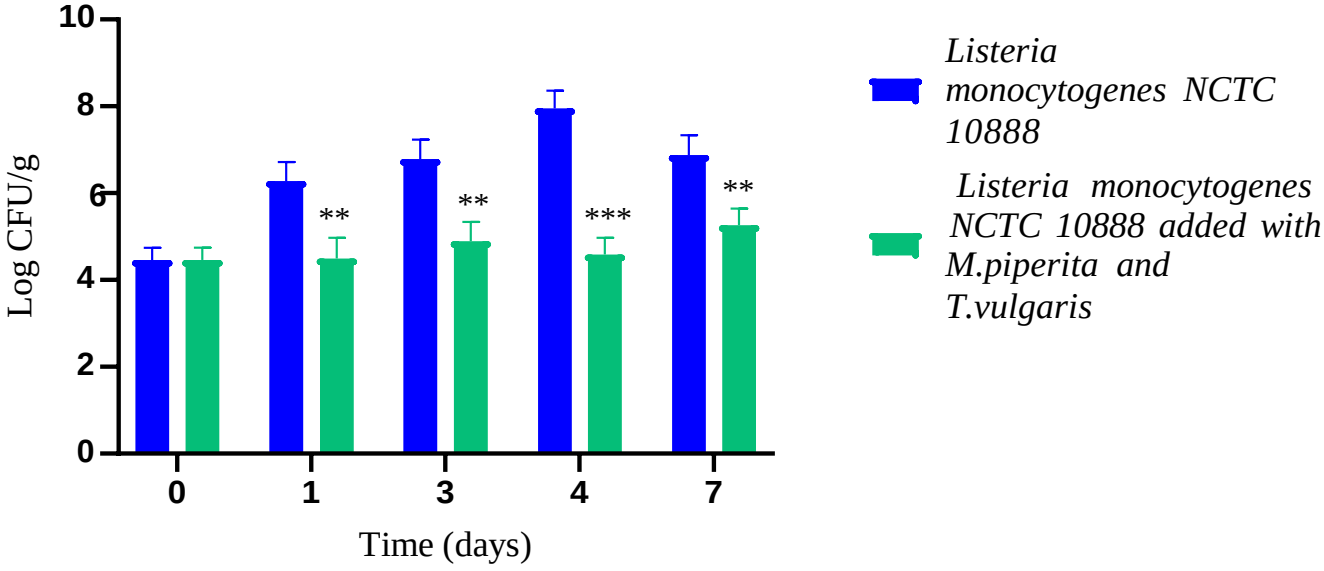

Figure 24: Antibacterial activity of *Mentha pip*erita and *Thymus vulgaris* against *Listeria monocytogenes* NCTC 10888.

p-values of < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered significant by t-test and ANOVA.

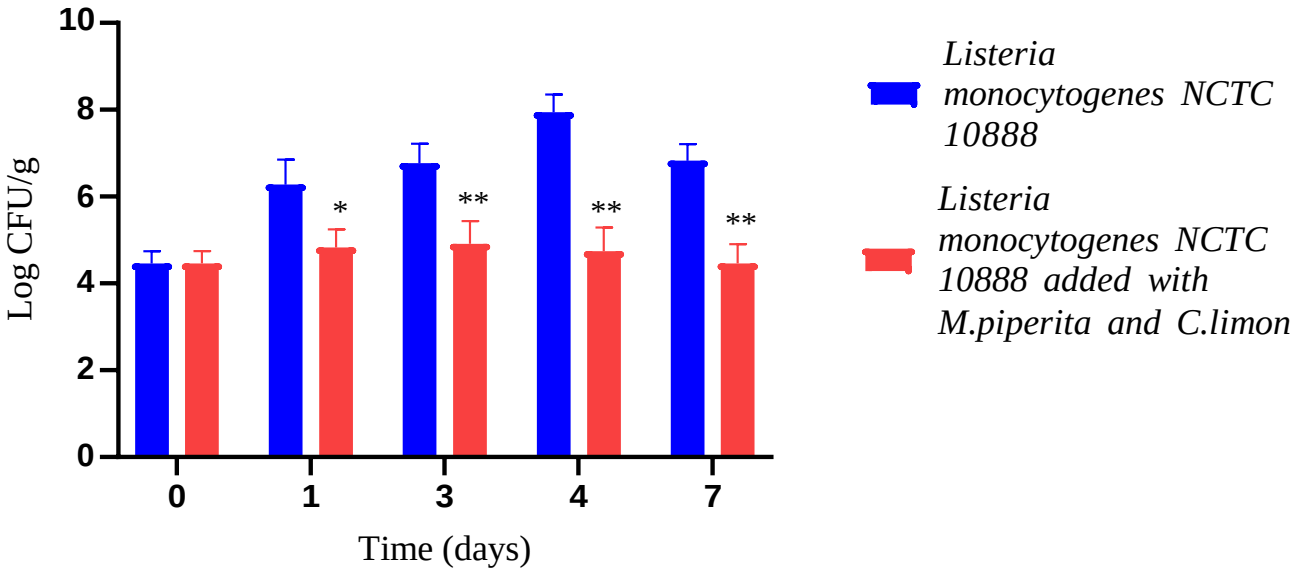

Figure 25: Antibacterial activity of *Mentha piperita* and *Citrus limon* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*) and p < 0.01 (**) were considered significant by t-test and ANOVA.

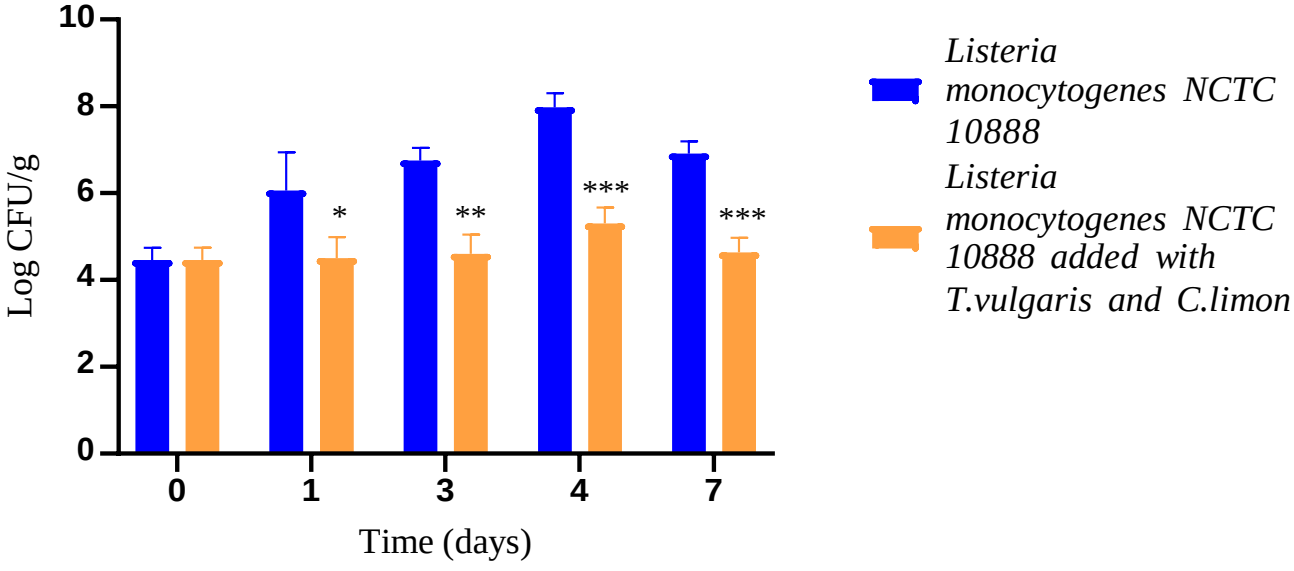

Figure 26: Antibacterial activity of *Thymus vulgaris* and *Citrus limon* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered significant by t-test and ANOVA.

Conclusion

The results obtained show that all the EOs confirm their anti-*L. monocytogenes* activity emerged in the above "in vitro" studies, even when incorporated into the coatings. The coating matrix has been found to be suitable for its use in food field, allowing a gradual release of the EOs on packaged food, thereby sustaining a marked antibacterial activity during the preservation at refrigerated temperature. At the same time, the EOs have shown to act synergistically even when incorporated in the coating, and the significant reduction in the amount of EOs (10-20 times) to be employed against *L. monocytogenes* overcomes the problem of their limited use due to the undesirable organoleptic impact when added to foods in high concentration. Hence, the inclusion of the EOs within the coating not only ensures the anti-listerial activity increasing the shelf-life of the food products, but it also mitigates even more the strong smell of EOs, improving the sensory properties of food, as already reported (Hemaiswaryaa et al., 2008).

Part 2 B. Edible antimicrobial coating with a mixture of Essential Oils and Bacteriocin bacLP17 against *L. monocytogenes* on shrimp samples

Results

All the EOs were active against *L. monocytogenes*, and the FIC-index showed a good synergy between the natural compounds tested, with consequent antilisterial activity detectable at much lower values than their single use. The best activity was observed for the mixture bacLP17 / *Salvia officinalis* (Tables 14- 16). Figures 27 - 37 report the mean values of the *L. monocytogenes* NCTC 10888 viable counts (log CFU/g) detected in the contaminated samples packaged either with doped (EOs and bacLP 17 in combination) or undoped (control) coatings, after storage at 4°C. The results showed a good antilisterial activity for the coating added with the four essential oils and bacLP17 in combination, with significant differences in *L. monocytogenes* NCTC10888 viable counts compared to the control. After 3 days of experimentation, all the single EOs combined with bacLP17 displayed a good anti-listerial activity, in particular the combination bacLP17/*S.officinalis* ($p<0.001$) (Figure 28). After 4 days the activity against *L. monocytogenes* of all the combinations bacLP17 / single EOs tested turns out to be excellent, in particular bacLP17 / *Citrus lemon* ($p<0.0001$) (Figure 31). Lastly, at the end of the study (7days), both bacLP17 alone and its combination bac

LP17 / *M. piperita* and bacLP17 / *C. lemon* displayed the best activity towards the viable cells of *L. monocytogenes* with a significant difference compared to the control ($p<0.001$) (Figures 27- 31). With regard the combinations between bacLP17 and two EOs, all these last compounds exerted synergistic effect with bacLP17 against *L. monocytogenes*. The mixture bacLP17/*M. piperita*/*S.officinalis* displayed a significant difference compared to the control after 24 h only ($p<0.01$) (Figure 32), with a gradual increase in the anti-listerial activity on the 3rd day of testing (Figure 32). The same trend has been observed for other mixtures in which *M. piperita*, *T. vulgaris* and *S. officinalis* were present (bacLP17/*M. piperita*/*C. limon*, bacLP17/*M. piperita*/*T. vulgaris*, bacLP17/*S. officinalis*/*T. vulgaris* ($p<0.01$) (Figures 36, 35, 33, respectively). After the 4th day of the study, the combinations of bacLP17 / *S. officinalis*/*T. vulgaris* (Figure 33) and bacLP17 /*M. piperita*/*T. vulgaris* (Figure 35) displayed a significant antibacterial effect on *Listeria* viable cells ($p<0.001$). This last combination maintains its potent anti-listerial activity ($p<0.001$) (Figure 35) until the end of the experiment (7 days). Lastly, the synergy bacLP17/*S. officinalis*/*C. limon* emerged on the last day with a considerable activity towards *L. monocytogenes* ($p<0.001$) (Figure 34).

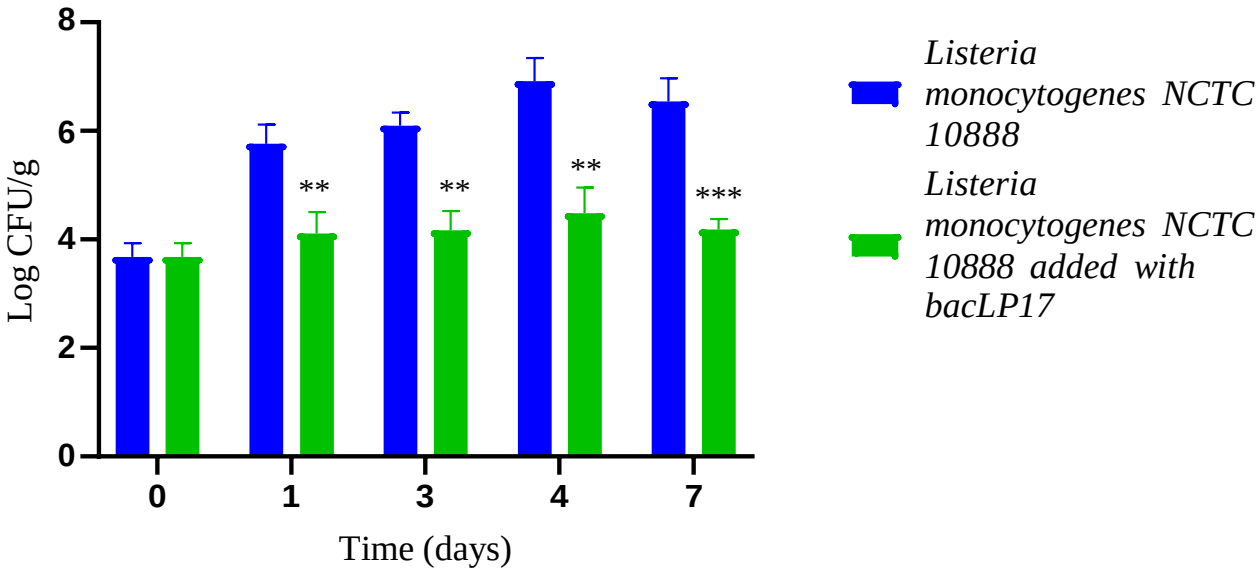

Figure 27: Antibacterial activity of bacteriocin bacLP17 against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered significant by t-test and ANOVA.

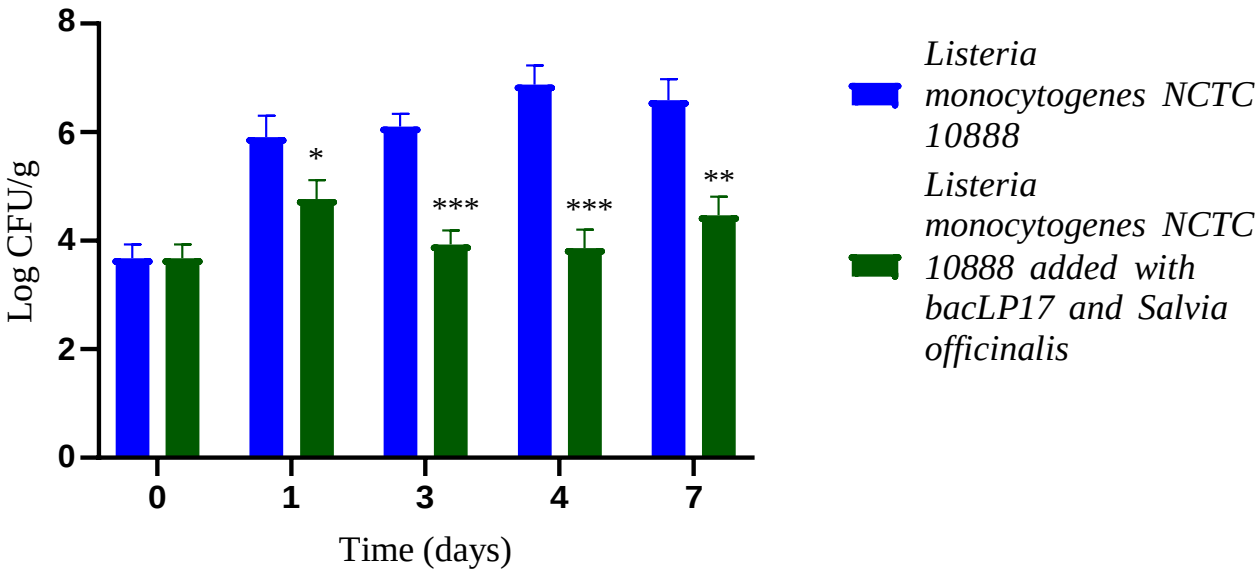

Figure 28: Antibacterial activity of bacteriocin bacLP17 added with *Salvia officinalis* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered significant by t-test and ANOVA.

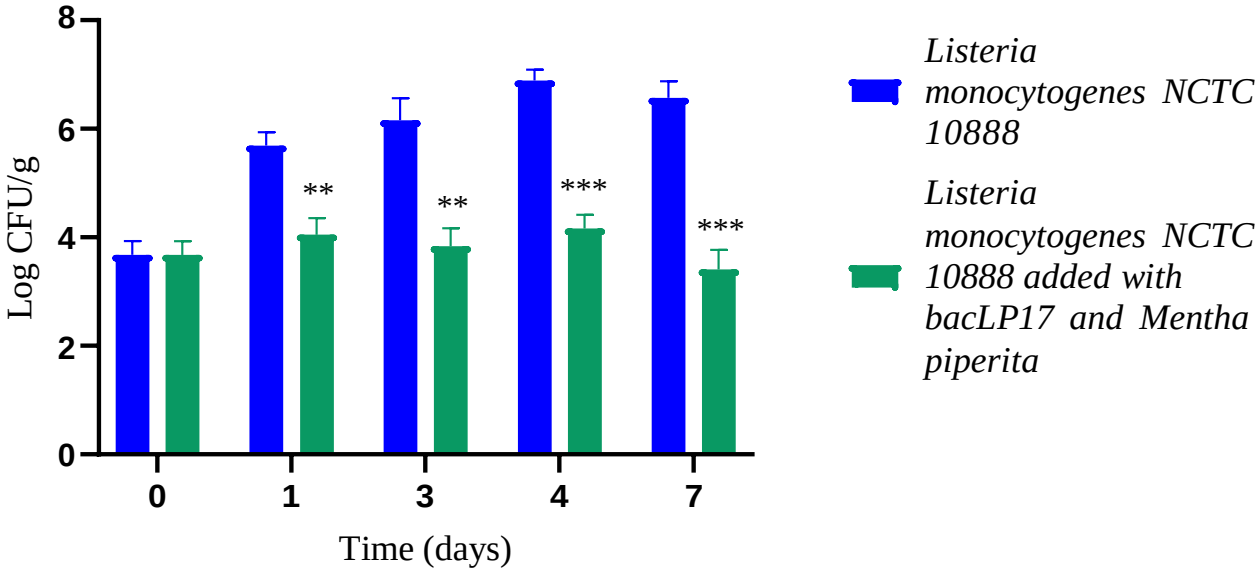

Figure 29: Antibacterial activity of bacteriocin bacLP17 added with *Mentha piperita* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered significant by t-test and ANOVA.

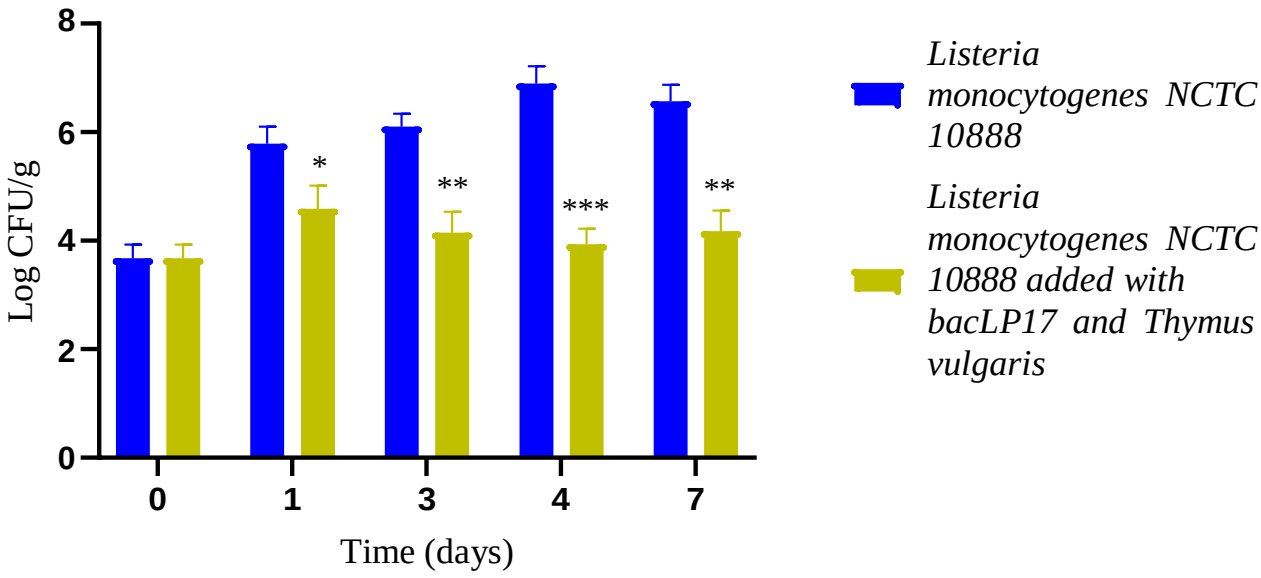

Figure 30: Antibacterial activity of bacteriocin bacLP17 added with *Thymus vulgaris* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered significant by t-test and ANOVA.

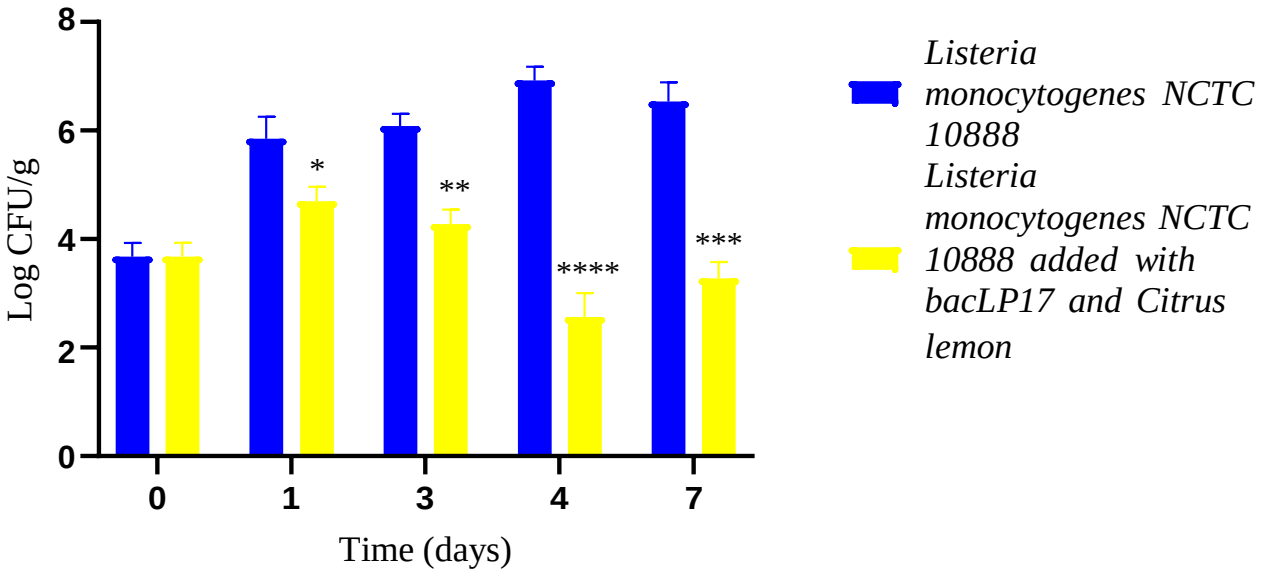

Figure 31: Antibacterial activity of bacteriocin bacLP17 added with *Citrus lemon* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered significant by t-test and ANOVA.

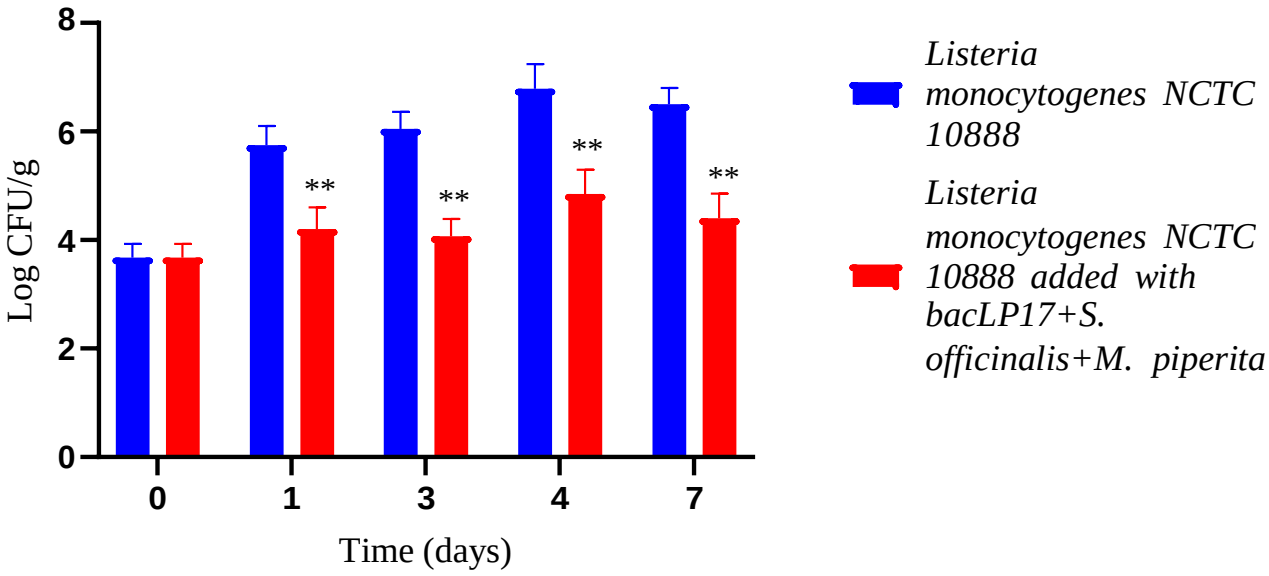

Figure 32: Antibacterial activity of bacteriocin bacLP17 added with *Salvia officinalis* and *Mentha piperita* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*) and p < 0.01 (**) were considered significant by t-test and ANOVA.

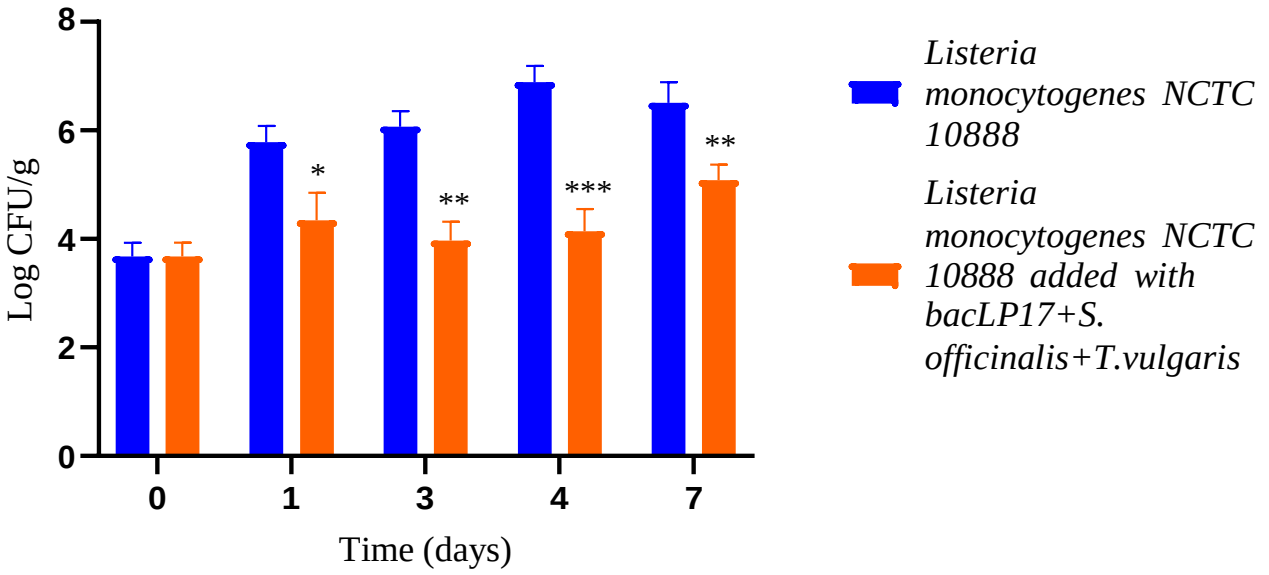

Figure 33: Antibacterial activity of bacteriocin bacLP17 added with *Salvia officinalis* and *Thymus vulgaris* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered significant by t-test and ANOVA.

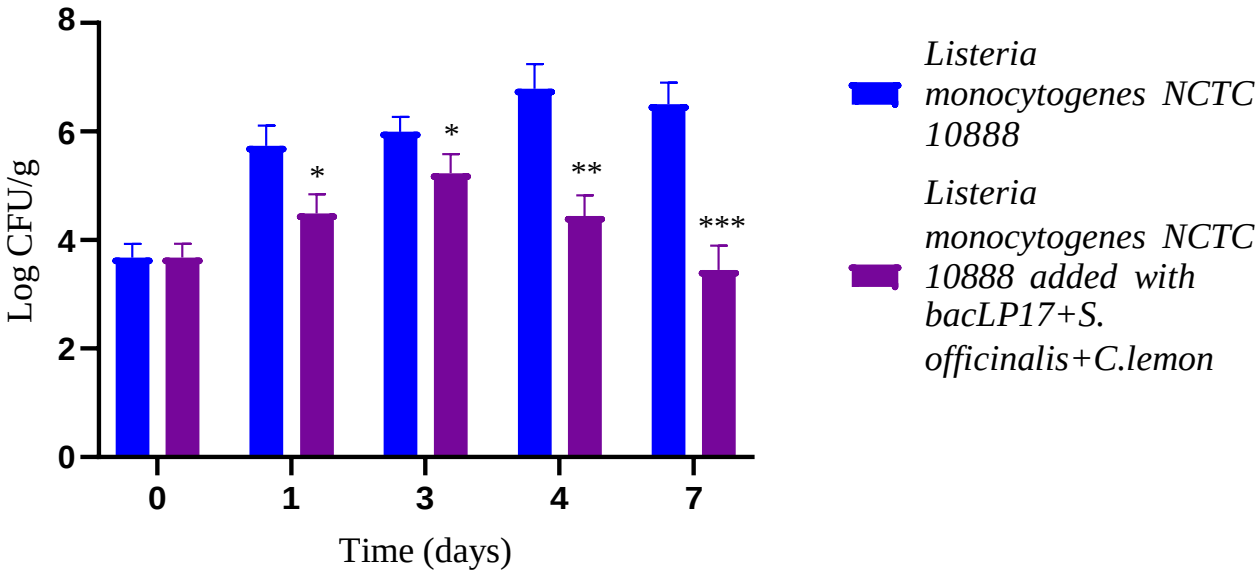

Figure 34: Antibacterial activity of bacteriocin bacLP17 added with *Salvia officinalis* and *Citrus lemon* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered significant by t-test and ANOVA.

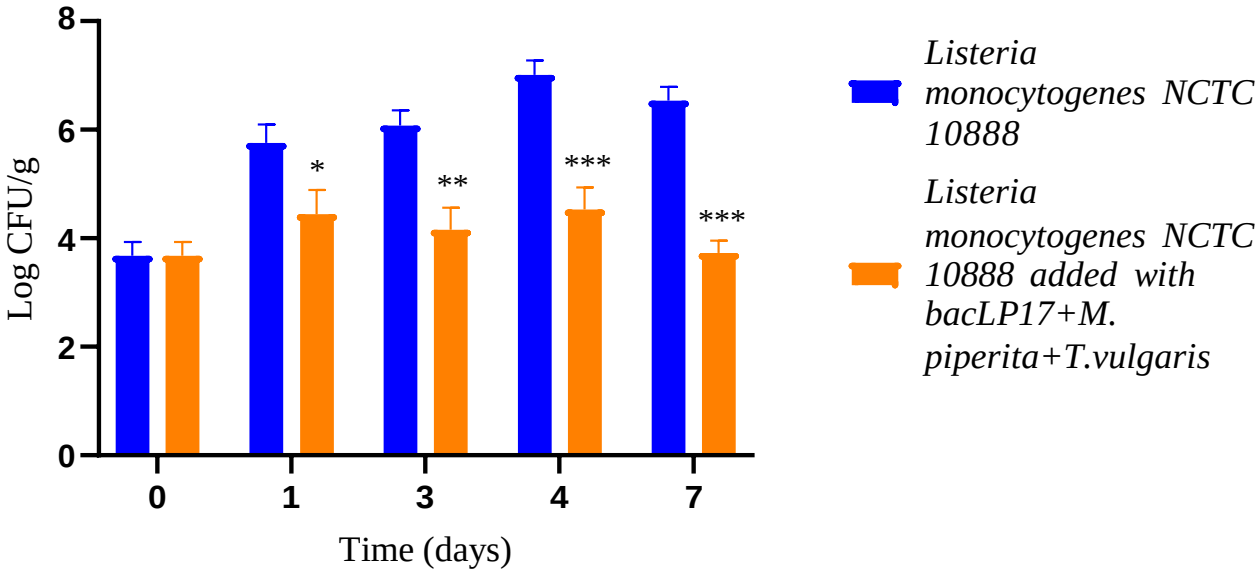

Figure 35: Antibacterial activity of bacteriocin bacLP17 added with *Mentha piperita* and *Thymus vulgaris* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered significant by t-test and ANOVA.

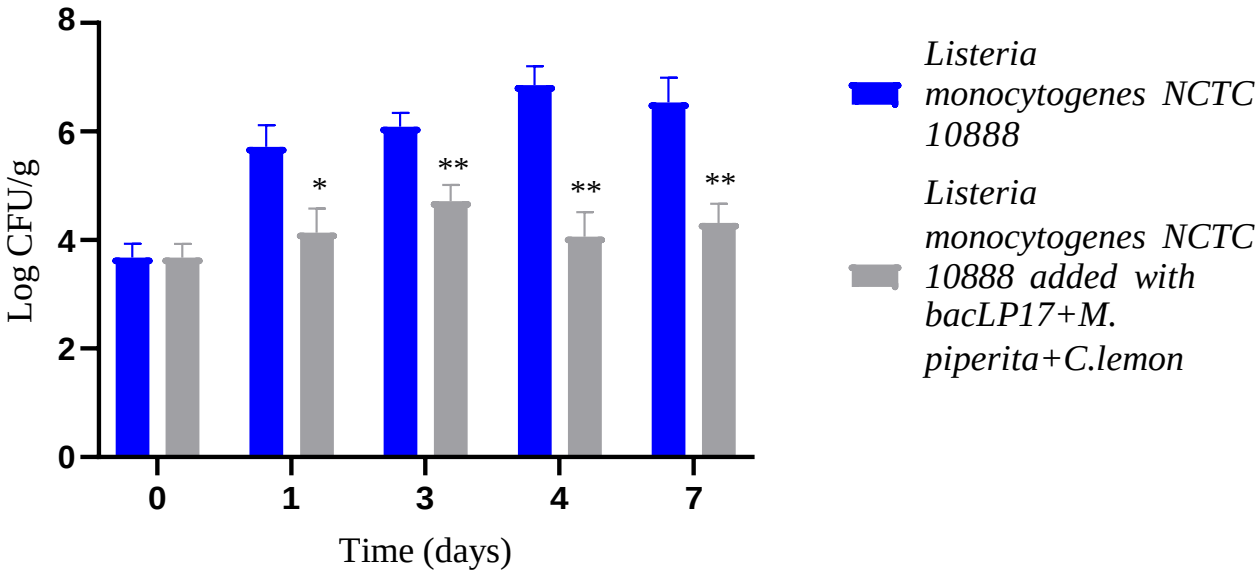

Figure 36: Antibacterial activity of bacteriocin bacLP17 added with *Mentha piperita* and *Citrus lemon* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*) and p < 0.01 (**) were considered significant by t-test and ANOVA.

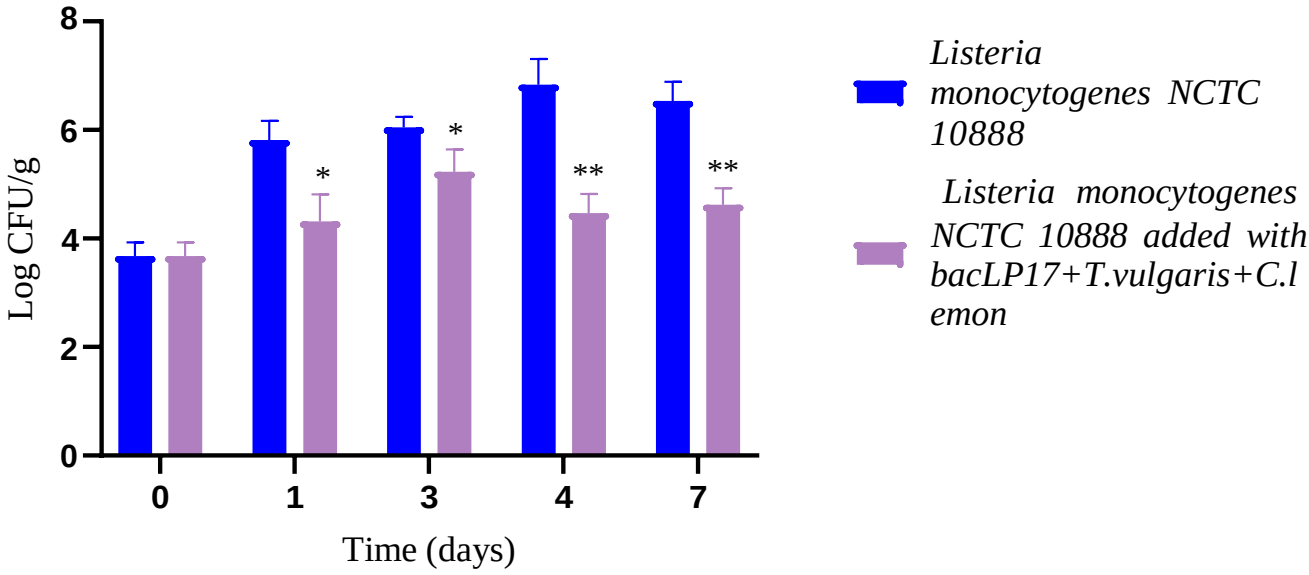

Figure 37: Antibacterial activity of bacteriocin bacLP17 added with *Thymus vulgaris* and *Citrus lemon* against *Listeria monocytogenes* NCTC 10888. p-values of < 0.05 (*) and p < 0.01 (**) were considered significant by t-test and ANOVA.

Conclusions

Even in this case, the results have shown the capability of the natural compounds to maintain unaltered inside the coatings their antibacterial activitiy against *L. monocytogenes*, with a gradual release from coating on food. The combined use inside an edible coating of natural substances of different origin and endowed with peculiar characteristics can overcome both some drawbacks associated with the use of chemical additives and same limitation due to the features of EOs and bacLP17 in the prevention of *L. monocytogenes* growth: bacLP17 do not have a broad host range, and the use of EOs is often limited for undesirable organoleptic impact. The synergistic effect observed in the present investigation led to obtain a more enhanced anti-*Listeria* activity of EOs at lower MIC values (that permits the use of EOs as preservative without affecting the sensorial quality of foods) when in combination with bacLP17, whereas the presence of EOs in the mixture ensure the bacteriocin a broader spectrum of activity (which also includes resistant strains).

Final Conclusions

The primary function of food packaging is to protect food from external contamination and thus improve its conservation, providing at the same time a support for the consumer. During the last few years, the changing demands of consumers and the market have induced the food industries to modify and innovate technologies and applications of food-packaging. Active food-packaging is therefore the most innovative system of interaction between packaging and food, to extend its shelf-life, increasing the quality, safety, and organoleptic characteristics during the storage period. The development of technological innovations in the agri-food field to expand in the time the "life" of the food, however, have not prevented the increased incidence of bacteria with high adaptability to survive in adverse environmental conditions. The most relevant pathogens are those endowed with psychrotropic characteristic, able to survive and even multiply at temperatures close to zero. Among the psychrotrophic bacteria *L. monocytogenes* appears to be the main human food-borne pathogen abundantly widespread in nature and isolated from a wide variety of foods. *L. monocytogenes* is a psychotropic microorganism causing listeriosis, an illness mainly affecting immunocompromised population, pregnant women, young and elderly individuals, especially in the age group over 64 years old. This pathogen causes septicemia, meningitis or other infections of the central nervous system and, in pregnant women, the infection can determine spontaneous abortion, still birth or fetal death. Listeriosis has the second-highest fatality rate (20%) and the highest hospitalization rate (90%) (FDA/USDA/CDC 2001), with 2536 European cases of listeriosis reported in 2016 (EFSA and ECDC 2016). This pathogen can contaminate foods at pre- and postharvest stages of production and its occurrence is highest in fish and fishery products (6%), followed by RTE salads (4.2%), RTE meat and meat products (1.8%), soft and semi-soft cheeses (0.9%), fruit and vegetables (0.6%) and hard cheeses (0.1%) (EFSA and ECDC 2017). Fish and seafood products, more commonly those consumed without further cooking and with extended shelf life at refrigeration temperatures (such as smoked fish), support the growth of the pathogen, that can both directly contaminate the raw seafood from the aquatic environment, or it can be also reintroduced as a post-processing contaminant (Guerra

et al., 2001). Outbreaks of listeriosis associated with smoked mussels, smoked trout and raw oysters, and gravid and cold-smoked fish have been reported (Ericsson et al., 1997; Brett et al., 1998; Tham et al., 2000; EFSA and ECDC 2019). The persistence of *L. monocytogenes* in food processing plants is an important factor both in the transmission of this foodborne pathogen and in the contamination of foods and food associated environments and, in this context, a primary role is played by the biofilm (Ferreira et al., 2014; Overney et al., 2017), a microbial community where microorganisms found both nutrients for growth and protection from different adverse conditions (Prakash et al., 2003; Garret et al., 2008), including preservation treatments. Within the biofilm, microorganisms are less susceptible to the conventional treatments than their planktonic counterparts, so biofilm poses a challenge in food processing facilities, where new strategies to eradicate this microbial structure are needed (Cortes et al., 2011; Galie et al., 2018). In recent years, the consumer's demands of natural foods and the environmental concerns have highlighted the necessity to preserve a highly perishable product like seafood using natural additives. The refrigeration is the most common way to increase the shelf life of foods, but it is unable to inhibit this psychrotrophic microorganism that survives and grows at 2-4 °C. The use of chemical preservatives has therefore become necessary but has often met the criticism and suspicion of consumers. To meet the consumers' requirements of high quality, minimally processed and additive free foods, new and natural technological approaches for food preservation must be found. Several natural preservatives from different sources have been widely studied, such as herbs and medical plants, microorganisms and animals, therefore gaining considerable attention (Gokoglu., 2019; Baptista et al., 2020). For all these reasons, two types of natural compounds are proposed in this study: bacteriocins and essential oils (EOs). Bacteriocins are ribosomal synthesized peptide or proteins produced by bacteria and able to inhibit or kill other related or unrelated microorganisms. The antibacterial activity of bacteriocins is due to their interaction with the bacterial cell surface and cell membrane, with cell permeabilization and pore formation as major mechanisms of action (Cintas et al., 2001). Bacteriocins have attracted attention as potential antibiotic alternative to prevent bacterial infections and they are widely studied for food preservation against

spoilage and pathogenic bacteria such as *L. monocytogenes* (Riley et al., 2002; Iseppi R et al., 2011; Iseppi R et al., 2019). Essential oils, a mixture of volatile compounds extracted from aromatic plants, have already shown in previous investigations an important activity against human pathogens such as *L. monocytogenes* (Barbosa et al., 2009; Gouveia et al., 2017). *Lamiaceae*, to which the two natural compounds used in the study belong, is one of the most important family, whose EOs are notoriously endowed with antimicrobial properties. Several lines of evidence support the bactericidal and bacteriostatic activity of *Salvia officinalis* against both Gram-positive and Gram-negative bacteria, even if Marino et al. (2001) indicates a weak bacteriostatic effect only. This activity is mainly due to the bioactive components, such as terpenes, phenolic acids and flavonoids, responsible for a variety of effects, such as disturbing the cytoplasmic membrane integrity of bacteria, affecting the electron transport chain, changing the pH homeostasis, disrupting the proton motive force, and coagulation of cell contents. A lot of studies demonstrated that the *Thymus vulgaris* EO has both a potent bacteriostatic and bactericidal effects against many against Gram-positive and Gram-negative human pathogens, having carvacrol and thymol as its main active components. Regarding the mode of action, thyme EO has the potential to cause the rupture of the cell membrane, by penetrating the phospholipids layer of the bacterial cell wall and blocking the enzyme systems (Afonso et al., 2019). Given the excellent result obtained in the "in vitro" studies using both the natural compounds alone and in association against *L. monocytogenes* we have secondarily added these substances in different combination to an edible coating, with the aim to produce a natural approch for both consumer and enviroment. The inclusion of the EOs within the coating not only ensures the anti-listerial activity and the increase the shelf-life of the food products, but it also can improve the sensory properties. In future studies it will be important to gain more knowledge on the spatial distribution and interactions of microbial species in food, on applications of active film and coating added with natural compounds like bacteriocins (antimicrobial compounds secreted by LAB group belonging to the probiotic category) and essential oils, discovery of new bacteriocins, and their advantageous use in association with other essential oils. Both these natural antimicrobials, in fact, present limits like the reduced

sensitivity of Gram-negative bacteria to LAB bacteriocins, and the strong smell of EOs. The synergism obtained with the combined use of EOs and bacteriocin may be a promising natural way to overcome both the narrow range of activity and the unpleasant sensory impact. Lastly, it is important underline that the use of edible coating obtained from food by-products is a great advantage for the environment because it is biocompatible and eco-friendly material. The use of EOs and bacteriocins together added to this type of edible packaging will be able to explore promising opportunities for the development of novel strategies based on active edible coatings effective in controlling *L. monocytogenes* growth in seafood products and in other minimally processed RTE foods. Further studies will be however necessary to improve the perspectives of active edible coatings for future applications in the food industry.

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14. Appendix: research activity in the training period and three-year publishedarticles

Ready-to-eat food (RTE) is a type of product that must not be cooked or reheated before serving. This includes salads, vegetables, fruits, cooked meats, smoked fish, desserts, sandwiches, cheese and food that are previously cooked in order to be later served cold. The trend towards consumption of minimally processed, ready-to-eat, refrigerated and frozen food products poses new problems to the identification and management of bacterial risks for consumers, mainly due to the inability to maintain a temperature-controlled environment. Even if properly stored, RTE foods can contain high risk ingredients that allow pathogens to grow and multiply. Recent modifications in food production and processing practices and the ever-changing eating habits of the consumers, who appreciate the readiness of RTE foods, have affected the incidence of pathogens that can multiply in such foods

14.1 Objectives and milestones of the training period

The **first objective** of the training period was to to isolate and characterize LAB strains from selected seafood, in order to demonstrate the ability of some of them to produce bacteriocins. Safety aspects of bacteriocinogenic strains and the properties of the best antibacterial substances were also investigated for their potential use in food preservation. The **second objective** was to determine the antibacterial activity of Essential Oils (EOs), used alone and in combination with other natural compounds like bacteriocins, nisin, chitosan, propolis and polyphenols, in order to demonstrate a possible synergistic antibacterial activity against pathogenic strains and their potential combined use in food storage. The **third objective** was to evaluate and characterize the bacterial load present in twenty four Ready-To-Eat (RTE) sandwiches, purchased at refrigerated vending machines and supermarkets in the province of Modena (Italy).

A1) **Bacterial strain:** Study and characterization of bacteriocin BacLP17 from *Enterococcus mundtii* LP17, isolated from commercial seafood product.

A2) **Determination of the antibacterial activity**: Bacteriocin production was screened by the deferred antagonism method using as indicators Gram-positive and Gram-negative bacteria belonging to different genera or species. The antibacterial ability of the best producer strains was confirmed by agar well diffusion assay.

A3) **Study of the antibacterial activity of Essential Oils by agar disk diffusion assay:** the test was used to screen the antibacterial activity of eight Essential Oils (EOs): *Mentha piperita, Melaleuca alternifolia, Salvia officinalis, Thymus vulgaris, Citrus limon, Citrus aurantium, Zingiber officinalis* and *Rosmarinus officinalis* against *Listeria monocytogenes* spp. Studies are underway on the antibacterial activity of other natural substances such as: chitosan, propolis, nisin and polyphenols.

A4) **Minimum Inhibitory Concentration (MIC) determination:** the MIC of the EOs endowed with the best antibacterial activity (*Thymus vulgaris, Salvia officinalis* and *Melaleuca alternifolia*) against *Listeria monocytogenes* spp. were determined using the microwell dilution method.

A5) **Fractional inhibitory concentration index (FICI):** The combined antibacterial activity between *Thymus vulgaris-Salvia officinalis* EOs and bacteriocins was determined with 96 microplates method and future studies with other essential oils will be performed.

A6) **Biofilm:** the activity of *Salvia officinalis* and *Thymus vulgaris* against a complex aggregation (biofilm) of *Listeria monocytogenes* spp. was evaluated.

A7) **RTE sandwiches**: objective of the study was to examine the microbiological quality of 24 ready-to-eat sandwiches, to evaluate the risk for consumers due to the presence of pathogenic strains, and the presence in the isolates of antibiotic resistance and other virulence factors.

14.2 Ready to eat sandwiches as source of pathogens endowed with antibiotic resistance and other virulence factors. RESULTS of a study carried out in the first year of PhD internship (published article: Camellini et al., 2021**)**

Introduction

Ready-to-eat food is a type of product that must not be cooked or reheated before serving. This includes salads, vegetables, fruits, cooked meats, smoked fish, desserts, sandwiches, cheese and food that you have cooked in advance to serve cold (Rocourt J., 1996). The trend towards consumption of minimally processed, ready-to-eat foods and refrigerated or frozen food products poses new problems relating to the identification and management of risks for consumers, mainly for the compliance with storage as, even if properly stored, they can contain high risk ingredients that allow pathogens to grow and multiply. Recent modifications in food production and processing practices and the ever-changing eating habits of the consumers, that appreciate speed and easy to use foods, have affected the incidence of pathogens, that can multiply in food cooked in advance and to serve cold. These products are usually characterized by a long shelf life at refrigerated temperatures, and they can be contaminated with spoilage bacteria and foodborne pathogens during all stages of the production process. Like many types of RTE food, sandwiches are tasty and easy to eat, especially for workers who are always in a hurry and eating meals away from home. Sandwich is a popular food that can contain some high-risk ingredients, such as raw vegetables, eggs and salad dressing and whose preparation usually involves manual handling. Many pathogenic bacteria like *Listeria, Staphylococcus, Shigella, Yersinia spp,* ubiquitous bacteria found in many environments like soil or water, can come into contact with these raw or fresh ingredients. Many human pathogens can also be transmitted through bare-hand handling, as *S. aureus*, which can be found on human skin and hair, or other pathogens colonizing the intestinal tract. The multi-ingredients used for the production and the sandwich fillings like cheese, chicken, eggs, ham, salad, sauce, and various types of fresh produce contribute to the microbial contamination and have been associated with foodborne illness outbreaks, the last reported in UK in 2019, where hospitalized patients died for the consumption of sandwiches contaminated by *L. monocytogenes* (Listeriosis in England and Wales: summary for 2019). The RTE foods preservation techniques are based on the use of low temperatures, which however do not

prevent some psychrotrophic bacteria, to multiply at temperatures close to those of refrigeration, such as *Listeria* (Ananchaipattana et al., 2016). These psychrotrophic bacteria might previously be present in the raw materials, but the risks associated with consumption of RTE foods (EFSA BIOHAZ Panel 2018) reflect the possible significant growth of these microorganisms in the processing plant equipment and during the food storage at refrigeration temperature (Tompkin, 2002). *Listeria* is a ubiquitous Gram-positive genus and can be isolated from a wide range of food products. *L. monocytogenes* is the most important specie responsible for food-borne outbreaks and severe illness, listeriosis, mainly in immunocompromised individuals and pregnant women (Swaminathan et al., 2007), even if other species like *L. ivanovii* and *L. seeligeri* have been found in sporadic cases. *L. ivanovii* has been isolated, although rarely, from infected humans, indicating pathogenic potential for humans (Guillet et al., 2010), whereas L. seeligeri although carries a not expressed virulence gene cluster, has been associated with rare cases of infection in humans (Rocourt et al., 1986; Snapir et al., 2006). The objective of the present investigation was to examine the microbiological quality of 12 ready-to-eat sandwiches and to evaluate the risk for consumers due to the presence of pathogenic strains, and the presence in the isolates of antibiotic resistance and other virulence factors.

Materials and Methods

Sampling

Twenty-two sandwiches in modified atmosphere packaging have been purchased on different days from automatic distributors and in supermarkets in the province of Modena, processed within 4 hours of the collection and stored at 4 °C for the duration of the experiment. The sandwiches contained different ingredient combinations: 1 tuna and tomato, ham and cheese, tomato and mozzarella cheese, tuna and eggs, turkey and vegetables, shrimp and pink sauce, cooked ham, raw ham, smoked cheese and tomatoes, cooked ham and mushrooms, tuna and onions, cooked ham and artichokes, raw ham and eggplants. The ingredients and the expiry date were recorded, to ensure that the samples were within their shelf-life period at the end of the study.

Microbiological Analysis

Sandwiches samples (25 g) were homogenized for 1 minute in sterile plastic bags, with 225 ml buffered peptone water added (Oxoid, Milan, Italy), in Stomacher (Lab Blender, Seward, London, UK). The homogenate was streaked with a 10 µl loop, on selective and non-selective plates and incubated at 37 ° C for 24-48 hours. Tryptic Soy Agar (TSA) was used as non-selective medium for the total bacterial count, while as selective plates the following media were used: MacConkey Agar (MK) for the isolation of *Enterobacteriaceae*, Listeria Palcam Agar Base (PAL), for the isolation of species belonging to the genus *Listeria*, Mannitol Salt Agar (MSA), for the isolation of *Staphylococcus spp*, Kanamycin Aesculin Azide Agar Base (KA) for *Enterococcus spp*. (all media from Oxoid, Milan, Italy). After incubation, colonies grown on selective plates were individually cultured to proceed with the identification and biological characterization. All bacterial counts were recorded as CFU/g.

Identification of Isolates

The identification of the isolates was obtained using the EnteroPluri-Test (Liofilchem, Italy) to identify microorganisms belonging to the Enterobacteriaceae family and the API®strips (Biomerieux, France) for the other genera. Subsequently the bacterial identification was definitively confirmed by by matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry (TOF/MS). All the strains were stored in phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 2.9 g Na2HPO4·12H2O, 0.2 g KH2PO4 with 1L of distilled water) supplemented with 30% (vol/vol) glycerine at − 80 °C.

Evaluation of virulence factors: hemolysin and gelatinase production

Hemolysin and gelatinase production were evaluated in all 54 isolates by spotting the plates added with the specific media with 10 µL of suspension, cultured in Tryptic Soy broth (Oxoid, SpA, Milan,

Italy) at 30 °C for 48 h. For the hemolysin production, all the strains were cultured on blood agar plates containing 5% of defibrinated horse blood (bioMérieux, Florence, Italy). After incubation at 37 ° C for 24 hours, the haemolytic activity was determined by observing a clear zone of haemolysis (b-haemolysis), a partial and greening haemolysis zone (a-haemolysis) or no activity (c-haemolysis) around the spots. Gelatinase production was assessed by inoculation of the strains in a Nutrient broth containing 10% gelatin. Positive gelatinase was recorded as degradation of the gelatin to liquid. Given that some microorganisms produce low amounts of gelatinase, all negative results were further incubated up to 15 days to observe any delayed positive reactions.

Determination of the Minimum Inhibitory Concentration (MIC)

The MICs of 30 out of 54 isolated and identified strains have only been evaluated, chosen on the basis of their ability to cause food poisoning. Following the guidelines of the EUCAST (European Committee on Antimicrobial Susceptibility Testing), for eight antibiotics: Amikacin, Ciprofloxacin, Ampicillin, Oxacillin, Imipenem, Tetracycline, Erythromycin and Vancomycin. The evaluation was carried out using the agar dilution method by adding double dilutions of antibiotics to the Mueller-Hinton agar medium (Oxoid, SpA, Milan, Italy). The results were then compared with the breakpoints from EUCAST 2020.

Results

Quantification and Identification of the isolates

A total bacterial count from 10^4 CFU/g to 10^6 CFU/g was found on the non-selective TSA medium for the most processed sandwiches produced with the greatest amount of ingredients and the most manipulated ones (meats, sauces and vegetables of different types). Fifty-four (54) strains were isolated from both samples and ingredients on selective media MSA, KA, PAL, MK with bacterial load ranging from 10^2 to 10^5 CFU/g, 10^2 to 10^4 CFU/g, 10^3 to 10^4 CFU/g, and 10^2 to 10^3 CFU/g, respectively. Of the 54 strains examined, twenty (37.04%) belonged to the genera *Staphylococcus,*

eight (14.81%) to *Leuconostoc*, five to (9.26%) *Listeria*, five (9.26%) to *Aerococcus*, four (7.41%) to *Lactococcus*, four (7.41%) to *Enterococcus*, four (7.41%) to *Acinetobacter*, two (3.70%) to *Aeromonas*, one (1.85%) to *Citrobacter*, one (1.85%) to *Yersinia* (the last two referred together in the figure as *Enterobacteriaceae*) (Figure 38).

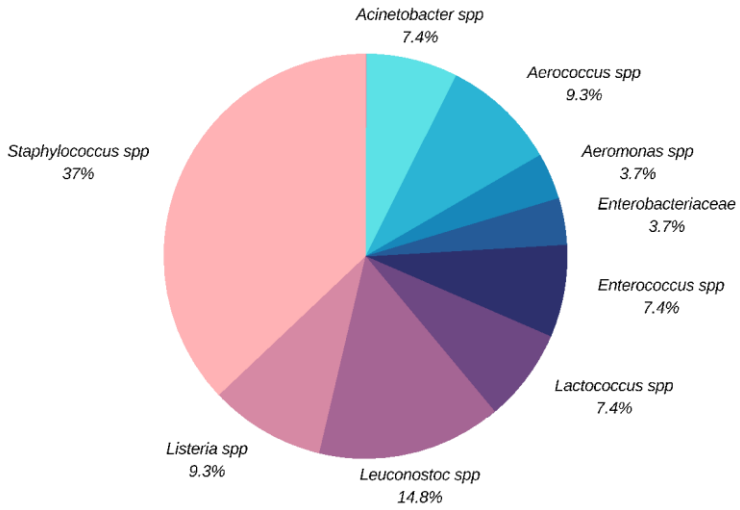

Figure 38: Bacterial species detected in the selective plates.

Staphylococcus is the most represented genus, with bacterial load ranging from 10^2 to 10^5 CFU/g. Notably, *S. aureus* was found on 14 samples and in 8 of these it was the only contaminant. With regard to the other Gram-positive strains, *Enterococcus* was the second isolated genus, found in three samples, and represented by *E. faecium* (2 strains), *E. avium* (1 strain) and *E. durans* (1 strain), followed by five *Aerococcus viridans* isolates. *Leuconostoc* and *Lactococcus* were also found, both genera used as commercial starters in the bakery sectors. Among the Gram-negative strains, in three samples bacterial loads between 10^3 and 10^4 CFU/g were found on MacConkey Agar (MK). *Aeromonas hydrophila* was isolated from one sample, and it was also present in another one together with *Citrobacter spp.*, whereas in the third sample the simultaneous presence of *Yersinia enterocolitica* and *Acinetobacter lwoffii* was observed. Lastly, three *Listeria ivanovii subsp.*

londoniensis and one *Listeria welshimeri* were isolated from three sandwiches, with bacterial loads around 10^2 CFU / g for all isolates.

Determination of the minimum inhibitory concentration

As reported in Table18, two species of *E. faecium* were resistant to all the antibiotics tested (4 µg/ml for Ciprofloxacin, 16 µg/ml for Ampicillin, 16 µg/ml for Erythromycin), except for Tetracycline. *E. avium* and *E. durans*, following EUCAST guidelines, were tested only against Ampicillin, to which they were sensitive. All the *Enterococcus* strains were resistant to Vancomycin, with high MIC values, which exceed the susceptibility breakpoint >256 µg/ml (Table 18).

Strains	Ciprofloxacin	Tetracycline	Ampicillin	Erythromycin	Vancomycin
E. faecium EC1	4 µg/ml	1 µg/ml	16 µg/ml	16 µg/ml	>256 µg/ml
E. faecium EC2	4 µg/ml	1 µg/ml	128 µg/ml	4 µg/ml	256 µg/ml
E. avium EC16	/	/	1 µg/ml	/	>256 µg/ml
E. durans EC17	/	/	2 µg/ml	/	>256 µg/ml
BREAK POINT	≥4 µg/ml	≥4 µg/ml	≥4 µg/ml	≥4 µg/ml	≥4 µg/ml

Table 18: MIC values for Enterococci strains and their Break Point.

All 13 *S. aureus* strains were sensitive to Amikacina. Only *S. aureus* S29 and S41 were sensitive to Ciprofloxacin, while the other 11 strains showed an intermediate profile (MIC value at the break point). It is important to note that the isolates are endowed with a multi-resistance profile since they are all resistant to Erythromycin (with values 2, 4, 32 and 256 µg/ml), Vancomycin (with values 4, 8, 256 and >256 µg/ml) and Oxacillin (with values 2, 16, 64, 256 and >256 µg/ml, respectively) (Table 19).

Strains	Amikacin	Ciprofloxacin	Erythromicin	Vancomiycin	Oxacillin
S. aureus S26	1 µg/ml	1 µg/ml	256 µg/ml	1µg/ml	>256 µg/ml
S. aureus S28	1 µg/ml	1 µg/ml	4 µg/ml	1µg/ml	>256 µg/ml
S. aureus S29	1 µg/ml	0,25 µg/ml	4 µg/ml	0.5µg/ml	>256 µg/ml
S. aureus S31	1 µg/ml	1 µg/ml	256 µg/ml	2µg/ml	256 µg/ml
S. aureus S32	1 µg/ml	1 µg/ml	32 µg/ml	2µg/ml	>256 µg/ml
S. aureus S33	1 µg/ml	1 µg/ml	256 µg/ml	µg/ml	>256 µg/ml
S. aureus S34	1 µg/ml	1 µg/ml	256 µg/ml	2µg/ml	>256 µg/ml
S. aureus S35	1 µg/ml	1 µg/ml	4 µg/ml	2 µg/ml	16 µg/ml
S. aureus S36	1 µg/ml	1 µg/ml	256 µg/ml	2µg/ml	64 µg/ml
S. aureus S37	1 µg/ml	1 µg/ml	2 µg/ml	2 µg/ml	16 µg/ml
S. aureus S41	1 µg/ml	0,25 µg/ml	2 µg/ml	2 µg/ml	2 µg/ml
S. aureus S42	1 µg/ml	1 µg/ml	32 µg/ml	1µg/ml	2 µg/ml
S. aureus S43	1 µg/ml	1 µg/ml	2 µg/ml	2µg/ml	2 µg/ml
BREAK POINT	≥8 µg/ml	≥1 µg/ml	≥1 µg/ml	≥2 µg/ml	≥2 µg/ml

Table 19: MIC values for Staphylococcus aureus strains and their Break Point.

With regard to the psychrotrophic bacteria, the five Listeria isolates (Table 20) were resistant to Erythromycin. As regards Tetracycline, all *L. ivanovii* londoniensis showed an intermediate profile, while *L. welshimeri* was resistant to the compound, with MIC value of 4 µg/ml. Four strains (3 strains of *L. ivanovii spp. londoniensis* with a value of 8 µg/ml and *L. welshimeri* with value of 128 µg/ml) was resistant to Ampicillin and one strain of *L. ivanovii spp. londoniensis* with MIC value equal to the susceptibility at the break point. The two strains of *A. hydrophila* were evaluated only against Imipenem to which they were found to be particularly resistant with values > 256 µg/ml, whereas *Y. enterocolitica* showed an intermediate profile for Ciprofloxacin and Tetracycline, with value of 0,25 µg/ml and 4 µg/ml, respectively (Table 21).

Strains	Tetracycline	Ampicillin	Erythromycin
L. ivanovii spp. *Londoniensis* 46L	1 μg/ml	8 μg/ml	32 μg/ml
L. ivanovii spp. *Londoniensis* 47L	1 μg/ml	8 μg/ml	32 μg/ml
L. ivanovii spp. *Londoniensis* 48L	1 μg/ml	8 μg/ml	32 μg/ml
L. ivanovii spp. *londoniensis* 49L	1 μg/ml	1 μg/ml	32 μg/ml
L. welshimeri 50L	4 μg/ml	128 μg/ml	256 μg/ml
BREAK POINT	≥1 μg/ml	≥1 μg/ml	≥1 μg/ml

Table 20: MIC values for *Listeria* strains and Break Point.

Strain	Imipenem	Ciprofloxacin	Tetracyclin
A. hydrophila 52EB	>256		
A. hydrophila 59EB	>256		
BREAK POINT	**≥ 1**		
Y. enterocolitica 58EB		0,25 μg/ml	4 μg/ml
BREAK POINT		≥0,25 μg/ml	≥4 μg/ml

Table 21: MIC values for *A. hydrophila* and *Y. enterocolitica* strains and Break Point.

Lastly, all the *Acinetobacter* species are sensitive to both the antibiotic Amikacina and Ciprofloxacin, and the only isolate belonging to the genus *Citrobacter* tested against Ampicillin showed a resistance profile with values > 256 μg/ml (Table 22).

Strains	Ampicillin	Amikacin	Ciprofloxacin
A. Iwofii 54EB		2	0,25
A. calcoaceticus 55EB		2	0,25
A. Iwofii 56EB		2	0,25
A. Iwofii 57EB		2	0,25
BREAK POINT		≥8	≥1
Citrobacter spp. 53EB	>256		
BREAK POINT	≥8		

Table 22: MIC values for Acinetobacter and Shigella strains and Break Point.

Phenotypical virulence factors

The results of the virulence tests (Figure 39a and b) shows that hemolytic and gelatinase activity was present in 40,74% and 18,52% of the isolates, respectively. Five strains (9.26%) showed α-hemolytic activity, seventeen strains (31.48%) β-hemolytic activity and thirty-two strains (59.26%) did not show any type of hemolytic activity. All *S. aureus* and 4 out 5 *Listeria* spp. (*L. ivanovii*) are endowed with β-hemolytic virulence factor (Figure 40). All *Listeria* and *Acinetobacter* spp isolates are capable of hydrolyzing the gelatin, keeping the culture medium liquid even at temperatures below the solidification point. Two strains (*Staphylococcus aureus* 28S and *Staphylococcus aureus* 37S) showed a partial gelatinase capacity, corresponding to an incomplete solidification of the media at low temperatures. (Figure 41).

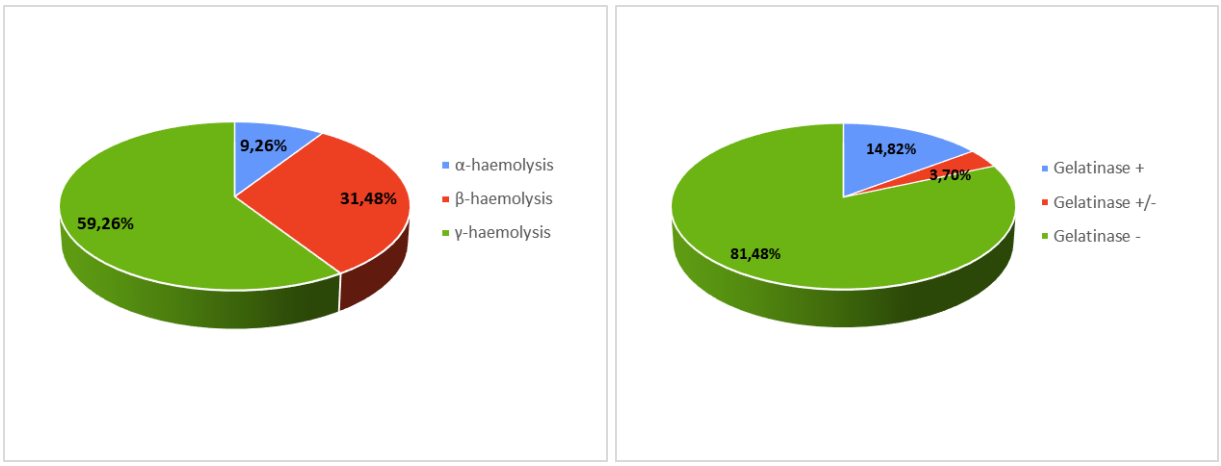

Figure 39: a and b: gelatinase and haemolysis test results.

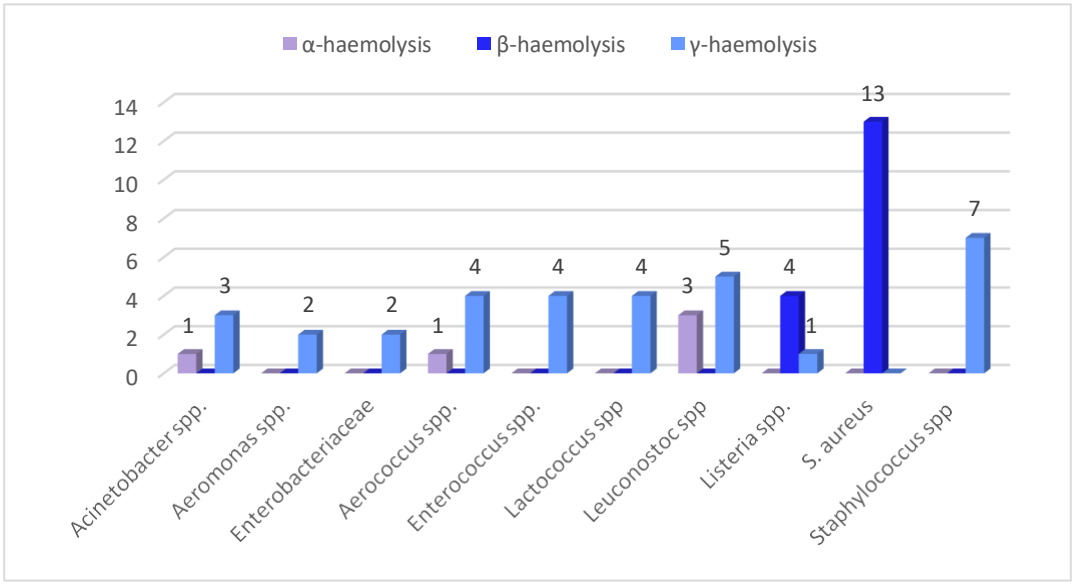

Figure 40: Haemolysis test results.

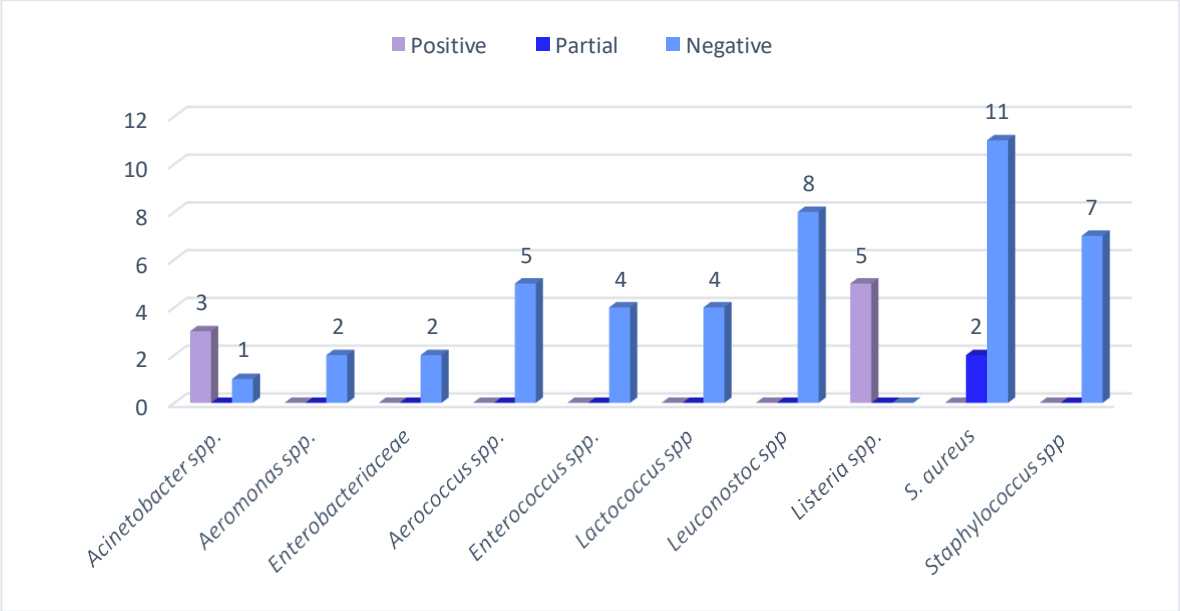

Figure 41: Gelatinase test results.

Discussion

Microbiological contamination is distributed throughout the processing chain, from its production to consumption. The risk is greater in foods that do not require further heating before being consumed, such as ready to eat (RTE) products. The European Food Safety Authority believes that, on most of the data collected from 2008 to 2015 cases of listeriosis (up to 90%) are due to consumption of RTE foods, such as smoked fish, preserved meat and cheeses, but also foods such as ready salads, where a third of the cases was due to the proliferation of *Listeria* spp., *Staphylococcus* spp., *Citrobacter* spp., *Y. enterocolitica* and other pathogenic bacteria, found in foods incorrectly stored in the refrigerator vending machine (EFSA 2015; El-Shenawy et al., 2011; Cossu et al., 2016). The present study showed that among the 54 isolates, fifty percent (50%) belongs to harmless microorganisms, including *Leuconostoc* and *Lactococcus,* lactobacilli used as starters in fermentation processes of cheese or bread, whereas the remaining fifty percent (50%) belongs to pathogenic bacteria (13 *S. aureus*, 5 *Listeria* spp., 4 *Enterococcus* spp., 4 *Acinetobacter* spp., one *Y. enterocolitica*, one *A. hydrophila*, and one *Citrobacter* spp.), pathogens contextually present in some samples and frequently found with a microbial load exceeding the limits of the EC Regulation 2073/2005 (El-Shenawy et al., 2011), as in the case of the four samples contaminated with *S. aureus* only. Other studies show the presence of *S. aureus* in RTE products, especially in cold served salads and sandwiches (Jang et al., 2013; Soares et al., 2020), tracing their presence to ingredients such as vegetables and / or fruit and products of animal origin (meat, sausages) (Fijałkowski et al., 2016). *S. aureus*, by the production of a thermostable toxin, is the species most frequently responsible for food poisoning (the third pathogen for food-borne diseases). *S. aureus* poisoning is a pathology of a modest clinical entity but has a strong socio-economic impact as it is highly widespread. The sample 3, product characterized by many components and therefore subjected to multiple manipulations, showed the presence not only of *S. aureus*, but also of *Listeria* and *Citrobacter* spp. In sample 6, a shrimp-based product, both *Y. enterocolitica* and *L. ivanovii* have been isolated. *L. ivanovii* has recently been associated with sporadic human infections, although it is usually linked to animal

infections, in particular ruminants, and is a milk and cheese contaminant (Alvarez-Ordóñez et al., 2015). *Citrobacter* species, belonging to the *Enterobacteriaceae* family, are considered opportunistic pathogens for humans, but for their wide distribution in the environment and intestinal tracts, they have the capability to be transferred from the farm to fresh produce destined for consumption, thereby constituting a public health risk (Iwu et al., 2019). Yersiniosis is the third most commonly reported zoonosis in the European Countries and *Y. enterocolitica* is the dominating species among human cases, mainly for the consumption of pig meat and products thereof (EFSA 2015). Being a psychrotrophic microorganism, a significant health threat is posed by refrigerated products where this pathogen, as well as *Listeria* and *Aeromonas,* can proliferate. *A. hydrophila* is a microorganism found in water, considered the most important infectious reservoir (Igbinosa et al., 2012), and consequently it is mainly isolated from seafood, meats and vegetables. It rarely causes food outbreaks, the most recent reported in Sweden and Norway (Hoel et al., 2019). *Acinetobacter* is rarely associated with diarrheal disease but *A. baumannii*, specie found in 4 samples, possess pathogenetic features, including antibiotic resistance, and could represent a risk for humans, linked to the consumption of contaminated foods (dairy products, raw fruit and vegetables) (Amorim et al., 2017). *Enterococcus* spp. have been recognized as emerging human pathogens in recent years, always endowed with antibiotic resistance and other virulence factors (Selleck et al., 2019; Bondi et al., 2020). They seem also to be involved in the production of biogenic amines in some fermented foods (Giraffa, 2002). The intoxication caused by the ingestion of these compounds can cause the onset of various symptoms, such as headache, vomiting up to the manifestation of severe allergic reactions. Relative to the virulence traits, the study has highlighted that all the *L. ivanovii* and *A. baumannii* are endowed with hemolytic and gelatinase activity, and 30 of the isolates showed a high profile of antibiotic resistance with MIC values exceeding the break point values defined by EUCAST. In particular, 11 *S. aureus* were resistant to ciprofloxacin, erythromycin, vancomycin and oxacillin. The isolation of resistant antibiotic strains is of concern not only for the consequent difficulty for therapeutic use, but

also because this characteristic can be transferred by conjugation, to different species within the food microbial community (Verraes et al., 2013; Wang et al., 2019).

Conclusions

The consumption of RTE foods that include sandwiches has continuously increased, consistent with the ever-changing eating habits of the consumers, and the variety of pathogens isolated in this study is a source of health concern. For this type of products, the preservation techniques are based primarily on the use of low temperatures, which however do not prevent some psychrotrophic bacteria from multiplying at refrigeration temperatures, and being eaten without cooking, there is no control of mesophilic pathogens. Hence the need to increase the level of attention and awareness throughout the food chain (from farm to fork), from producers of ready-made food to the most vulnerable consumer groups, which can significantly reduce the risk by following good hygiene practices. The increasing importance attributed to the quality and safety of food has prompted the European Union in 2004 to draw up a series of regulations, which entered into force in Italy in 2006, known as the "Hygiene Package". The EC regulation 852/2004 (EC Regulation 2073/2005) is responsible for ensuring the hygiene and safety of food products "from farm to table", that is, from the production area (farm) up to consumption (table). It sets both general and specific hygiene requirements to be applied to personnel, means of transport and places where food is processed and sold. It also requires the implementation of the HACCP (Hazard Analysis and Critical Control Points) system, applying the principle of self-control which incentives food business operators to adopt various measures to monitor the healthiness of the last product. In conclusion, our results show that sandwiches could be a health risk, in particular for susceptible individuals, as they can be a vehicle for the transmission of food-borne pathogens. The quantification and identification of bacterial strains isolated from the various sandwiches shows how products with more components, and consequently more manipulated, are also the most contaminated samples. In particular, the isolation of *S. aureus* from a lot of samples leads us to believe that food handling is the most likely cause of bacterial

contamination, followed by contamination caused by an inaccurate washing of the vegetables present in the food (Jang et al., 2013), as evidenced by the presence of other species like *Listeria*, *Enterococcus*, *Enterobacteriaceae* and bacteria belonging to the genera *Acinetobacter*, *Aerococcus* and *Aeromonas*. These results underline once again the need for an improvement in hygienic conditions during food processing and the choice of quality raw materials. Given the presence of possible hygienic deficiencies in the production chain of some products examined, careful controls are of fundamental importance, both during the food processing and marketing, in order to avoid the onset of infections difficult to treat, especially in weaker subjects.

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