Use of natural antimicrobials for the control of *Listeria monocytogenes* in foods

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*Listeria monocytogenes* is a gram positive, psychrotropic, facultative anaerobic bacterium and it is the etiological agent of listeriosis, a severe foodborne disease of major public health concern. The bacterium can survive under relatively extreme conditions such as low or high temperatures, low pH, reduced water activity and high salt content. Due to its psychrotropic character it is a pathogen of concern in refrigerated food products. One strategy used to overcome the low temperature tolerance is the addition of antimicrobial agents. The objective of this chapter is to review the bibliography concerning the use of natural antimicrobials to control the growth of *L. monocytogenes* in foods. With special emphasis it will be analyzed the effect of environmental factors and composition of the food on the effectiveness of antimicrobials. It will also explore the combinations that show a synergistic action. This information will help to choose the antimicrobials together with other environmental conditions that assure the control of *L. monocytogenes* in foods, aiding to improve the safety of foods.

**Keywords** natural antimicrobials; *Listeria monocytogenes*

1. Introduction

*Listeria monocytogenes* represents the *Listeria* species most commonly associated with disease in both animals and humans. As a facultative pathogenic saprotroph, *L. monocytogenes* can live in soil and decaying vegetation, but once it enters an animal or human host, it can cause severe disease [1]. The majority (99%) of the infections caused by *L. monocytogenes* are thought to be foodborne [2]. Being ingestion of contaminated food the origin of infection, governments and food safety agencies worldwide have taken serious measurements to reduce the occurrence of *L. monocytogenes* in the food production chain. Major listeriosis outbreaks have been related to contaminated ready to eat meat and poultry products [3]. For example, the US has adopted a so-called ‘zero-tolerance’ policy for ready to eat foods, which means that the detectable presence (≥1 CFU in 25 g of sample) of *L. monocytogenes* in ready-to-eat foods requires a recall. This has great economical implications for the food industry, leading to recalls of the contaminated product and temporary shutdown of food processing plants until the contamination problem has been solved. The estimated annual cost of recalls related to *L. monocytogenes* for the food industry may be as high as $1.2 billion to $2.4 billion in the US [4].

Controlling *Listeria* in foods and in food processing facilities implies an ongoing effort from academia, government agencies and the food industry. The latter actors are aimed at developing new and improved methods to prevent the survival and growth of *Listeria*. The ability of *L. monocytogenes* to survive a wide range of adverse conditions, including acidic pH, low temperatures, and high sodium chloride concentrations [5] make this organism difficult to control in food. Several studies that utilize various preservation techniques for the control of *Listeria* in foods are being conducted. Most of them aim at achieving food safety without compromising the sensory and nutritional qualities of foods [6]. Among them, the use of natural antimicrobials appears as a suitable tool which can offer several benefits [7]: (i) extend shelf life of foods; (ii) provide extra protection during temperature abuse conditions; (iii) decrease the risk for transmission of foodborne pathogens through the food chain; (iv) ameliorate the economic losses due to food spoilage; (v) reduce the application of chemical preservatives; (vi) permit the application of less severe heat treatments without compromising food safety; better preservation of food nutrients and vitamins, as well as organoleptic properties of foods; (vii) permit the marketing of “novel” foods (less acidic, with a lower salt content, and with a higher water content), and (viii) serve to satisfy industrial and consumers demands. Therefore, some of the current trends of the food industry could be satisfied, at least in part, by the application of natural antimicrobials. Numerous antimicrobials exist in plants, animals, and microorganisms where they often take part in defense mechanisms. Typical examples of these compounds are bacteriocins, organic acids, essential oils and chitosan, among others. Most of them are considered GRAS (generally recognized as safe) compounds. Although it is known that antimicrobials do not provide the magic bullet for the inhibition of spoilage and pathogenic microorganisms, they are used as one stress factor within the hurdle technology [8]. The use of the main natural antimicrobials to control *L. monocytogenes* growth in foods will be discussed.
2. Bacteriocins

Bacteriocins are ribosomally-synthesized peptides or small proteins with antimicrobial activity, produced by different groups of bacteria, active against bacteria related to the producer strain. The latter is typically immune against its bacteriocin. The structural bacteriocin genes are present in operon-like gene clusters that also harbor genes encoding proteins for immunity, processing, regulation and translocation functions. In general, bacteriocins are heat resistant, but they are inactivated by proteolytic enzymes such as trypsin, pepsin and other proteases. Several structural groups may be distinguished, ranging from small peptides (molecular weight < 10 kD) including lantibiotic type bacteriocins to large proteins (molecular weight > 30 kD) and also including one group of complex molecules with a lipid or polysaccharide moiety essential for activity. Bacteriocins have been classified into classes (class I, type A and type B lantibiotics, class IIA, IIB, IIC and class III) based on their genetic and biochemical characteristics [9]. The bacteriocins produced by lactic acid bacteria (LAB) offer several desirable properties that make them suitable for food preservation: (i) are GRAS substances; (ii) are not active and nontoxic on eukaryotic cells; (iii) become inactivated by digestive proteases, having little influence on the gut microbiota; (iv) are usually pH and heat-tolerant; (v) they have a relatively broad antimicrobial spectrum, against many foodborne pathogenic and spoilage bacteria; (vi) they show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane: no cross resistance with antibiotics, and (vii) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation [10].

Although several bacteriocins from LAB have been characterized to date, their use as food preservatives is still very limited. Among bacteriocins produced by LAB, nisin, a class I bacteriocin which has demonstrated antilisterial activity, was the first bacteriocin to be characterized and is the only one approved worldwide for use in food applications. The inhibition of *Listeria* by nisin has been demonstrated in culture media as well as in different foods. As an illustration, cottage cheese, ricotta-type cheeses [11], fresh pork sausages [3], cold-salmon [12], Turkish fermented sausages (sucuk) [13], raw and cooked pork meat [14], can be mentioned. In addition, the class IIA group, the largest group of bacteriocins which includes pediocin-like peptides, has attracted much of the attention due to their anti-Listeria activity. Pediocin PA-1 is the most extensively studied class IIA (or pediocin family) bacteriocin, due to its broad antibacterial activity, stability in foods, and potential for use as a food bio-preservative [15]. The stability of pediocin PA-1 in foods such as cheese, frankfurters, Spanish dry fermented sausages and chicken sausage, has been demonstrated [16]. In vitro studies have shown that the pediocin PA-1 produced by *Pediococcus acidilactici* UL5 has strong inhibition activity against a wide variety of *L. monocytogenes* strains [17]. Moreover, narrow-spectrum bacteriocins can be used to selectively inhibit certain high-risk bacteria in foods like *L. monocytogenes* without affecting harmless microbiota. Together with the available commercial preparations of nisin and pediocin PA-1/AcH, other bacteriocins (like for example lacticin 3147, enterocin AS-48 or variacin) also offer promising perspectives. Regarding enterocins, the ability to inhibit growth of *Listeria* is common to most *Enterococcus* bacteriocins [18, 19], being this ability related to the close phylogenetic relationship of enterococci and listeriae [20]. Enterocins have proved to be strong inhibitors of foodborne pathogens such as *L. monocytogenes*, *Clostridium tyrobutyricum* and *Staphylococcus aureus* [21]. Several enterocins proved to be effective inhibitors of *L. monocytogenes*, namely enterocin 416K1 in frankfurters and fresh cottage cheese [22], enterocin MR-10A in pickled cucumber [23], dry fermented Hornad salami [24].

Foods can be supplemented with *ex situ* produced bacteriocin preparations, or by inoculation with the bacteriocin-producer strain under conditions that favor production of the bacteriocin *in situ* [25]. *Ex situ* produced bacteriocins are preparations obtained by cultivation of the producer strain followed by adequate recovery and processing which can be added as partially purified or purified concentrates. In order to be added as food preservatives, bacteriocins require specific legal approval. Nisin is the only bacteriocin hitherto accepted as a food preservative (E234). *Ex situ* produced bacteriocins can also be applied in the form of immobilized preparations, in which the partially-purified bacteriocin or the concentrated cultured broth is bound to a carrier. *In situ* bacteriocin production implies the use of bacteriocinogenic cultures. Selected strains must be well-adapted to the particular food environment in which they will be used and able to grow under the food processing and/or storage conditions and to produce enough bacteriocin amounts as to inhibit the target pathogenic or spoilage bacteria. Bacteriocinogenic strains can be used either directly as starter cultures, as adjunct or co-cultures in combination with a starter culture, or as protective cultures (especially in the case of non fermented foods) [10]. Having GRAS status, bacteriocinogenic LAB cultures can be safely added to foods which revised their major advantage regarding food preservation.

Chemical nature of bacteriocins makes them prone to be involved in a number of food-related events, namely interaction with food components, precipitation, inactivation, or uneven distribution of bacteriocin molecules in the food matrix. Moreover, the efficacy of bacteriocins in foods will greatly depend on some other limiting factors such as the food microbiota and the target bacteria.

Gänzle *et al.* [26] stated that bacteriocin activity may be affected in food matrices by (i) changes in solubility and charge of the bacteriocins; (ii) binding of the bacteriocins to food components; (iii) inactivation by proteases, and (iv) changes in the cell envelope of the target organisms as a response to environmental factors. Furthermore, food processing conditions, storage temperature, pH, and bacteriocin instability to pH changes constitute environmental factors that play a crucial role on the effectiveness of bacteriocins.
Application of LAB bacteriocins in foods is currently intended as a part of multiple-hurdle technology. This concept must consider the effects of possible antagonistic interactions between different preservatives or food components [8]. For example, nisin activity decreased by 50% when it was added to milk containing 12.9% fat whereas the addition of Tween 80 significantly increased the activity of nisin against *L. monocytogenes* in milk regardless the fat content [27]. The interaction of nisin with surfactants in a food matrix containing nisin is relatively ambiguous and it still needs to be elucidated [28]. Nevertheless, interactions between bacteriocins and additional hurdles in food can also be additive or synergistic. As an illustration, potassium sorbate combined with nisin decreased bacterial counts on a vegetarian food [29] and had a listericidal effect in buffered broth (pH 5.5) during incubation at 4°C [30]. These combinations proved to have the best preservative effect when compared to results for each preservative agent when used alone. The combination of several antimicrobials is also advantageous for prolonged storages specially for preventing the regrowth of *L. monocytogenes* [10].

The use of chemical preservatives together with enterocin AS-48 effectively inhibited *Listeria* in cooked ham in a concentration-dependent way at 5°C and 15°C [31]. However, even at the higher concentration used (60 μg.g⁻¹) it was not possible to avoid the regrowth of *Listeria* after 15-30 storage days at 5°C when the bacteriocin was used alone. The authors attributed the lower effectiveness of AS-48 in cooked ham compared to broth to: (i) a higher retention of the bacteriocin molecules by meat and fat components; (ii) a slower diffusion, and (iii) the irregular distribution of the bacteriocin molecules and the bacterium in the meat matrix with a higher dry matter content compared to liquid media. Results from bacteriocin extraction experiments revealed that bacteriocin levels decreased markedly after day 7, which could explain regrowth of surviving bacteria. The combination enterocin AS-48-nitrite/nitrate (0.007%) used to overcome this situation reduced *Listeria* below detection level from the first sampling. Other combinations of AS-48 (40 μg.g⁻¹) with other preservatives as sodium pyrophosphate, pentasodium tripolyphosphate (STPP), sodium benzoate or potassium sorbate were also effective in reducing *Listeria* during storage at 5°C to a lesser extent. Although the combined effect of AS-48 and several of the antimicrobial compounds had been previously tested for *Listeria* inactivation on vegetable foods [32], the results derived from those studies could not be extrapolated to meat since one of the most effective compound in the combined treatments of raw vegetables, sodium lactate, was inefficient when combined with AS-48 in increasing the inhibition of *Listeria* in cooked ham. The anti-listeria effect of nisin at 6°C has also been reported in cooked ham [33]. However, after an immediate bactericidal effect in the first day of storage, *L. monocytogenes* rapidly regrew to reach viable counts similar to those of the control. In this case, although the combination with sodium lactate (1.8%) did not eliminate the *Listeria*, it reduced the population levels by at least one log cycle below initial values (approximately 4 unit log) along 75 storage days. These results emphasize the need to test the effectiveness of the enterocin in each food system to establish the precise mode and concentration to be applied [31]. This study also shows that application of sub-lethal heating at 60°C for 2 min in combination with enterocin AS - 48 (20 μg.g⁻¹) significantly improved the antimicrobial effect against *L. monocytogenes* in model cooked ham. When the sub-lethal heat treatment was applied in combination with AS - 48 (20 μg.g⁻¹) and STPP (0.5%), the results improved remarkably with respect to the separate application of each antimicrobial compound.

Similar to the use of antibiotics, the concern with the use of bacteriocins is the development of resistance in foodborne pathogens. Gravesen *et al.* [34] investigated the frequency of resistance development in *L. monocytogenes* to pediocin PA-1 and nisin A along with the effects of strain differences and environmental conditions. The resistance frequencies for pediocin investigated in about 20 strains were approximately 10⁻⁶, irrespective of the environmental conditions, while the frequency of resistance to nisin was strain-specific and varied with environmental conditions from 10⁻² to 10⁻³. The development of resistance to bacteriocins in a food system and the influence of a number of environmental factors such as low temperature, acidic pH and presence of sodium chloride on the frequency of resistance development show several limitations that curb the bacteriocin application in foods. Introduction of the hurdle technology concept combined with bacteriocins has become a valuable tool to fight against pathogen bacteriocin resistance. Multiple hurdle technology targets the bacterial cell in different ways resulting in better control of the pathogen. Nilsson *et al.* [35] investigated the combined action of nisin and carbon dioxide on *L. monocytogenes* cells grown at 4°C. Nisin brought about a two-log reduction in wild type *L. monocytogenes* cells and acted synergistically with carbon dioxide to give a four-log reduction in cell count. Nisin had no effect on nisin-resistant cells grown in the presence of air or carbon dioxide. Carbon dioxide increased the lag phase of *L. monocytogenes* by six days and was more effective against nisin-resistant cells compared to the wild type strain. The presence of carbon dioxide increases the membrane permeability and the proportion of short-chain fatty acids in the cell membrane, which helps in the pore formation by nisin [35]. The combined effect of heat and nisin on wild type and nisin-resistant *L. monocytogenes* cells had been studied [36]. The heat sensitivity of wild type and nisin-resistant strains was the same in the absence of nisin. The synergistic effect of heat and nisin on nisin-resistant cells caused a 3.7 log reduction in the first 7 minutes of treatment. The authors postulated that sub-lethal heat treatment alters the membrane permeability along with nisin that causes poration of the cell membrane. The use of bacteriocins with other treatment methods to achieve food preservation requires the use of lower concentrations of the bacteriocin, and this helps to prevent the risk of development of bacteriocin resistant population of cells [10].

Branen and Davidson [37] investigated the effect of ethylenediaminetetraacetic acid (EDTA) and lactoferrin on the antimicrobial activity of nisin. Low levels of EDTA used in the study synergistically enhanced the activity of nisin
against *L. monocytogenes*. EDTA functions as a chelator of divalent cations interfered with ions located on cellular membrane. Lactoferrin alone did not show any bacteriostatic effect against the organisms tested, but in a combination treatment with 50% less nisin, lactoferrin totally inhibited *L. monocytogenes*. A study by Ettyayebi et al. [38] investigated the synergistic action of nisin and thymol. The results showed that nisin Z and thymol resulted in only a partial inhibition of both pathogens when used alone. But the two agents acted synergistically in combination treatment and sub-inhibitory concentrations of both nisin Z and thymol being sufficient to reduce the growth of both pathogens. Thymol alters the bacterial membrane structure resulting in greater permeability for nisin. This results in a higher concentration of nisin within the bacterial cells, thus permitting the use of lower nisin concentrations when used synergistically to obtain the same level of antibacterial activity [38].

### 3. Organic acids

Organic acids such as acetic, lactic, malic, and citric are natural constituents of many foods and they have been used in food preservation since ancient time. Its antimicrobial action is based mainly on their ability to reduce the pH of the aqueous phase of the food. In the case of weak lipophilic organic acids such as acetic or sorbic, the undissociated form is also able to penetrate the cell membrane. The latter exerted its inhibitory action by dissociating and acidifying the cytoplasm. Additionally, other mechanisms take place such inhibition of enzymes, nutrient transport and overall metabolic activity [39]. Although naturally present in certain plant tissues, lipophilic organic acids are manufactured for commercial use by chemical synthesis since it is more convenient from the economical point of view. Due to its higher solubility, salts are most commonly used than the organic acids [39].

The antimicrobial effectiveness of organic acids and their salts in laboratory media and in foods depends on pH, temperature, water activity, the type of acid used, its concentration and method of application. Moreover, interactions with other additives or ingredients may increase or decrease the antimicrobial activity. In general, the activity is enhanced at low pH and/or when they are combined with additional stress factors. Based on equal molar concentration, organic acids showed the following order of effectiveness: citric > malic > lactic > acetic > HCl against *L. monocytogenes* inhibition [39]. Several studies demonstrated that inhibitory effects of acids are greater at lower temperature [40]. In addition, the combined effect of temperature, pH and organic acid through the development of predictive models was studied [41, 42] showing that interaction between stress factors played an important role on inhibition.

Most important applications of organic acids are linked with the preservation of ready to eat meat products in order to prevent post process contamination [3, 43]. Processors usually include sodium or potassium lactate up to 2.0% with 0.05-0.15% of sodium diacetate in the formulations. Dipping or spraying solutions in combination with other stress factors are not currently applied in the industry but they are options that have demonstrated good results. Surface application can be more effective than addition in the formulation since bacteria are at the product surface, place where contamination occurs. Consequently, as a small amount of the antimicrobial is necessary, no additional changes in food product formulations should be made. For example, dipping frankfurters with a solution containing 2.0% of acetic acid, 1.0% of lactic acid, 0.1% propionic acid and 0.1% of benzoic acid followed by a steam prevented the growth of *L. monocytogenes* for 14 weeks at 7ºC [43].

The successful use of mixtures of lactates and diacetates is probably linked to the fact that combination of lactates and sodium diacetate exerts a strong synergistic effect especially at low pH [44] and also they do not adversely affect sensory characteristics [44, 45]. Other mixtures that showed a synergistic action were: (i) potassium propionate in combination with potassium lactate or diacetate in pork scapple [45]; (ii) potassium lactate, sodium diacetate and nisin in smoked sausage [3]; (iii) lactate-diacetate blend and lauric arginate in cooked ham [46]; (iv) sorbates and nisin in broth [30]. In the case of cured meat products, the use of sorbates, is not widely accepted since there are some reports about the possible mutagenic products formed by the reaction of sorbic acid with sodium nitrite [47]. Regarding the use of lactates, their addition depresses water activity of the product contributing by this mechanism to the inhibitory activity [44].

Cured meats, such as sausage, ham and frankfurters are more susceptible to the listericidal effects of organic acids probably as a result of the presence of salt, nitrite and other preservatives [40]. Regarding the effect of fat on organic acids, the inhibitory activities of lactate and propionate increase with the fat content. Conversely, potassium sorbate exhibited a less antilisterial action [48]. Probably, the latter tends to migrate to the fat as a result of its higher partition coefficient.

Devlieghere *et al.* [49] studied the growth of *L. monocytogenes* in modified atmosphere packed cooked meat products and developed a predictive model for the effect of temperature, water activity, concentration of sodium lactate and dissolved CO2 in the aqueous phase. They found a strong synergistic effect between CO2 and lactate. Probably, the pH decreasing effect that CO2 exerted in the medium increased the amount of undissociated molecules of lactic acid and as a consequence enhanced the inhibitory activity of lactate. This combination of hurdles is useful to avoid the proliferation of *L. monocytogenes* in meat products with water activity lower than 0.97.

Another way of using organic acids is immobilized in gels or into edible films. It was reported that lactic and acetic acids applied in immobilized calcium alginate gels were more effective than acids applied alone on meat tissue.
Probably, alginate coating increased the contact time of the acid with the meat surface [50]. Moreover, sliced Bologna and summer sausage packaged in films containing 0.5 to 1.0% p-aminobenzoic acid and sorbic acid promoted a 3 log reductions of inoculated \textit{L. monocytogenes} [51].

Finally, it should be stressed that \textit{Listeriae} cells exposed to organic acids may survive during storage at refrigeration and begin to multiply if other barriers are not present. Moreover, exposure to acids also induces stress responses in \textit{Listeriae} which make the bacteria more tolerant to other stress factors [39].

4. Essential oils

Essential oils (EOs) are oily liquid mixes of volatile and complex compounds extracted from different part of aromatic plants (flowers, buds, leaves, seeds, bark, stems, twigs, fruits, herbs, wood and roots). They are synthesized by plants as secondary metabolites and can be obtained mainly by steam distillation or supercritical fluid extraction [52, 53]. Essential oils can contain 20-60 components depending on the material they come from and the extraction method used. They are terpenes and terpenoids, as a majority group, and aromatic and aliphatic compounds of low molecular weight, as a minority group [53].

The historical use of EOs in foods is as flavorings. Although it is known that EOs have a wide spectrum of antimicrobial activity [54], their use as preservatives in food have not yet been extended. In the last few decades, the need to consume healthy foods reemerged the search of the minimal inhibitory concentrations (MIC) of EOs and the study of the effect of food composition on their effectiveness.

Numerous EOs have been examined for their activity against \textit{Listeria} growth in laboratory media. In general, it was reported that EOs of bay, coriander, cinnamon, clove, liquorice, nutmeg, pepper, oregano, \textit{Satureja montana}, spruce and thyme showed the highest inhibitory activity. The effectiveness of oils of basil, lemon balm, marjoram, \textit{Pistacia lentiscus}, rosemary and sage was lower than the ones mentioned above, whereas \textit{Listeria} showed high resistance to EOs of aniseed, caraway, fennel, garlic, ginger, onion and parsley [55, 56, 52, 57-66].

The antimicrobial activity of EOs mainly depends on their composition. The inhibitory action is more related to the main than to the minor components. However, the latter might modulate the antimicrobial action of main components since it was demonstrated that several components of EOs are involved in the fixation on cell walls and cellular distribution [53]. Among the main components that have been associated with the high action of EOs, carvacrol, thymol, linalool, eugenol, trans-cinnamaldehyde, p-cymene, 1.8-cineole and \textit{\gamma}-terpinene can/could be mentioned [54]. The mechanism of antimicrobial action of EOs is not well known. It has been reported that EO components may degrade cell wall, damage cytoplasmic membrane and proteins of membrane, leak vital intracellular compounds, coagulate cytoplasm and deplete the proton motive force [54].

The existence of interactions among oils was also reported. de Azeredo \textit{et al}. [55] suggested a synergistic effect between oils of oregano and rosemary. Marjoram or thyme EOs showed additive interaction with oils of basil, rosemary or sage. Zhang \textit{et al}. [57] reported that the joint presence of rosemary and liquorice EOs suppressed the growth of \textit{L. monocytogenes} more than when these oils were used alone.

As it was mentioned above, most of the studies of antimicrobial activity of EOs against \textit{Listeria} are carried out in laboratory media. However, to transfer this information to a food matrix is necessary to consider that food components, processing and storage conditions and physical structure of a food [54, 60, 61, 63, 67] may modify the in vitro effectiveness of EOs. In most of the studies, the effective doses of EOs in food systems are higher than in laboratory media. Firouzi \textit{et al}. [61] reported that higher levels of oregano and nutmeg oils would be necessary to reduce \textit{L. monocytogenes} growth in ready to cook barbecued chicken than in broth culture. The great antilisterial effect of clove and thyme oils in culture media was significantly reduced in hotdogs [64]. Higher levels of EOs of \textit{Pistacia lentiscus} and \textit{Satureja montana} were necessary to reduce the \textit{Listeria} growth in refrigerated minced beef than in culture medium [56]. Therefore, the evaluation of EOs effectiveness in food products or in model systems simulating food composition is a previous step to the correct application of EOs in real foods.

The activity of EOs may be reduced by the protein content of the food, probably by complex formation between phenolic compounds of oils and protein. These complexes would compete with the ones formed with protein of cell membrane of target bacteria [66]. In the study of the effect of relevant factors to cheese ripening on antilisterial activity of spruce essential oil, Canillac and Mourey [63] observed that the addition of sodium caseinate in a culture broth significant increased the MIC. However, Gutierrez \textit{et al}. [58] reported that the effectiveness of oregano and thyme oils increased with the protein level in beef extract culture medium being the oregano oil the most effective one. These authors have suggested that hydrophobic proteins as peptones may facilitate EOs dissolution, increasing the preservative action.

The presence of high levels of fat reduces the action of EOs against \textit{L. monocytogenes} growth. The effectiveness of oregano and thyme EOs was diminished by high concentrations of sunflower oil [58]. Singh \textit{et al}. [64] found that antimicrobial action of clove and thyme oils reduced as fat level increased in hotdogs. Clove was more effective than thyme, but these authors do not advise their use alone to complete protection against pathogens in hotdogs. Cinnamon and clove oils showed antilisterial activity in refrigerated soft cheese with different levels of fat [66]. The former was
more effective at low fat contents whereas clove had a strong inhibition at high fat content. All these works have suggested that the lower activity of EOs at high fat content may be due to: (i) EOs dissolution in the lipid fraction of the food, decreasing the concentration in the aqueous phase together with their antimicrobial action; (ii) the reduced water content in foods, particularly in fatty foods, in relation to culture media which may slow down the movement of the preservative to the active site in the microbial cell, and (iii) the presence of fat in the food which may produce a protecting layer around the bacteria.

In general, bacteria are not so protected from EOs action by carbohydrates than by fat or protein in foods. Antilisterial actions of oils of oregano and thyme improved due to the increase in sugar level up to 2.3% in beef extract and tomato serum model media. However, higher concentrations of sugars did not show any effect on the EOs effectiveness [59]. On the contrary, the increase in levels of potato starch from 1 to 10% slightly decreased the antilisterial activity of these oils. At the highest potato starch level the activities were similar to the observed in absence of EOs [58].

The antimicrobial activity of EOs increases at acidic pH where the hydrophobicity of EOs is higher than at more alkaline pH levels. Thus, the dissolution of the oils in the lipids of the cell membrane of target bacteria would increase, while at high pH levels repulsion between negative charges of amino compounds from food and cell surface could happen. Therefore, the cell wall would be less protected by amino compounds increasing EOs action [63]. Gutierrez et al. [58] did not observe growth of L. monocytogenes at pH 4.0 in Tryptic Soy Broth while the higher antilisterial effect of these oils was found at pH 5.0.

The temperature of storage is another factor to be considered in the study of effectiveness of EOs against L. monocytogenes since this microorganism has the ability to grow in a wide range of temperatures (2-45°C) [6]. Several studies reported that EOs action against Listeria increases as temperature decreases. As an example, Mytle et al. [68] have shown that clove oil was more or equal effective at 5 than 15°C in controlling the growth of L. monocytogenes in chicken frankfurters.

The physical structure of the food also has a role in the antimicrobial action of EOs. Probably, bacteria may enter in the pore of the surface of certain foods such as beefs reducing the chance and time of direct contact with the antimicrobial [69]. Gill et al. [65] reported great antilisterial action of cilantro oil in broth with MIC values in the range of 0.018-0.074% depending on the strain. However, a level of 6.0% was ineffective when it was included into a gel with glycerol monolaurate or gelatin coating a ham. The increase in the viscosity of culture medium due to agar addition increased cell resistance to antilisterial activity of spruce oil [63].

It must be taking into account that sensory properties of EOs may be a limiting factor to their use in food and it should be considered. Chicken frankfurters treated with 2%v/w of clove oil were unacceptable to the consumer whereas samples with 1% were sensory accepted. The last level had effective antilisterial activity in the food [68]. Djenane et al. [56] showed that sensory properties of minced beef meat treated with oils of P. lentiscus and S. montana were acceptable at two fold MIC values (0.20 and 0.06% v/v, respectively) after 8 days of storage. The oils showed bactericidal activity against Listeria at these concentrations and the use of the combination of EOs increased the individual antimicrobial action. Thus, combining EOs would allow the use of lower levels to reduce Listeria growth minimizing the unacceptable sensory changes in the food. The indirect uses of Eos have also been suggested, for example in water to wash vegetables similar to the use of chlorine [66] or in the impregnation of porous surface of wood in cheese ripening to improve sanitary safety [63]. Other alternative would be the use of the microencapsulation technology that not only may protect the EOs from factors such as heat but it also allows the controlled release of the antimicrobials [67].

### 5. Chitosan

Chitin is the major component of the shells of crustacean such as crabs, lobsters and shrimps. It is also an important part of exoskeleton of invertebrates and can be found in the cell walls of some fungi. Due to its structure, it may be compared to cellulose. This biopolymer is a polysaccharide composed of β(1→4) linked units of N-acetylglucosamine. Chitosan, another biopolymer which has received considerable attention, is obtained from the deacetylation of chitin [70, 71].

Chitosan has numerous properties that make it applicable to the food industry. Regarding to the extension of food shelf life, its antimicrobial action and its ability to form edible films are the most outstanding. Furthermore, chitosan is obtained from natural sources, nontoxic, and presents anti-oxidative activity, biocompatibility and biodegradability [72, 73]. Antibacterial activity of chitosan depends on several factors such as the deacetylation degree, molecular weight, temperature, pH of the medium and other components presence [74].

The mechanism of the antimicrobial activity of chitosan is not completely known, but different hypothesis have been proposed: (i) interactions between the positively charged chitosan molecules and the negatively charged microbial cell membranes produce a change of permeability which leads to the leakage of proteinaceous and other intracellular constituents; (ii) interaction with microbial DNA interferes with the mRNA and proteins synthesis; (iii) chitosan acts as a water and metal binding agent and it can also inhibit different enzymes [70, 71].
Edible films have been extensively investigated; they are able to maintain the chemical, sensory and microbiological quality of food [75, 76]. Many studies have been carried out to determine the ability of chitosan edible films to inhibit growth of *L. monocytogenes* in different kinds of food such as ham steaks [77], cold smoked salmon [78] and red meat [75, 79].

Chitosan is able to control *Listeria* in laboratory liquid media. However, different results are obtained when it is in the form of films, in contact with a solid medium. Chitosan edible films are unable to control *Listeria* in foods. There are two possible explanations for the observed behaviour of chitosan when is used as a film forming agent: (i) it is not able to migrate from the film to the food matrix [77, 78], and (ii) its high affinity for food components such as proteins, may cause the formation of complexes that not allow chitosan to interact with microorganisms [79]. Moreover, when other antimicrobials, such as sodium lactate, potassium sorbate or nisin are added to the chitosan film, growth of *Listeria* can be controlled due to the ability of the antimicrobials to migrate from the chitosan film to the food matrix [78].

The ability of chitosan films to inhibit *Listeria* is retained when films are incorporated into liquid foods, e.g. soups [80]. Furthermore, chitosan inhibitory effect is evident in food where it can be dispersed and incorporated throughout the matrix such as bovine pâté [81].

In conclusion, it is necessary to incorporate chitosan into the food matrix to achieve an inhibitory effect on the growth of *Listeria*. Although antimicrobial activity of chitosan films is negligible, addition of other preservatives such as bacteriocins, organic acids or essential oils may be promising, since it allows gradual release of them into the food.

### 6. Examples of the combined use of natural antimicrobials with other stress factors

Compilation of evidence of the effective use of antimicrobials and hurdle technologies to control *L. monocytogenes* in food is shown in Table 1. The synergy between antimicrobials and chemical preservatives and/or heat and/or other treatments can be explained by the fact that bacterial cells sub-lethally injured by different stressing conditions become sensitive to different physical and chemical agents to which healthy cells are resistant. Anyway, the synergistic effects observed in complex solid environments are frequently lower compared to results obtained in liquid media [8, 39, 54] reinforcing the suggestion that interaction of antimicrobials with food components can interfere with their efficacy. The food composition seems to play a key role on the final effect of antimicrobials against *L. monocytogenes*. Thereby, the adequate combination of hurdles will be dictated by the specific type of food to be preserved.
Table 1 Compilation of some uses of antimicrobials and hurdle technologies to control *L. monocytogenes* in food.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Additional hurdle</th>
<th>Food Application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterocins A and B</td>
<td>High Hydrostatic Pressure (HHP)</td>
<td>Cooked ham</td>
<td>Marcos <em>et al.</em> [82]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low acid dry fermented sausages</td>
<td>Jofré <em>et al.</em> [83]</td>
</tr>
<tr>
<td>Nisin</td>
<td>EDTA and lysozyme</td>
<td>Ham and bologna</td>
<td>Gill and Holley [84]</td>
</tr>
<tr>
<td>Nisin</td>
<td>Heat (60 or 65°C)</td>
<td>Cold-pack lobster meat</td>
<td>Budu-Amoako [85]</td>
</tr>
<tr>
<td></td>
<td>Moderate heat</td>
<td>Milk</td>
<td>Maisnier-Patin [86]</td>
</tr>
<tr>
<td>Nisin</td>
<td>Irradiation</td>
<td>Frankfurters</td>
<td>Chen [87]</td>
</tr>
<tr>
<td></td>
<td>Low-dose irradiation (2.3 kGy)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium lactate</td>
<td>Nisin</td>
<td>Cold-smoked rainbow trout</td>
<td>Nykänen <em>et al.</em> [12]</td>
</tr>
<tr>
<td>and refrigeration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium lactate, potassium sorbate, curing salts and refrigeration</td>
<td>Nisin</td>
<td>Meat modeled system</td>
<td>Buncic <em>et al.</em> [30]</td>
</tr>
<tr>
<td>Sorbate and refrigeration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic acid solutions</td>
<td>Steam surface pasteurization</td>
<td>Fully cooked frankfurters</td>
<td>Murphy <em>et al.</em> [43]</td>
</tr>
<tr>
<td>Mint essential oil</td>
<td>High Pressure Processing (HPP)</td>
<td>Yogurt</td>
<td>Evrendilek <em>et al.</em> [88]</td>
</tr>
<tr>
<td>Cone essential oil</td>
<td>Nisin</td>
<td>Milk</td>
<td>Yoon <em>et al.</em> [89]</td>
</tr>
<tr>
<td>Spice extracts</td>
<td>Modified atmosphere packaging Vacuum</td>
<td>Fresh pork</td>
<td>Zhang <em>et al.</em> [57]</td>
</tr>
<tr>
<td></td>
<td>packaged</td>
<td>Ham slices</td>
<td></td>
</tr>
<tr>
<td>Thyme essential oil</td>
<td>Nisin</td>
<td>Minced beef</td>
<td>Solomakos <em>et al.</em> [90]</td>
</tr>
<tr>
<td>Chitosan films</td>
<td>Nisin, sodium lactate, sodium diacetate,</td>
<td>Ham steaks</td>
<td>Ye <em>et al.</em> [77]</td>
</tr>
<tr>
<td></td>
<td>potassium sorbate, sodium benzoate</td>
<td>Cold-smoked salmon</td>
<td>Ye <em>et al.</em> [78]</td>
</tr>
<tr>
<td>Chitosan films</td>
<td>Temperature</td>
<td>Fish soup</td>
<td>Fernandez-Saiz <em>et al.</em></td>
</tr>
<tr>
<td></td>
<td>Storage at 37°C, 12°C or 4°C</td>
<td></td>
<td>[80]</td>
</tr>
<tr>
<td>Chitosan</td>
<td>Refrigeration (4°C)</td>
<td>Bovine pâté</td>
<td>Bento <em>et al.</em> [81]</td>
</tr>
</tbody>
</table>
7. Conclusions

Natural antimicrobials in combination with other stress factors are a valuable tool to control the growth of *L. monocytogenes* in foods. Besides, the uses of multiple stress factors decrease the possibility of appearance of resistance strains. Since the efficacy of mentioned antimicrobials can be influenced by the chemical composition and the physical conditions of foods it is necessary to validate the antimicrobial activity in each particular food system to establish the effective concentration and the most adequate combination of additives or preservative treatments to be applied with the antimicrobial.

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References


[29] Fang T J, Chen CY, Chen HHL. Inhibition of *Staphylococcus aureus* and *Bacillus cereus* on a vegetarian food treated with nisin combined with either potassium sorbate or sodium benzoate. *Journal of Food Safety*. 1997; 17: 69–87.


[64] Singh A, Singh RK, Bhuniaa AK, Singh N. Efficacy of plant essential oils as antimicrobial agents against Listeria monocytogenes in hotdogs. LWT - Food Science and Technology. 2003; 36: 787-794.


