Bacteriocin activity and resistance in livestock pathogens

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Since their discovery in the first half of the 19th century, antibiotics have been extensively used in livestock production as therapeutic agents and growth promoters. In many countries, antibiotic therapy is still the first choice to combat microbial infections in livestock animals, and their efficacy and cost-effectiveness contribute to their popularity. Nevertheless, the continuous use of antibiotics has resulted in the emergence of multidrug-resistant microbial strains that no longer respond to antibiotic therapy. A number of strategies have been explored to control microbial pathogens and to improve growth and feed efficiency in livestock animals without the use of antibiotics. Among these, bacteriocins, probiotic micro-organisms and bacteriophages have been more extensively studied and proposed as potential alternatives to classic antibiotics in animal husbandry. Bacteriocins are antimicrobial peptides ribosomally synthesized by many species of Bacteria and some strains of Archaea. Many bacteriocins are active at small concentrations, and exhibit bactericidal or bacteriostatic activity toward sensitive cells. The main mechanisms of bacteriocin activity vary from pore formation in cytoplasmic membranes to the inhibition of cell wall biosynthesis and enzyme activities (RNAse or DNAse) in target cells. Nisin, the most well known bacteriocin, is a potential candidate for use in prophylaxis and control of pathogens associated with bovine mastitis, and promising results have been demonstrated in in vivo experiments. However, nisin-resistance is a phenotype that has been already demonstrated for some gram-positive bacteria, including Staphylococcus aureus, Streptococcus bovis and Listeria monocytogenes. The mechanisms that modulate the co-evolution between pathogens and resistance to antimicrobial peptides, although very important, are still limited and less documented than the resistance to antibiotics. Previous studies indicated that several mechanisms might be involved in the resistance phenotype, varying from physiological adaptations in a bacterial population, such as changes in membrane lipid composition, modulation of the cell surface hydrophobicity and net charge, to genetic modifications (mutations) in individual cells that contribute to select for stable phenotypes. Although some genes involved in nisin resistance have been identified, there is little evidence that bacteriocin resistance could be spread among sensitive bacteria.

Keywords: Nisin; Animal therapy; Mastitis; Cattle; Antimicrobial Peptides

1. Introduction

Antimicrobial peptides (AMPs) synthesized by many species of Bacteria and some Archaea members are known as bacteriocins. Bacteriocins exhibit bactericidal or bacteriostatic activity [1], and differ from each other especially in terms of size and mode of action [2]. Bacteriocin-producing bacteria belong to different systematic groups and occupy various ecological niches, such as soil, dairy products, meat products, fermented plant products and the gastrointestinal tract of warm-blooded animals. Bacteriocins usually function as anti-competitor compounds that allow the producer cells to compete with other micro-organisms in their natural environments, enabling bacteriocinogenic strains to invade complex and established bacterial communities. Alternatively, antimicrobial peptides can ensure the survival and perpetuation of bacteriocinogenic strains into an occupied niche [3-5]. Additional roles have been proposed for some bacteriocins produced by Gram-positive bacteria, such as chemical mediators in quorum sensing and communication molecules in bacterial consortia [6, 7].

The inhibitory spectrum of bacteriocins can be narrow and confined to closely related species, or it can be relatively broad, inhibiting a range of target organisms, including food-spoilage and pathogenic bacteria, such as Listeria monocytogenes, Bacillus cereus, Clostridium tyrobutyricum, methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococci [8, 9]. In general, bacteriocins act mainly by pore formation in target cell membranes, or by inhibiting cell wall synthesis or enzyme activities in the cytosol (RNAs or DNAs) [2].

Bacteriocins produced by Gram-positive and Gram-negative bacteria differ into several ecological and evolutionary aspects. In Gram-positive bacteria, the biosynthesis of bacteriocins is self-regulated and bacteriocin production is not a lethal event. In addition, the spectrum of antimicrobial activity is broader than the peptides from gram-negative species and bacteriocin release is controlled by specific regulatory mechanisms. In Gram-positive bacteria the gene clusters for bacteriocin production are generally organized in the chromosome and include genes encoding the pre-peptide, and proteins responsible for post-translational modifications, regulation, immunity and transport across the cytoplasmic membrane. In contrast, Gram-negative bacteria are often killed by bacteriocin production, the release of the peptide is controlled by common regulatory mechanisms (e.g. SOS system), and specific genes encoding proteins responsible for cell lysis are common [10].

Bacteriocins produced by lactic acid bacteria (LAB) are the most studied and promising bacteriocins produced by Gram-positive bacteria, especially because many LAB are considered “generally recognized as safe” (GRAS). Many bacteriocin-producing LAB, including the genera Lactobacillus, Lactococcus, Enterococcus, Streptococcus,
Pediococcus, Leuconostoc and Bifidobacterium, have been isolated from different food matrices, such as fermented dairy products, vegetables, fruits, meat and fish and also from the human and animal gastrointestinal tract [11].

The isolation and biochemical characterization of bacteriocins indicated that some of these antimicrobial peptides had mechanisms of action similar to ionophores and classical antibiotics, which stimulated the research for applications in livestock production.

2. Mode of action

Nisin, produced by Lactococcus lactis subsp. lactis, is the most well known bacteriocin produced by LAB. Nisin is a small (3.5 kDa), cationic and hydrophobic peptide containing five lanthionine rings, that belong to the lantibiotic class of bacteriocins. Nisin provides a paradigm for studies of lantibiotic structure, biosynthesis and mode of action of antimicrobial peptides, and is often referred to as the “prototypical” lantibiotic [12].

Discovered in 1928, nisin received GRAS status in 1988, being approved by the US Food and Drug Administration (FDA) for food applications [13]. In 1995, nisin (code E234) was authorized for food preservation in the European Union by Directive 95/2/EC. In 2001, the FDA affirmed nisin as GRAS for use as an anti-microbial agent on cooked meat and poultry products [14]. So far, nisin is the only bacteriocin which has been approved for use in over 50 countries as a food preservative [15].

Nisin has different antimicrobial activities based on both high-affinity targets and low-affinity membrane interactions [16]. Nisin binds with high affinity to the Lipid II molecule, a hydrophobic carrier for peptidoglycan monomers, using this compound as a specific receptor to integrate into the bacterial membrane and to form pores that increase membrane permeability; nisin-Lipid II interaction compromises the incorporation of precursor units, blocking the biosynthesis of bacterial cell wall [17-19]. Lipid II has also been recognized as the primary target for antibiotics (e.g. ramoplanin and vancomycin), and other bacteriocins, including mutacin 1140, pediocin, subtilin, galidermin, epidermin, mersacidin and bovicin HC5.

The interaction between nisin and Lipid II starts specifically with the high affinity binding between nisin’s N-terminus with the pyrophosphate from Lipid II, while the C-terminal region of the bacteriocin inserts into the cell membrane [20]. The interaction between nisin-Lipid II complexes with the cell membrane results in the formation of complexes that consist of several nisin and Lipid II molecules, which assemble further into larger complexes; the conversion of the large complexes into a pore requires the cooperative insertion of several nisin molecules into the lipid bilayer. The final pore structure is believed to have a stoichiometry of eight nisin and four Lipid II molecules [21]. The loss of membrane integrity caused by nisin’s pore-forming ability induces a passive efflux of small intracellular metabolites through the lipid bilayer. Because of the loss of ions (potassium, phosphate), amino acids and ATP, the proton-motive force is reduced or dissipated and the cell dies.

Independently of Lipid II binding, nisin can impair microbial membranes at micromolar concentrations [18] or displace cationic autolytic enzymes from their anionic binding sites in the target bacterial cell wall, resulting in premature lysis of nascent cell-wall septa [22]. Nisin can also promote the release of some enzymes, such as N-acetylmuramoyl-L-alanine amidase and N-acetylglucosaminidase, which hydrolyze the cell wall by binding to teichoic, teichuronic and lipoteichoic acids [23]. Nisin also inhibits the outgrowth of bacterial spores, by uncoupling the establishment of oxidative metabolism or membrane potential and the shedding of external spore structures [24].

Besides nisin, other lantibiotics have also been described and characterized. Mersacidin, produced by Bacillus HILL Y-85/54728, is a globular lantibiotic containing three lanthionine rings [25] that interacts with Lipid II in a region that is different from the binding site of vancomycin. Previous studies indicated that mersacidin interferes with the bacterial cell wall biosynthesis by inhibiting the transglycosylation reaction [17]. Lacticin 3147, produced by Lactococcus lactis, is the most studied of the two-component lantibiotics [26], and consists of two peptides, a compact A1 peptide and a flexible, elongated A2 peptide; the former has four lanthionine rings and the later comprises three rings and an a-ketoamide capped N terminus [27]. Both mersacidin and lacticin 3147 have potential to treat drug resistant micro-organisms, such as methicillin-resistant S. aureus [28, 29].

Bovicin HC5, an elongated lantibiotic produced by Streptococcus bovis HC5, contains post-translational modified amino acids residues and three putative lanthionine rings [30]. It is stable at high temperatures and at low pH, and has a broad spectrum of activity, including food-borne pathogens, such as Listeria monocytogenes [31, 32]. Bacterial adaptation has not yet been demonstrated and the primary mode of action of bovicin HC5 is based on the interaction with its specific target, the Lipid II molecule. However, the pore-forming activity of bovicin HC5 is dependent on the membrane thickness. Independent on the membrane thickness, bovicin HC5 maintains its antibacterial activity by binding to Lipid II molecules and quenches them into domains, inhibiting the bacterial cell wall biosynthesis [33].

Although the mechanism of action of many bacteriocins has not yet been fully elucidated, in vitro and in vivo studies indicated at least some of these antimicrobial peptides could be useful in livestock production.
3. Applications of bacteriocins in animal production

Over the years, bacteriocin research has been primarily moved by applications in food preservation and food safety. Over the last 20 years, more than 700 patents based on bacteriocins produced by LAB have been registered, and approximately 400 were linked to food preservation and to animal probiotics (http://www.freepatentsonline.com). However, many bacteriocins also show potential for biotechnological and agroindustrial applications. Some bacteriocins show desirable properties for in vivo application, such as stability to low pH and heat, simplicity for production and extraction and little, if any, inhibitory activity towards eukaryotic cells. Therefore, bacteriocins have been evaluated as the most promising class of antimicrobial peptides to be used as antibiotic substitutes in the field of animal and human medicine or for design and production of new antimicrobials [34]. Particularly on animal trials, bacteriocin and bacteriocin-producing bacteria may be useful to improve animal nutrition and health through the manipulation of ruminal fermentation, the control of animal infections and the inhibition of enteric pathogens [35].

Antibiotic therapy has been a valuable tool used in animal research, as growth promoters or therapeutic agents, and their efficacy and cost-effectiveness contribute to their popularity. Nevertheless, treatment of animals with antibiotics leads to antibiotic residue problems in the environment and veterinary products [36] as well as an increase in the frequency of resistance among bacterial species [37]. A number of strategies not dependent on antibiotics have been proposed to improve growth and feed conversion and novel strategies to reduce or eliminate animal pathogens have been tested by different research groups. The alternatives include bacteriocins, probiotic micro-organisms and bacteriophages [38, 39].

Bacteriocins produced by different Gram-positive bacteria have been tested both in vitro and in vivo. The peptides that have been tested for livestock production differ in their physicochemical characteristics (Table 1) and spectrum of activity, but preliminary studies indicated that they might be a potential and effective alternative to classical antibiotics used in animal husbandry.

<table>
<thead>
<tr>
<th>Bacteriocin</th>
<th>Class</th>
<th>Common amino acids</th>
<th>Mass (Da)</th>
<th>Net charge</th>
<th>Basic/Acidic/Hydrophobic residues</th>
<th>Half life</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin A</td>
<td>Lantibiotic, type A</td>
<td>CT</td>
<td>3516.78</td>
<td>+5</td>
<td>5/0/8</td>
<td>Mammalian: 20 h Yeast: 30 min E. coli: &gt; 10 h</td>
</tr>
<tr>
<td>Nisin Z</td>
<td>Lantibiotic, type A</td>
<td>CT</td>
<td>3493.74</td>
<td>+4</td>
<td>4/0/8</td>
<td>Mammalian: 20 h Yeast: 30 min E. coli: &gt; 10 h</td>
</tr>
<tr>
<td>Mersacidin</td>
<td>Lantibiotic, type B</td>
<td>CGT</td>
<td>1980.63</td>
<td>-1</td>
<td>0/1/5</td>
<td>Mammalian: 1.2 h Yeast: &gt; 20 h E. coli: &gt; 10 h</td>
</tr>
<tr>
<td>Lacticin A1</td>
<td>Lantibiotic, type B</td>
<td>CT</td>
<td>3449.32</td>
<td>0</td>
<td>2/2/8</td>
<td>Mammalian: 1.2 h Yeast: &gt; 20 h E. coli: &gt; 10 h</td>
</tr>
<tr>
<td>Lacticin A2</td>
<td>Lantibiotic, type B</td>
<td>T</td>
<td>3005.92</td>
<td>+2</td>
<td>2/0/8</td>
<td>Mammalian: 7.2 h Yeast: &gt; 20 h E. coli: &gt; 10 h</td>
</tr>
<tr>
<td>Bovicin HC5</td>
<td>Lantibiotic, type A</td>
<td>n.a</td>
<td>2449.14</td>
<td>n.a</td>
<td>n.a</td>
<td>n.a</td>
</tr>
<tr>
<td>Lichenin</td>
<td>unclassified</td>
<td>I</td>
<td>1413.68</td>
<td>-1</td>
<td>1/2/5</td>
<td>Mammalian: 20 h Yeast: 30 min E. coli: &gt; 10 h</td>
</tr>
<tr>
<td>Enterocin A</td>
<td>Class IIa</td>
<td>G</td>
<td>4851.38</td>
<td>+5</td>
<td>6/1/12</td>
<td>Mammalian: 7.2 h Yeast: &gt; 20 h E. coli: &gt; 10 h</td>
</tr>
</tbody>
</table>


n.a.: data not available

Modern husbandry systems usually involve large herds of young animals confined into limited spaces and fed similar diets. In order to maximize feed efficiency and maintain high levels of productivity, antimicrobial agents are often incorporated into animal feeds and water to improve feed digestion and prevent the occurrence of microbial diseases. Many substances affect animal performance indirectly, and the modification of the ruminal fermentation is suggested as the main effect of antimicrobials on ruminant animals.
3.1 Ruminal fermentation

The rumen is a complex ecosystem composed by a variety of bacteria, protozoa, fungi, archaea and viruses that have established a symbiotic relationship with the animal host. The ruminant animal does not secrete digestive enzymes in their forestomach, but the microbial communities that colonize the rumen produce hydrolytic enzymes required for fiber digestion and utilization of soluble and insoluble substrates. Because the rumen is an anaerobic environment, the microbes must diverted their reduction equivalents to metabolic intermediates in order to re-oxidize the pool of coenzymes. The volatile fatty acids produced during the fermentation (e.g. acetate, propionate and butyrate) are absorbed in the rumen epithelium and used as the main energy supply to the ruminant animal [40].

However, ruminal fermentation is somewhat inefficient and up to 11% of the feed energy can be lost when reducing equivalents could be used for propionate synthesis are diverted to methane production. Methane is a thermogenic gas related to global warming and intensive ruminant production is often considered to have a high environmental impact. Additionally, if ruminant diets are protein-rich and amino acid deamination is dissociated from carbohydrate metabolism, excess ammonia can be lost as urinary urea. This not only represents a loss of dietary protein to the ruminant but also has an environmental impact, especially due to the contamination of subsurface aquifers.

Growth promoters are used in cattle rations to manipulate the ruminal microbial community composition with the aim to alter ruminal fermentation and to increase feed efficiency. The inhibition of hydrogen-producing bacteria and amino acid deamination decreases the production of hydrogen (a precursor of methane) and the accumulation of ammonia, which increases the retention of energy and dietary protein by the ruminant animal [41]. Nisin was able to decrease the methane production in vitro, and combinations of this bacteriocin with nitrate have been reported to reduce methane emissions in sheep [42].

The inhibitory effect of nisin against common rumen anaerobes has also been demonstrated [43,44] and in vivo experiments indicated that nisin affected ruminal fermentation in a way similar to monensin, the most common ionophore used as feed additive in cattle rations [45].

Jalal and Laukove [46] introduced nisin into an artificial rumen system and detected some changes in fermentation parameters, such as an increase in hemicellulose degradation and acetate and propionate production, which contributed to the improvement of microbial balance in this environment. In experiments with ruminants, nisin applied with other additives also modified several ruminal fermentation parameters [42, 47, 48]. Kišidayová and co-workers [49] evaluated the influence of nisin and monensin against the prevalence of lactobacilli, enterococci, amylolytic streptococci, and E. coli in an artificial rumen. According to the authors, the population of enterococci and the growth of amylolitic streptococci were not influenced by the treatment with nisin and monensin. However, monensin had strong anti-protozoal effects, which contrasted with the stimulatory effects of nisin. Nisin also showed a tendency to decrease the populations of lactobacilli and the concentration of E. coli, while an increase of E. coli was observed upon treatment with monensin.

However, it should be noted that nisin is not a bacteriocin native from the rumen and preliminary results indicated that this peptide is not stable in the rumen environment [50, 51]. Nonetheless, other bacteriocins naturally produced by rumen bacteria have a similar spectrum of activity as nisin and appears to be stable in the rumen environment [52]. In this regard, bovicin HCS, a lantibiotic produced by Streptococcus bovis HCS, was isolated from the bovine rumen and shown to suppress the in vitro methane production by 50% [50] and adaptation was not observed. Similar results were reported when ruminal hyper-ammonia producing bacteria were tested for their sensitivity to bovicin HCS [52], which indicate that these antimicrobial peptides might have an ecological role in vivo.

3.2 Bovine mastitis

Bovine mastitis is a major disease in dairy cattle [53] and Staphylococcus aureus is one of the most frequently isolated pathogen implicated in clinical and subclinical mastitis infections [54]. Several bacteriocins have been tested against mastitis pathogens in vitro and positive results have been reported for in vivo studies performed with intramammary formulations containing bacteriocins.

The efficacy of therapeutic formulations containing nisin was assessed for the treatment of bovine mastitis, and considerable reduction in viability of S. aureus and E. coli were observed (3.9 and 4.2 log cycle, respectively) [55]. Wu and co-workers [56] demonstrated significant increase in cure rates of infections caused by S. agalactiae, S. aureus, and other mastitis pathogens (90.1%, 50% and 65.2%) when cows were treated with nisin Z, via intramammary administration; moreover, after 48 hours of treatment, no bacteriocin residue was detected in milk.

The addition of the two-component bacteriocin lactacin 3147 to teat seals also offered protection against mastitis infection in vivo [28], being effective to control mastitis in experimentally induced as well as in naturally infected animals [57]. Twomey and co-workers [58] evaluated a formulation containing lactacin 3147 in order to determine the concentration of bacteriocin required to reduce the incidence of mastitis. A dosage of 8,200 AU/g of teat seal was shown to reduce the incidence of mastitis in approximately 45%, when compared to control treatments (non treated animals). The therapeutic potential of intramammary infusions containing viable cells of L. lactis subsp. lactis DPC3147 (the producer of lactacin 3147) was evaluated in cows with sub-clinical and clinical mastitis. Animals treated
with *L. lactis* showed a rate of cure of 47 % and 35 % for sub-clinical and clinical mastitis, respectively, while the rate of cure in animals treated with the conventional antibiotics was 61 % [59].

Bacteriocins and BLIS produced by *Bacillus* species with a history of use in food and industry have also been assayed for potential applications in livestock. Mersacidin, produced by *Bacillus* HIL Y-85/54728, showed strong antimicrobial activity against *S. aureus* both in vitro and in vivo [29, 60]. Barboza-Corona and co-workers [61] assessed the in vitro antimicrobial activity of several bacteriocins produced by *Bacillus thuringiensis* (morrincin 269, kurstacin 287, kenaycin 404, entomycin 420 and tolworthcin 524) against *S. aureus* strains obtained from cows diagnosed with mastitis. Despite the differences in sensitivity, all *S. aureus* strains showed susceptibility to the bacteriocins tested, and morrincin 269 and kurstacin 287 showed the greatest inhibitory activity against the *S. aureus* strains tested.

These in vitro and in vivo experiments indicate that bacteriocins are useful to control mastitis pathogens in dairy cattle and could also be used to prevent other bacterial infections in livestock animals. However, further studies are needed to address the stability of these antimicrobial peptides in complex ecosystems (e.g. the gastrointestinal tract) and to investigate the development of resistance among target bacteria. Moreover, very little toxicological data is currently available for bacteriocins suggested for use in animal production, but the safety of these antimicrobial agents against eukaryotic cells must be determined.

### 3.3 Probiotics

Some *Bacillus* strains can also be used as probiotics in livestock to inhibit pathogenic bacteria, to improve the health status and the performance of farm animals and poultry (e.g. BioPlus2B, containing a mixture of *B. licheniformis* and *B. subtilis* strains) [62]. *Bacillus licheniformis*, a strain that produces the bacteriocin lichenin, was reported to inhibit the growth of *Streptococcus bovis* and *Eubacterium ruminantium*, and was shown to have polysaccharide-hydrolytic activities [63].

General strategies have been proposed to reduce or eliminate *Campylobacter* contamination in poultry production systems, and bacteriocins have been proposed as an alternative agent in the on-farm control of *Campylobacter* [64]. The prevention and control of *C. jejuni* in the poultry reservoir is an important issue in the control of human campylobacteriosis [65]. Several commensal bacteria active against *Campylobacter* have been isolated from chicken intestinal tract and potent bacteriocins have been purified and characterized, such as SRCAM 602 from *Paenibacillus polymyxa* [66, 67], OR-7 from *Lactobacillus salivarius* [68], and E-760 and E 50–52 from *Enterococcus* spp. [69, 70]. Previous reports indicated that the oral administration of these bacteriocins reduced *C. jejuni* colonization in poultry, decreasing the risk of campylobacteriosis in humans [69].

The administration of bacteriocin-producing *B. circulans* NRRL B-30644 and *P. polymyxa* strains (NRRL B-30507, NRRL B-30508 and NRRL B-30509) were also used to control *Campylobacter jejuni* in animals carrying zoonoses [67]. *B. amyloliquefaciens* CECT 5940, that produces a BLIS, was used as a probiotic in poultry systems and reduction of pathogenic bacteria, such as *C. perfringens*, *E. coli* and *Yersinia*, was observed [71].

Strompfova et al. [72] described the influence of a BLIS produced by *Enterococcus faecium* EF55 on the microbiota of the gastrointestinal tract of Japanese quails. After 24 hours, a single treatment with crude extracts containing BLIS reduced the level of fecal colonization by *Escherichia coli*, *Enterococcus*, *Staphylococcus*, and *Lactobacillus* when compared to the control groups. In another study, Laukova et al. [73] investigated the effect of enterocin A, produced by *Enterococcus faecium* EK13, on the development of *Salmonella* infection in gnotobiotic Japanese quail, and a reduction of the colonization by *Salmonella enterica* in the cecum and ileum was observed when enterocin A was used therapeutically.

### 4. Resistance

Despite the promising results obtained with the use of bacteriocins, the large scale application of antimicrobial peptides remains limited due to the lack of data regarding the stability in different pH values, the destiny of the bacteriocin after ingestion, the loss of antimicrobial activity, the cytotoxicity and immunogenicity of the peptide [74]. Moreover, bacteriocin resistance has also been identified and should be addressed for future regulatory approval and public acceptability of the use of bacteriocins [75].

The issue of resistance to bacteriocins is less documented than for conventional antibiotics, probable because of the mode of action of the bacteriocins. Unlike antibiotics that act on a specific, high-affinity target, most bacteriocins act in different ways to inhibit or kill sensitive bacteria [76], and this complex mode of action is not favorable for the development of bacterial resistance [77-79]. The mechanism of bacteriocin resistance appears to be complex and probably involves various structural and physiological changes in the bacterial cell envelope [2, 34, 80, 81].

The selection of the resistant phenotypes can limit the use of antimicrobials, including bacteriocins. In the presence of antimicrobial compounds, it is observed that some micro-organisms can adapt to these substances, however the current knowledge regarding the mechanisms that modulate the co-evolution between pathogen and the acquisition of bacteriocin resistance, remains very limited and is much less documented than the resistance to classical antibiotics.
Thus, understanding the mechanisms that govern these adaptations is essential for the development of new strategies to control current and emerging pathogens [81].

Among the bacteriocins with potential applications in livestock animals, nisin is the only peptide to which mechanisms of resistance have already been studied to some extent in different target organisms. Even though nisin resistance seems to be the result of multiple factors, at least some physiological and molecular mechanisms appear to occur among different nisin-resistant phenotypes.

Nisin resistance has been reported for several species of bacteria, including Bacillus cereus [82], Lactobacillus casei [83], Listeria innocua [84], Listeria monocytogenes [85, 86], Clostridium botulinum [87], Pediococcus acidilactici [88], Streptococcus bovis [89], Streptococcus thermophilus [90], Lactococcus lactis [91] and S. aureus [92, 93]. The nisin resistance phenotype (NisR) is a complex phenomenon, which results from modifications in cellular components and metabolism. Alterations in the cell envelope have been pointed out as the main mechanism for bacteriocin resistance in bacteria, however the exact molecular mechanism involved is not yet been fully explained.

Figure 1 compile information regarding reported phenotypes of nisin resistance. Nisin typically goes through the cell wall of nisin-sensitive cells and reaches the cytoplasmic membrane (probably via electrostatic interactions with a net negatively charged surface). Nisin molecules then interact specifically with lipid II, disrupting membrane integrity by pore formation. In the NisR cells, the interaction between nisin and the cell envelope can be hampered by several phenotypes, such as changes in phospholipids and fatty acid composition, decrease in negative surface charge, changes in cell hydrophobicity, cell wall thickening, extracellular nisin degradation, differential gene expression and mutations.

Nisin is a cationic compound with a hydrophobic portion; therefore, reduction of the cell wall hydrophobicity and negative surface charge can prevent the interaction between the bacteriocin and the cell surface (Fig. 1A and 1B). Streptococcus bovis, a bacterium frequently associated with ruminal acidosis in animals fed high grain diets quickly developed resistance to nisin when exposed to sub-lethal doses of the bacteriocin. Nisin-resistant S. bovis JB1 cultures were less negatively charged, had lower hydrophobicity and were more lysozyme resistant than nisin-sensitive cultures. S. bovis JB1 cultures had more lipoteichoic acids (LTA) that migrated more slowly through a polyacrylamide gel compared to the LTA of sensitive cultures, which indicated that changes in teichoic acids could increase the resistance of S. bovis to nisin [89]. Changes in hydrophobicity and surface charge related to the resistant phenotype have also been reported for S. aureus [92, 94, 95] and L. innocua [84].

The teichoic acids have an important role in controlling cell shape, cation balance and the activity of autolytic enzymes. Teichoic acids also contribute to a net negative charge of the cell wall, which facilitates the interaction with positively charged compounds [96, 97]. The reduction in negative charge surface appears to be an important feature in the resistance phenotype to bacteriocins and other cationic compounds [89, 94]. The change in surface charge in vivo seems to be mediated by D-alanylation, a process where D-alanine is incorporated into cell wall teichoic acids. This process is mediated by proteins encoded by the dltABCD operon [94, 95, 98, 99]. Inactivation of the dlt operon reduced the occurrence of the NisR phenotype among S. aureus [94], L. lactis [100] and B. cereus cells [99]. In addition, the content of D-alanine incorporated into the teichoic acids was higher in L. lactis NisR [101].

Removal of the cell wall from L. monocytogenes NisR resulted in the loss of nisin resistance, suggesting that differences in cell wall composition contributed to resistance (Fig. 1E) [85]. The L. innocua NisR showed altered susceptibility to antibiotics and enzymes that target the cell wall, indicating that the cell wall plays an important role in nisin resistance. L. innocua NisR also showed cell wall thickening [84], a characteristic that has also been observed in S. thermophilus NisR [90]. Kramer et al., studying nisin resistance in spheroplasts of Micrococcus flauus and L. monocytogenes suggested that cell wall alterations may prevent the access of nisin to Lipo II, its primary target in sensitive bacteria [102].

Changes in membrane composition were reported for variants NisR of L. monocytogenes that decreased the content of anionic phospholipids (phosphatidylglycerol and cardiolipin) (Fig. 1C). Because the cell membrane became less negatively charged, the ability of nisin to form pores also decreased [103]. Similar results were reported for nisin-resistant L. monocytogenes ScotA cultures. The NisR cells had higher percentage of straight-chain fatty acids and lower percentage of branched chain-fatty acids and these modifications were consistent with a less fluid cytoplasmic membrane, which reduced nisin’s ability to insert into the cell membrane (Fig. 1D) [104].

Nisin degradation was one of the first mechanisms to be suggested for nisin resistance in bacteria [82], but later studies did not support this idea. More recently, the proteolytic degradation of nisin was described in L. lactis as being mediated by a nisin resistance protein (NSR), coding by the nsp gene localized in the 47 kb plasmid pTSS0. In vitro, the NSR protein inactivated nisin by removing six amino acids residues from its carboxy-terminal portion [91]. This truncated nisin molecule had reduced affinity for the cell membrane and decreased ability to form pores. It was demonstrated that the NSR protein was localized at the cell membrane but the exactly mechanism of nisin resistance by NSR protein in vivo is still poorly understood (Fig. 1F) [91].

Another issue investigated is the differential expression of genes related to the microbial metabolism. Transcriptome analyses of L. lactis NisR revealed that 92 genes were differentially expressed in DNA microarrays compared to nisin sensitive cells. From these, 62 genes were more highly expressed in NisR cells and encoded proteins that were involved in a plethora of mechanisms, including cell wall biosynthesis, energy metabolism, fatty acid and phospholipids metabolism, regulatory functions, stress-related proteins and nutrient transport (Fig. 1G) [98]. However, a more recent
work indicated that the *L. lactis* Nis\(^R\) phenotype might be primarily related to cell wall alteration. These alterations included thickening of the wall at the cell division sites and increased concentration of D-alanyl esters and galactose in lipoteichoic acids [101].

Recently, DNA mutation has been linked to the nisin resistant phenotype. In *S. aureus*, mutation of the putative gene SAOUHSC\_02955 appears to contribute to the resistance phenotype. Bioinformatics analyses revealed that the SAOUHSC\_02955 gene encodes a sensor histidine kinase, which was designated as the “nisin susceptibility associated sensor (*nsaS*)”. How *nsaS* participates in nisin resistance is yet to be determined, but regulation of several genes is probably involved (Fig. 1H) [93].

Because several pathogenic and commensal bacteria quickly develop and maintain stable nisin resistance phenotypes *in vitro*, the efficacy of nisin in livestock production might be compromised. However, many other bacteriocins have been isolated and characterized from the livestock environment and at least some of these peptides seem to be more stable and less susceptible to resistance. Further studies are needed to determine the occurrence of bacteriocin resistance *in vivo* and the combined effect of bacteriocins and other antimicrobial agents might be useful to improve their efficacy and safety in animal husbandry. Because bacteriocins are usually degraded by proteolytic enzymes, it is anticipated that little, if any, residues will be present in agricultural products as a consequence of bacteriocin utilization. Moreover, there is little indication that bacteriocin resistance can be transferred by horizontal gene transfer, a feature highly desirable for antimicrobial agents used for therapeutic and prophylactic purposes.

![Fig. 1 Mechanisms of nisin resistance in bacteria.](image)

A scheme showing the mechanism of nisin resistance of. Nisin disrupts membrane integrity through pore and prevent peptidoglycan synthesis due to sequestration of lipid II (not shown). Letters A and B indicate the primary alterations observed in Nis\(^R\) variants. In some cases, more than one kind of alteration has been reported for the same bacterium. A) net surface charge alteration; B) changes in hydrophobicity; C) changes in phospholipid composition; D) changes in membrane fatty acid composition; E) cell wall thickening; F) proteolytic degradation of nisin; question mark indicates that the mechanism of nisin degradation *in vivo* by NSR protein is not known; G) differential gene expression; H) DNA mutation. WTA - Wall Teichoic Acid and LTA - Lipoteichoic Acid. See text for details.
Acknowledgements

The support by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasilia, Brazil) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasilia, Brazil) to this work is gratefully acknowledged.

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