Minireview

Antibacterial Activity of Lactic Acid Bacteria to Improve Shelf Life of Raw Meat

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Lactic Acid Bacteria (LAB) are generally recognized as safe. It has been used to increase the shelf-life of fermented products, and its antimicrobial action is based on the metabolites secretions, such as lactic acid, hydrogen peroxide, reuterin, bacteriocins and the likebacteriocins substances. It has been proven that LAB are able to inhibit deteriorating bacteria of raw meat, but improper handling of live cultures could lead to spoilage. So, the use of their bacteriocins, small antimicrobial peptides, could be an alternative. Besides reducing the number of spoilage bacteria, it seeks to inhibit pathogenic bacteria such as Salmonella, enterohemorrhagic Escherichia coli and Listeria. The food industry uses few bacteriocins and now bacterial resistance has been reported. For that reason, the search of novel bacteriocins produced by LAB is a priority. Moreover, the natural microbiota of meat could be a reservoir of LAB.

Key words: Bacteriocin / spoilage / inhibition / pathogenic / Salmonella, Escherichia coli.

INTRODUCTION

Spoilage of meat and meat by-products by microbial activity is a common problem in the meat industry (Devlieghere et al., 2004). The food industry applies several processing techniques for inhibition and/or inactivation of microorganisms to guarantee safe products with a long shelf-life (Deak, 2014). Most techniques used are based on temperature manipulation (heat and cold treatment), and chemical treatment (acids, bases and salts) (Buncic et al., 2014). However, consumers demand tasty, nutritious, natural, and non-chemically treated products. Thus, the food industry has developed new preservation techniques, such as pulsed electric fields (PEF), high hydrostatic pressure (HPP), modified atmosphere packaging (MAP), active packaging, natural antimicrobial compounds and bio preservation (Devlieghere et al., 2004). In bio preservation, shelf-life is extended and meat safety is enhanced by using natural or controlled microbiota (Devlieghere et al., 2004). For these purposes, LAB are the most used microbiota, because these bacteria and their metabolites are generally recognized as safe (GRAS) (Cleveland et al., 2001). LAB and their metabolites are capable of inhibiting various spoilage microorganisms (Nychas et al., 2008; Buncic et al., 2014), and even pathogens such as Escherichia coli O157:H7 (Roldán et al., 2012) and Salmonella sp. (Smith et al., 2005a). In recent years, some strains of LAB and their growth-inhibitor factors were isolated, characterized and recommended to extend product shelf-life. However, this situation could contribute to selective and accelerate resistance of spoilage bacteria (Cleveland et al., 2001). To avoid bacterial resistance, some researchers have proposed manipulating the meat-native microbiota for increasing the number of beneficial bacteria. This technique could inhibit a spoilage bacterium with less resistance risk (Zaher and Fujikawa, 2011). This review explores the possibilities of using natural microbiota to isolate...
bacteriocin LAB producer strains, and its application for extending meat shelf-life.

**BACTERIAL SPOILAGE**

This can be defined as any change in a food product that makes it unacceptable from the sensory point of consumer view (Dias et al., 2013). Beef meat is one of the most perishable foods, because its composition is ideal for growth of a wide variety of microorganisms (Mayr et al., 2003), some of them may cause the spoilage of meat (Ercolini et al., 2006). The physiological status of animals and spread of contamination during slaughter, and, the storage and distribution conditions, like temperature, affect the microbiological quality of slaughter, and, the storage and distribution conditions, and, the spread of contamination during storage, and, the storage and distribution conditions, the status of animals and spread of contamination during slaughter, and, the storage and distribution conditions, like temperature, affect the microbiological quality of meat (Nychas et al., 2008). The signs of microbial spoilage of meat are organoleptic changes like physical damage, oxidation or appearance of off-flavors and off-odors, resulting from microbial growth and metabolism in the product; principal genera are *Pseudomonas* spp., *Enterobacteriaceae*, *Brochothrix thermosphacta* and LAB (Gram et al., 2002; Ercolini et al., 2006). Thus, the natural microbiota and the storage conditions of the meat have an influence on the final organoleptic properties (Ercolini et al., 2006) and shelf-life of meat and meat products (Borch et al., 1996).

Furthermore, preservation parameters of meat affect microbial selection, hence the spoilage microorganism dominant of a product could be predicted (Gram et al., 2002). The fact of modifying some factors, such as temperature, gaseous atmosphere, and meat pH can result in the extension of product shelf-life (Borch et al., 1996). Consequently, the importance of these parameters for bacterial growth should not be underestimated. Different spoilage bacteria species could colonize meat surface through different mechanisms (Ercolini et al., 2009) and interact, mainly could as competition (Bruhn et al., 2004). Also, interactions among microorganisms of microbiota can determine selection and/or metabolism of spoilage bacteria (Ercolini et al., 2009).

Low temperature is the most common applied strategy for preservation of stored meat, mainly from carcasses after slaughter, primal cuts, during transportation to distributors for storage of meat at various wholesale and retail food sites, and finally to the consumer's kitchen (Gram et al., 2002; Ercolini et al., 2006). High growth of bacteria is a prerequisite for meat spoilage, so during all of these phases, chill temperature are used to delay growth of bacteria, and hence meat alteration (Borch et al., 1996). The expected shelf life is related to day range, whilst at the time of rejection, typical odor is putrid and slime is visible on the surface (Bruhn et al., 2004).

**LAB IN MEAT**

Generally considered as natural strains of LAB in meat and meat products are: *Camobacterium piscicola* and *C. divergens*; *Lactobacillus sakei*, *Lb. curvatus* and *Lb. plantarum*, *Le. mesenteroides* subsp. *mesenteroides*, *Le. gelidum* and *Le. carnosum* (Hugas, 1998). The LAB plays an important role in food fermentations causing flavor and texture changes together with a preservative effect, resulting in an increase in the shelf life of transformed product (Hugas, 1998). The chill-storage under modified atmosphere of red meats has an effect on the meat microbiota, triggering a change from spoilage Gram-negative bacilli to fermentative LAB. This change produces an effect on the shelf life extension (Borch et al., 1996). In fresh meat, LAB bring about a mild fermentation process, without producing any changes in the sensorial characteristics, because of the low carbohydrate content and the buffering capacity of meat. In the same way, the growth of LAB in naturally fermented meats after the addition of sugar transform the products through the production of lactic acid by LAB, and then, the subsequent decrease in pH denatures the meat proteins favoring the decrease of water activity (a_w) which ends up in a microbial stabilization of the transformed product (Borch et al., 1996; Hugas, 1998; Favaro and Todorov, 2017).

The metabolic products of LAB and the bacteria itself play an important role in preservation of foods, although the uncontrolled growth of some species of LAB could spoil meats and meat products; *Leuconostoc* and *Lb. sakei* have been described as slime producing organisms in processed meats, sulfide-producing strains of *Lb. sakei* have been described as spoiling vacuum-packaged meat (Wills et al., 1987). Likewise, the growth of heterofermentative LAB can also cause off-odors and holes after the production of CO2 (Kandler, 1983).

LAB growth in meat might cause microbial interference to spoilage and pathogenic bacteria, through several mechanisms like nutrient and oxygen competition, also with competition for attachment/adhesion sites and production of a wide range of inhibitory substances, primarily lactic acid or lactic and acetic acids, acetoin, diacetyl, hydrogen peroxide, reuterin, bacteriocins and bactericins-like (Hugas, 1998).

**LAB INHIBITION POTENTIAL**

In food production and commercialization, it crucial that suitable actions are taken to guarantee safety stability during food shelf life. Consumers require foods of high quality, without chemical preservatives, safe, and possessing long shelf-life. The spoilage and
short shelf-life is a major part of microbial activity. The deterioration and short shelf life of the meat is mainly due to the spoilage microorganisms. The natural meat microecosystem is very important, because any bacteria could cause spoilage, even LAB. In fact many types of LAB can be considered deteriorating bacteria because they change the sensory characteristics, such as Lactobacillus sp., Leuconostoc sp., Lactococcus and Enterococcus (Ammor et al., 2006; Hamasaki et al., 2003). However, several antagonistic activities may occur in microbiome and control of the microbiota is the key to avoiding these problems. LAB are capable of showing antagonism effects by synthesizing antimicrobial compounds and these productions depends on some factors such as temperature, atmosphere, humidity or interaction with other bacteria (Geremew, et al., 2015; Awojobi et al., 2016, Ammor et al., 2006).

The modification of atmospheric CO₂ between 20-40% benefits to Lb. sakei CTC 372 or CTC 711 applied to meat, by reducing the growth of spoilage bacteria almost 80% and also inhibiting spiked L. monocytogenes growth, even at refrigeration temperatures (4 °C) (Djenane et al., 2005). Also, temperature is another important factor because at ambient temperatures (20 °C) the LAB fulfills its bioprotective function. On the contrary, at refrigeration temperatures and under vacuum, they are not as efficient and grow at a lower speed (Signorini et al., 2006).

In addition, antifungal capacities have been reported, under laboratory conditions and using culture medium. Several LAB have shown inhibitory capacity against molds of the genus Penicillium at 72 h (Kivanc et al., 2014). Also, some species of genus Lactobacillus can inhibit Aspergillus fumigatus (Kim, 2005), A. niger (Svanström et al., 2013), A. flavus and P. expansum (Dalié et al., 2010).

Additionally, multiple bacterial genera are causing diseases outbreaks throughout the world, and the most important bacteria in the meat industry have been Salmonella and Enterohemorrhagic E. coli (EHEC). The presence of these pathogens is mainly due to poor handling of feces during slaughter. It has been reported that the use of mixed cultures of L. acidophilus in TSB medium and ground beef inhibit the growth of Salmonella and EHEC in a period of 5 days, maintaining the sensory quality within acceptable ranges (Smith et al., 2005b). Also, in broth, lactic acid produced by different species of Lactobacillus inhibited S. enterica ser. Typhimurium (Makras et al., 2006) and if there is oxygen the LAB can produce hydrogen peroxide which increases the effect (Ammor et al., 2006). Although LABs show a clear inhibitory potential, its use as live cultures is very delicate, which is why the bad selection or bad handling of strains can produce adverse effects such as slime production, off-odors or color changes, even if initial dose are low (10 CFU g⁻¹) (Iulietto et al., 2015; Hamasaki et al., 2003).

An alternative to avoid poor outcomes using live cultures, is use of crude extracts or purified metabolites, like bacteriocins (Yong et al., 2015). The use of crude fractional extracts of LAB has become an inhibitor against different meat spoilage bacteria such as Leuconostoc spp. (Yong et al., 2015; Lee and Kim, 2011), pathogens such as Staphylococcus aureus (Yong et al., 2015), EHEC (Smith et al., 2005b), Salmonella sp. (Makras et al., 2006; Smith et al., 2005b) and L. monocytogenes (Djenane et al., 2005).

Meanwhile, it has been reported that the use of bacteriocins is more effective if dosed in the appropriate amount. However, these substances are specific and cannot be used massively (Iulietto et al., 2015). Search of new technologies for food conservation and the use of substances produced by microorganisms deserves more research.

**BACTERIOCINS**

Bacteriocins are proteinaceous toxins produced by bacteria in ribosomes for inhibiting growth of closely related bacterial strains (Hugas, 1998). These are polypeptides which are functionally, structurally, and ecologically diverse. Each bacterium synthesizes a different kind of bacteriocin (Table 1). The regulation is by operons, which are responsible for synthesis and autoimmunity (Deegan et al., 2006).

Five classes of bacteriocins have been proposed, and most of them are classified in Classes I and II. (Deegan et al., 2006; López et al., 2008). Class I. These bacteriocins are heat-stable and small (<5 kDa) peptides and generally known as lantibiotics due to presence of non-proteogenic thioether amino acids lanthionine (Lan) or methyllanthionine (MeLan) (McAuliffe et al., 2001; Lee and Kim, 2011). The lantibiotics typically have no antibacterial effect against Gram-negative bacteria, while conversely in closely related Gram-positive are most effective (Lee and Kim, 2011; Wiedemann et al., 2001). The lantibiotics are divided into two subclasses based on chemical structure and functionality. Subclass A: included peptides relatively elongated, flexible and positively charged, these act by disrupting cytoplasmic membrane forming pores of sensitive target species (McAuliffe et al., 2001). Subclass B: these peptides are more rigid, have a globular structure and they are either negatively charged or have no net charge. These act by interfering with essential enzymatic reactions of sensitive bacteria, like cell wall biosynthesis (McAuliffe et al., 2001; Lee and Kim, 2011; Bierbaum and Sahl, 2009).

Recently, the based on action mode of lantibiotics
sub-classification has been questioned, by the discovery that mutant variant of nisin can act through both mechanisms (pore formation and inhibition of cell wall biosynthesis), this due which nisin can bind of precursor protein wall, lipid II, and then proceed to pore formation (Wiedemann et al., 2001).

Class II. Non-lantibiotics, these bacteriocins are small (<10 kDa) and heat-stable peptides composed of 30 to 60 amino acids and do not contain Lan or MeLan (Deegan et al., 2006; López et al., 2008). Between these bacteriocins the chemical structure is very heterogeneous, and their sub classification is difficult. However, they can be sub classified in some groups. The subclass IIa: pediocin-like (or Listeria-active), is composed of 37 to 48 amino acid with a terminal portion, and contains one or two α-helix (Lee and Kim, 2011).

The subclass IIb: two-component bacteriocins. Pediocin-like peptides show a high degree of homology (40–60%), when they are aligned on corresponding amino acid sequences. In particular, the cationic N-terminal domain contains the homologous region pediocin box (YGNGVXCCCCXCV), with the two cysteine residues forming a disulphide bridge (Deegan et al., 2006; López et al., 2008). Class IIb refers to two-component bacteriocins that require two peptides in order to work synergistically. These peptides themselves have little or no activity, and there appear to be no sequence similarities between complementary peptides. Both lactacin F and lactococcin G are members of this subgroup (Deegan et al., 2006). The subclass IIc: these bacteriocins have a covalent union between C and N terminal portion, which result in a cyclic structure (Cotter et al., 2012).

### MODE OF ACTION

The bacteriocins act binding on specific receptors located on cell wall of target bacteria, after which several isolated or adjuvant mechanisms act to kill the bacteria. (Souza et al., 2005; Hernández-Dominguez et al., 2008). Bacteriocins are positively charged molecules with hydrophobic patches. Electrostatic interactions, with negatively charged phosphate groups on target cell membranes are thought to contribute to the initial binding with the target membrane (Hugas, 1998; Cleveland et al., 2001). Microbial cell killing due bacteriocin action could occur as the consequence of unbalanced cytoplasmic membrane function (affecting energy use and permeability), inhibition of nucleic acid synthesis, interference on the protein synthesis and changing cell translator mechanism. There are still some bacterial lineages that could suffer cell lysis (Basanta et al., 2009), especially lantibiotics, that inhibit target cells by forming pores in the membrane, depleting the transmembrane potential and/or the pH gradient, resulting in the leakage of cellular materials (Cleveland et al., 2001). Nisin and pediocin AcH are examples of bacteriocin owner of prominent antimicrobial spectrum, which are able to exert inhibitory activity on the growth of bacteria (Ramírez and Ulloa, 2011).

### USE IN MEAT

Nisin is one of the few bacteriocin that is used in the industry as a biopreservative. However, it has not yet been implemented in meat. Nisin can inhibit the growth of Gram-positive bacteria so they help reduce the contamination problem in meat. Also, the greatest interest in the use of bacteriocins is inhibition of meat pathogens (Devlieghere et al., 2004). Some

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**TABLE 1. Bacteria and bacteriocins produced.**

<table>
<thead>
<tr>
<th>Bacteriocin</th>
<th>Producer microorganism</th>
<th>Potential application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin (IA)</td>
<td>Lactococcus lactis</td>
<td>Food biopreservative and bovine mastitis prevention</td>
</tr>
<tr>
<td>Nukacin 3299 (IA)</td>
<td>Staphylococcus simulans</td>
<td>Prevention of streptococcal bovine mastitis</td>
</tr>
<tr>
<td>Mutacin, 1140 (IA)</td>
<td>Streptococcus mutans</td>
<td>Prevention of dental caries</td>
</tr>
<tr>
<td>Hyacin 3682 (IA)</td>
<td>Staphylococcus hyicus</td>
<td>Control of phytopathogens</td>
</tr>
<tr>
<td>Mersacidin (IB)</td>
<td>Bacillus spp.</td>
<td>Prevention and control of methicillin-resistant S. aureus</td>
</tr>
<tr>
<td>Lactacin 3147 (IC)</td>
<td>Lactococcus lactis</td>
<td>Bovine mastitis prevention</td>
</tr>
<tr>
<td>Pediocin PA-1 (Iia)</td>
<td>Pediococcus acidilactici</td>
<td>Food biopreservative</td>
</tr>
<tr>
<td>Aureocin A53 (IId)</td>
<td>Staphylococcus aureus</td>
<td>Prevention and control of bovine mastitis</td>
</tr>
<tr>
<td>Lysostaphin (IIla)</td>
<td>Staphylococcus aureus subsp.</td>
<td>Prevention and control of human and animal infections caused by S. aureus</td>
</tr>
<tr>
<td>Enterocin AS-48 (IV)</td>
<td>Enterococcus faecalis</td>
<td>Food biopreservative</td>
</tr>
</tbody>
</table>

(Adapted from Cleveland et al., 2001; Devlieghere et al., 2004; Bastos et al., 2015)
bacteriocins of genus *Lactobacillus* have an antimicrobial effect against varieties of *Salmonella*, such as Enteritidis, Heidelberg, Newport and Typhimurium, and can inhibit their growth for several days (Kim et al., 2015). The bacteriocin of *Pediococcus acidilactici* can inhibit *L. monocytogenes* in meat for a period of 28 days, under refrigeration conditions (Nielsen et al., 1990). Also, *Lb. sakei* 706 and BacFL31 bacteriocins besides inhibiting the growth, they presented a bioprotective effect avoiding a new colonization of *Listeria and Salmonella* (Schillinger et al., 1991; Chakchouk-Mtibaa et al., 2017). Otherwise, *B. thermosphacta* bacteriocin was used to avoid cross-contamination of meat with the plastic containers, reducing the pathogen population from log_{10} 7 to 3, after two days (Siragusa et al., 1999). Similarly, the bacteriocin application from spoilage meat bacteria, *Leuconostoc carnosum*, in the meat vacuum packaging is able to reduce the number of viable cells of pathogen at 21 days period (Budde et al., 2003).

Although, some studies have reported efficacy in use of pure bacteriocins, that is not the only use way. Other investigators have reported good results reducing viable cell of *Salmonella, E. coli, L. monocytogenes* and EHEC, using associations of bacteriocins with chelators, such as nitrates, citrates or EDTA (Cutter and Siragusa, 1995; Moon et al., 2002; Belfiore et al., 2007). And, combinations of other methodologies such as active-package, controlled atmosphere and HPP (Zhang and Mustapha, 1999). Though this in practice would increase the cost of meat preservation.

**MICROBIAL RESISTANCE**

Bacteriocin-producing strains generally are immune to their own antibacterial products through immunological systems, whose encoding genes are in bacteriocin gene cluster (Deegan et al., 2006). Furthermore, bacteriocins may also have cross-immunity, as some subclass Ila and subclass IIb, and lantibiotics such as Pep5 and epicidin 280. However, the cross-immunity for at least some bacteriocin subclass IIb depends on the receptor in the target cell (Ahmed and Shimamoto, 2015). Mechanisms involved in immunity are not known for most bacteriocins. In most immunological systems, self-protection depends on a single small protein (Bastos et al., 2015). These proteins have been detected either anchored to the membrane surface or embedded in the membrane (Ahmed and Shimamoto, 2015; Bastos et al., 2015). Some are largely exported, remaining trapped at the membrane, and within the cell wall compartment (Van Reenen and Dicks 2011). The roles proposed for these proteins include blocking the insertion of the bacteriocin into the membrane or protection of a specific target, shielding it from the bacteriocin (Bastos et al., 2015).

Another immunity mechanism reported for some class II bacteriocins depends on the activity of multi-drug transporter proteins, which participate in bacteriocin immunity by removing bacteriocin that enters the cytoplasmic membrane from the outside (Bastos et al., 2015). The frequency at which susceptible organisms develop resistance to a given bacteriocin is therefore a very important issue to consider when bacteriocin-based biocontrol strategies are proposed. Bacteriocins have not been extensively used in the clinical setting (Deegan et al., 2006; Bastos et al., 2015). Nisin has long been used as a food biopreservative, but nisin resistance has not been reported among food-spoilage microorganisms in the food industry (Deegan et al., 2006).

Therefore, understanding the potential for bacteriocin resistance development has been possible primarily from experiments performed under laboratory conditions (Deegan et al., 2006). Once a new preservative is found safe and effective, it is critical to ensure the longevity of its use by preventing the proliferation of resistant cells. Nowadays, cells exhibit resistance to several antibiotics and the transfer of resistance between microorganisms have been documented. Nonetheless, bacteriocins are not antibiotics, there is concern that exposure to bacteriocins will render cells more resistant to antibiotics (Hugas, 1998; Cleveland et al., 2001). There is substantial variation in the frequency of spontaneous mutations resulting in bacteriocin resistance, depending on the microorganism, the bacteriocin and the strain tested, and the assay used, including the bacteria/bacteriocin ratio as well as environmental conditions. The mechanisms involved in acquired resistance are also quite diverse (Bastos et al., 2015).

The frequency of nisin (100 IU mL\(^{-1}\)) resistance development in *L. monocytogenes* strain ScottA was found to drop (at least, 100-fold) when the strain was grown at 10 °C and in the presence of decreasing pH (5.5 and 6.0) and NaCl concentration (2.0 and 0.5%). Growth on NaCl (6.5%) at 30 °C also reduced the frequency of nisin (500 IU mL\(^{-1}\)) resistance development. Nisin resistance seems to have little impact on bacterial fitness, and the resistance seems to be stable in most mutants in the absence of selection. That indicates how some bacteria develop resistance to bacteriocins (Bastos et al., 2015).

**TOXICITY**

Bacteriocins have been consumed for a long time, principally through fermented food. The approval of some bacteriocins as nisin was based on data regarding its safety, not on the history of common use. Acute, subchronic, and chronic toxicity studies, as well as
reproduction, sensitization, in vitro, and cross-resistance studies showed that bacteriocins are safe for human consumption at an acceptable daily intake of 2.9 mg person$^{-1}$ day$^{-1}$ (Cleveland et al., 2001).

These substances to be used as food additives must fulfill such requirements some characteristics as: all proposed additives must be toxicologically tested and evaluated in all pertinent aspects, including accumulative, synergistic and potential effects. Only those additives considered safe at the intended level of insertion must be released to use; all food additives must be again evaluated when arisen new information about their use and safety. Food additives must be kept in conformity with specifications approved by Codex Alimentarius Commission. Justification of additive use must be based strongly in the requisites of food safety of different characters of consumers, and it also must present an economically and technically feasible alternative; the temporary or permanent approval of use of a food additive must consider the limitation for specific foods, the purpose, use conditions, decrease of necessary level to reach the desirable effects and the acceptable daily intake for human, and still must consider the probable intake of special consumers (Souza et al., 2005; Deegan et al., 2006).

Even though antimicrobial roles of bacteriocins have been recognized, their application in food conservation has still a rare application in food conservation. This fact is probably due to the absence of detailed studies of their particularities (Zhou et al., 2010). Among major studied bacteriocins are nisin, acidocin, bavaracin, curavaticin, and sakacin, although they are not still well characterized. Nisin is the single bacteriocin commercially used, as a biopreservative, and considered safe by World Health Organization, and has received the denomination of GRAS by Food and Drug Administration (Souza et al., 2005). Nisin or its combination with lower levels of nitrate can prevent the growth of Clostridium (Ávila et al., 2014). However, some researchers concluded that nisin is not effective in meat applications, due to high pH (Hugas, 1998; Cleveland et al., 2001; Zhou et al., 2010), and inability to be uniform distributed, as well as interference by meat components such as phospholipids; whilst other studies find contradictory results (Zhou et al., 2010).

**CONCLUSIONS**

LAB have inhibitory potential and represent an alternative to avoid spoilage and increase the shelf-life of raw meat. Native microbiome can be a good LAB reservoir to isolate bacteria with inhibition capacity. However, bad handling, bad use or meat microbiome variation can limit the use of live cultures, and these factors can be different among geographic regions and what could favor inappropriate results, such as lime, off-odors, off-flavor and color changes. Therefore, the best way to use as a biopreservative on meat is in the form of extracts or pure antimicrobial substances.

The bacteriocins can also be used as a biopreservative because it does not produce organoleptic changes in the meat and reduce the growth of deteriorating bacteria, and some can inhibit pathogens. But it is necessary to characterize and evaluated novel bacteriocins and develop efficient conservation methodologies to avoid resistance.

**CONFLICT OF INTEREST STATEMENT**

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

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