

## Bacteriocins: Nomenclature, detection, mechanism of action and potential use in poultry production

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### Abstract

The indiscriminate use of antibiotics as growth promoters might alter the effectiveness of products of competitive exclusion, minimizing the efficacy of protection in newborn poultry. Subtherapeutic doses of these chemotherapeutic agents administered almost throughout the life of fowls have some counter indications due to the eventual development of resistant bacterial populations. On the other hand, probiotics do not leave residues in products of animal origin or develop bacterial resistance because they are essentially natural, thus benefiting all segments of the animal production chain. Consumers of the 21st century are interested in foods with additional health benefits and those that contribute to the prevention of illnesses. The action of probiotics mainly lies in the inhibition of intestinal colonization by pathogenic bacteria such as *Salmonella* spp. Bacteria to be used as probiotics are selected based on the evaluation of their metabolites, colonization potential and multiplication capacity. Many probiotic bacteria produce substances that inhibit or kill intimately related species or even different strains of the same species. These substances, called bacteriocins, consist of an ample and diverse group of antimicrobial proteins, which differ from antibiotics since they are peptides and their structural genes are frequently found on plasmids and transposons. Until the last decade, research regarding bacteriocins was centered on those derived from Gram-negative bacteria, mainly colicin-producing *Escherichia coli*. However, although these bacteria are undesirable in food, studies on colicins have been important because they have permitted the development of the basic methods currently used for the detection and isolation of other classes of bacteriocins produced by Gram-positive bacteria (lactic acid bacteria). At present, bacteriocins produced by lactic acid bacteria call particular attention because of their potential application to the food industry as natural antimicrobial substances for food preservation and in probiotics for use in poultry production.

**Key words:** Bacteriocins, probiotics, chickens, lactic acid bacteria, *Salmonella* spp., *Lactobacillus* spp., inhibition and exclusion competitive.

### Introduction

Over the last few decades, industrial poultry rising has demonstrated a constant technical evolution based on its high competitiveness in market acquisition compared to other agricultural activities because it offers low-cost, healthy and highly nutritious products. The repeated and abusive application of commercial antibiotics as growth promoters using subtherapeutic doses almost throughout the animal's life has raised concern in various countries. Within this context, a new concept of additive has emerged which could replace antibiotics in the production of broiler chickens without causing damage to the normal intestinal microbiota and leaving no residues in animal carcasses<sup>1</sup>. These products containing beneficial bacteria are called probiotics and consist of live microorganisms able to balance the intestinal microbiota, thus having beneficial effects on the host's health<sup>2</sup>.

Several lactic acid bacteria such as *Lactobacillus*, *Pediococcus*, *Bacteroides*, *Bifidobacterium* and *Enterococcus* have been used as probiotics alone or in combination<sup>3</sup>. These and other bacteria are found in different quantities throughout the segments of the gastrointestinal tract of fowls. The efficacy of the spectrum of action of lactic acid bacteria against pathogenic microorganisms is determined by the production of metabolites with an antimicrobial action, including organic acids such as lactic and acetic acid<sup>4</sup>, hydrogen peroxide which inhibits the growth of pathogens based on a strong oxidant effect on bacterial cells<sup>5</sup> or

molecular destruction of nucleic acids and cell proteins<sup>6</sup> and the production of specific proteins or protein complexes called bacteriocins.

Since lactic acid bacteria occur naturally in almost all fermented products, their bacteriocins can be easily accepted as additives by inspecting agencies and mainly by consumers<sup>7</sup>. The sensitivity of bacteriocins to proteolytic enzyme degradation is highly interesting in terms of food safety, since their ingestion does not cause alterations in digestive tract ecology, with these substances thus not posing the same risks associated with antibiotic use<sup>8</sup>.

### Bacteriocins

The first record of bacteriocins dates back to 1925, when André Gratia demonstrated that an inhibitory substance, today known as colicin, produced by *Escherichia coli*, had a bactericidal activity against other *E. coli* strains<sup>9</sup>. Colicins constitute a diverse group of antimicrobial proteins that inhibit bacterial growth through the inhibition of cell synthesis, permeabilizing the cell membrane or inhibiting RNase or DNase activity<sup>10</sup>.

According to Tagg et al.<sup>11</sup>, the term bacteriocin refers to proteins with the following characteristics: a narrow inhibitory action spectrum, the essential presence of a biologically active protein fraction, a bactericidal mode of action, binding to specific cell receptors and plasmid genetic information that determines the

production and the immunity of bacteriocin's target cells, for the production and target cell immunity of bacteriocins is located on plasmids. This definition was based on colicin-type proteins, which have been used as a model system for the investigation of the general ecology of bacteriocins<sup>12</sup>. However, studies have demonstrated that few antagonistic substances, especially those produced by Gram-positive bacteria, adequately fit the classical definition of bacteriocins produced by Gram-negative bacteria<sup>11</sup>. Sahl and Bierbaum<sup>13</sup> and Jack et al.<sup>14</sup> demonstrated that the genetic organization of bacteriocins produced by Gram-positive bacteria highly differs from that of colicins. There are typically eight to 12 genes compared with two to three genes required for colicin.

Montville and Kaiser<sup>15</sup> suggested that the definition of bacteriocins should be based on only two basic requisites, i.e. their protein nature and the absence of lethality to the producer cells. These authors confirmed that few antimicrobial proteins fit the classical definition proposed by Tagg et al.<sup>11</sup>.

### **Bacteriocins Produced by Lactic Acid Bacteria**

Among Gram-positive bacteria, lactic acid bacteria have been widely explored and found to be a large reservoir of antimicrobial proteins that show a "natural" effect on food preservation<sup>10</sup>. Other advantages are that these proteins are non-toxic, easily digestible, leave no residues in foods and are resistant to heat and acidity, and can therefore be used to increase the safety and lifetime of many foods<sup>16</sup>. They are biologically active proteins that show antimicrobial properties against species intimately related to the producer organism<sup>17,18</sup>.

Bacteriocins produced by lactic acid bacteria are generally small heterogenous cationic proteins consisting of 30 to 60 amino acid residues with a high isoelectric point, which vary markedly in terms of producer microorganism, spectrum of action, molecular weight and biochemical properties<sup>19,20</sup>.

Nisin, produced by some strains of *Lactococcus lactis* subsp. *lactis*, currently is the best characterized bacteriocin. Discovered in the 1920s and classified as a 34-amino acid lantibiotic, nisin only inhibits the multiplication of Gram-positive bacteria, including *Listeria monocytogenes*. It is the only bacteriocin produced commercially and legalized for use in foods<sup>20-22</sup>, being considered non-toxic because it is rapidly inactivated by  $\alpha$ -chymotrypsin, an enzyme produced in the pancreas and released into the small bowel.

Many other bacteriocins produced by lactic acid bacteria are still being characterized<sup>23-25</sup>. *Lactobacillus reuteri*, a natural inhabitant of the gastrointestinal tract of humans and animals including fowls, is able to synthesize and secrete an antimicrobial substance called reuterin which shows an antagonistic action against Gram-negative and Gram-positive bacteria, yeast, fungi, protozoa and viruses<sup>26,27</sup>. Some strains of *Lactobacillus salivarius* produce salivaricin B which shows a broad spectrum of action, inhibiting the growth of *Listeria monocytogenes*, *Bacillus cereus*, *Brochothrix thermosphacta*, *Enterococcus faecalis* and *Lactobacillus*<sup>24</sup>.

**Nomenclature:** The nomenclature of bacteriocins in general is subjective because it is based on the addition of the suffix "cin" to the genus or species name to denote bacteriocinogenic activity. Whereas some bacteriocins receive their denomination based on the species (plantaricin, sakacin, caseicin) or genus name (lactococcin, lactocin, pediocin) of the producer microorganism,

other bacteriocins produced by different *Lactococcus* species are generically called lactostrepcin, nisin and diplococcin. Sequential letters placed in the order of discovery are used after the name of the bacteriocin for the differentiation of those produced by different strains of the same species, such as lactacin F which refers to the sixth bacteriocin reported for a *Lactobacillus* species<sup>11, 15, 28, 29</sup>.

According to Tagg et al.<sup>11</sup>, the most adequate procedure would be to call bacteriocin-like inhibitory substances or BLIS antimicrobial substances that are still not completely characterized, and bacteriocins those showing a protein nature and bactericidal action. McCormick and Savage<sup>30</sup> also suggested the same term for all inhibitory substances of a protein nature.

At times, the nomenclature for bacteriocins is contradictory, since the same species might produce more than one bacteriocin, and in this case they can be designated by the addition of consecutive letters of the alphabet. For a more precise specification of a bacteriocin, in addition to its usual designation, the name of the producer strain should be added<sup>11, 31, 32</sup>.

Another factor that makes the nomenclature of bacteriocins even more difficult is that there are at least six examples of independently isolated bacteriocins which received different denominations, but which were later shown to be same substance based on their amino acid sequences. The confusion created by many different names for the same molecule can impair the development of studies<sup>32</sup>. Jack et al.<sup>14</sup> proposed that a new name should only be assigned to a bacteriocin after identification of the amino acid sequence proves that it is indeed a new bacteriocin.

**Classification:** Four general classes of antimicrobial proteins have currently been recognized and are divided according to their structural and physicochemical properties: class I bacteriocins, called lantibiotics, which are characterized by the presence of rare amino acids in their chemical structure such as lanthionine,  $\beta$ -methyl lanthionine and dehydrated residues. Class II, the largest group of bacteriocins, is divided into three subclasses, i.e., anti-*Listeria* peptides containing an YGNGV amino acid sequence close to the N-terminal portion, pore-forming peptides, and thiol-activated peptides which require cysteine residues for their activation. Classes III and IV comprise heat-labile proteins with a molecular weight higher than 30 kDa and complex bacteriocins consisting of a protein fraction and one or more functional groups required for activity<sup>33</sup>.

An alternative system based on the presence of sulfhydryl groups has been proposed by Jack et al.<sup>14</sup>, in which bacteriocins containing lanthionine rings are called lantibiotics, those containing disulfide bonds are designated cystibiotics, and those requiring sulfhydryl groups are called thiolbiotics. The research area of bacteriocins is very dynamic and, according to Montville and Winkowski<sup>32</sup>, more time is necessary for the creation of a definitive classification system.

Genetic and biochemical studies of bacteriocins have been conducted mainly for members of classes I and II due to the potential commercial use of these peptides<sup>34</sup>.

**Mechanism of action:** The mechanism of action of bacteriocins occurs in two distinct phases. The first phase consists of adsorption of the bacteriocin to specific and nonspecific receptors on the cell membrane of the target bacterium. During this phase,

the bacteriocin is sensitive, for example, to proteolytic enzymes. The second phase is irreversible and involves lethal changes in the sensitive strains. The idea that bacteriocins act on the cell membrane has been well accepted, but the exact mechanism involved is still unclear<sup>35</sup>.

Bruno et al.<sup>36</sup> and Bruno and Montville<sup>17</sup> have demonstrated that one of the common mechanisms of action is collapse of the proton motive force. This mechanism resembles that described for other antimicrobial proteins of Gram-negative bacteria, such as colicins and defensins. Identification of common characteristics in these peptides is important to better understand their mode of action. Most bacteriocins are cationic and amphiphilic substances with an  $\alpha$ -helix,  $\beta$ -sheet or screw-like secondary structure<sup>32,35</sup>. On the basis of the amphiphilic characteristics of these molecules, at least two mechanisms can be proposed that would permit membrane permeabilization, i.e., the formation of a complex with membrane components, leading to pore formation, or destabilization of cytoplasmic membrane integrity with a detergent-like effect. However, no receptor or region in the bacteriocin molecule that would act as a receptor-binding site has been identified thus far. *In vitro* studies using lipid membranes have provided evidence that the mechanism of action most probably involves pore formation<sup>35</sup>.

Lantibiotic bacteriocins act on energized membranes and do not require protein receptors for their action. For example, nisin is active against a variety of microorganisms, including strains of *Lactococcus*, *Streptococcus*, *Staphylococcus*, *Listeria* and *Mycobacterium*, as well as spore-producing vegetative cells of *Bacillus* and *Clostridium*<sup>37,38</sup>. On the other hand, most non-lantibiotic bacteriocins act on non-energized membranes and may require a protein receptor<sup>32</sup>.

The outer membrane of Gram-negative bacteria acts as a cell permeability barrier, but the presence of chelating agents, hydrostatic pressure or cell damage might render this group of microorganisms sensitive to bacteriocins. In many cases, sensitivity is also due to membrane rupture<sup>21</sup>.

**Biosynthesis conditions:** Various environmental conditions, including pH, temperature, aeration, sugar concentration, buffering capacity of the medium and time of incubation, affect the production of bacteriocins<sup>9</sup>.

Desmazeaud<sup>39</sup> suggested that the biosynthesis of bacteriocins occurs at the end of the exponential phase of bacterial growth. These molecules are then excreted into the culture medium, but might remain bound to some bacteria, as is the case of nisin. The fact that nisin only starts to be synthesized after half the biomass is formed suggests that its biosynthesis is mediated by a multienzyme system that transforms a 57-amino acid ribosomal pre-nisin into the active nisin consisting of 34 residues.

On DeMan-Rugosa-Sharpe (MRS) medium supplemented with Tween 80, glucose, peptone, yeast extract and a pH ranging from 6.5 to 7.5, *Lactobacillus casei* produces the highest concentration of lactacin 705<sup>40</sup>. Brink et al.<sup>24</sup> observed that the production of acidocin B, a bacteriocin produced by *Lactobacillus acidophilus* M46, was eight times higher when the bacteria were cultured in 5-fold concentrated MRS broth at pH 5.5 compared to bacteria grown in normal MRS broth without pH control. The production of acidocin B depended less on bacterial growth than on the presence of sugar, amino acids and vitamins, which were essential

for the biosynthesis of this bacteriocin.

Mortvedt-Abildgaard et al.<sup>41</sup> observed a higher bacteriocin activity at the beginning of the stationary growth phase at 30°C. When *Lactobacillus sake* was cultured at pH 5, production of 2000 to 3000 arbitrary units of bacteriocins per mL was observed, corresponding to an 8- to 10-fold increase compared to the culture fermented without pH control. Fermentation at pH 6 produced less than 10% bacteriocins.

According to Tagg et al.<sup>11</sup>, the physiological status of the sensitive strain has a great influence on its susceptibility to the lethal action of bacteriocins, with metabolically active cells being more sensitive. In this respect, cells in the exponential growth phase were found to be more sensitive to lactacin 481<sup>42</sup>, lactostrepcin 5<sup>43</sup>, nisin<sup>44</sup> and diplococcin 3466<sup>45</sup>.

**Methods for the detection of bacteriocins:** Several methods for the study of bacteriocins are available, which are based on the demonstration of their antagonistic activity<sup>11</sup>. The simplest and most commonly employed method used to initially test a culture for bacteriocin production consists of the growth of the producer bacteria on agar overlaid with another layer of agar containing the sensitive organism. Other methods are based on the turbidity of an indicator culture when grown in the presence of different bacteriocin concentrations, on the release of cell material that absorbs ultraviolet light, on the ability of survivors (indicator culture) to cause a reduction in the indicator dye<sup>11</sup>, or even, as proposed recently by Giraffa et al.<sup>46</sup>, based on conductance measurements. It is important to remember that inhibition can be caused by other factors such as the production of organic acids or the presence of bacteriophages, hydrogen peroxide or other nonspecific inhibitors. Thus, additional experiments are necessary to exclude an influence of these factors<sup>32</sup>.

Lewus and Montville<sup>47</sup> analyzed techniques for the detection of bacteriocins produced by lactic acid bacteria based on the diffusion of bacteriocins through solid or semisolid culture medium, inhibiting the growth of a sensitive organism. In the flip-streak method (inoculation and flipping of agar), supposedly bacteriocin-producing bacteria are streaked onto an agar plate and incubated, and the bacteriocin-sensitive organism is streaked perpendicularly on the opposite side of the plate. In the spot on the lawn method, the test organism is seeded in point-form on an agar plate and after incubation the resulting colony is overlaid with an agar layer containing the sensitive indicator microorganism. Another assay used is the well-diffusion method, in which holes are cut into the agar seeded with the sensitive (indicator) organism and filled with the culture supernatant of the test organism. After a period of incubation, inhibition zones can be observed around the wells, thus demonstrating bacteriocin-mediated inhibition of the sensitive organism.

The well-diffusion method consists of the simultaneous growth of producer strains such as *Lactobacillus* spp. and indicator (sensitive) strains such as *Salmonella*, *Listeria* and *Lactobacillus*, aimed at the simultaneous growth of producer and indicator strains and the subsequent production and diffusion of bacteriocins, thus demonstrating that the antagonism observed based on the inhibition zone depends on an inhibitor produced immediately at the beginning of growth of the indicator microorganism. Bacteriocin-producing strains of *Lactobacillus reuteri*, *Lactobacillus salivarius* and *Lactobacillus* spp. isolated from

**Table 1.** Action of bacteriocins produced by lactic acid bacteria against indicator microorganisms determined by the well-diffusion method.

Producer strain	Origin	Indicator microorganism	Inhibition	Reference
<i>L. reuteri</i>	Cecum/chicken	<i>S. typhimurium</i>	Positive	Lima <sup>50</sup>
<i>L. salivarius</i>	Cecum/chicken	<i>S. enteritidis</i> phag.4	Positive	Lima <sup>50</sup>
<i>L. salivarius</i>	Cecum/chicken	<i>E. faecalis</i>	Positive	Lima <sup>50</sup>
<i>L. reuteri</i>	Crop	<i>L. monocytogenes</i>	Positive	Lima <sup>50</sup>
Lactic acid bacteria	Cheese	<i>S. typhimurium</i>	Positive	Alexandre et al. <sup>51</sup>
Lactic acid bacteria	Meat	<i>L. monocytogenes</i>	Positive	DeMartinis et al. <sup>52</sup>
Lactic acid bacteria	Sausage	<i>L. helveticus</i>	Positive	Prado et al. <sup>53</sup>
<i>L. sake</i>	Sausage	<i>S. typhimurium</i>	Positive	DeMartinis and Franco <sup>54</sup>
<i>L. salivarius</i>	Wako-Japan	<i>L. helveticus</i>	Positive	Arihara et al. <sup>55</sup>
<i>L. acidophilus</i>	Feces	<i>L. helveticus</i>	Positive	Toba et al. <sup>56</sup>

the crop and cecum of chickens have demonstrated an antagonistic action against Gram-positive and Gram-negative indicator microorganisms (Table 1).

The presence of bacteriophages as well as organic acids and hydrogen peroxide can be responsible for the inhibition of sensitive strains. Tagg and McGiven<sup>48</sup> demonstrated that one way to prevent diffusion of bacteriophages is to seed the indicator (sensitive) culture on the side of the agar opposite to the side where the producer strain was seeded. To exclude inhibition due to the production of organic acids, the culture medium should be prepared without glucose or any other fermentable sugar<sup>9</sup>. Incubation under anaerobic conditions and application of the enzyme catalase may reduce the production of hydrogen peroxide<sup>9,49</sup>.

The activity of a bacteriocin can be estimated based on the size of the inhibition zones produced in the diffusion test using agar plates overlaid with an organism sensitive to the bacteriocin to be tested. Activity is determined based on the radius or diameter of the inhibition zone<sup>32</sup>.

### Conclusions

In poultry production, *Lactobacillus* species used as probiotic bacteria produce a variety of antimicrobial substances, which are mainly of protein origin, called bacteriocins. The well-diffusion method using cell-free supernatants of *Lactobacillus* strains permits the determination of the antagonistic action of bacteriocins against Gram-positive and Gram-negative indicator microorganisms. In view of their technological importance due to their potential "natural" effect especially on food preservation, these protein substances derived from lactic acid bacteria should be widely explored by researchers, mainly in terms of definition, nomenclature, classification, mechanism of action, and genetic studies.

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