Chapter

An Overview of *Salmonella* Biofilms and the Use of Bacteriocins and Bacteriophages as New Control Alternatives

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Abstract

Salmonella is a major food-borne pathogen around the world. In the European Union (EU), this pathogen is responsible of more than 90,000 human cases of salmonellosis every year. Salmonellosis in normally linked to the consumption of contaminated food, especially poultry products as meat, eggs and the products elaborated with them. Several control measures have been implemented in the EU to reduce the prevalence of *Salmonella* in the food chain. However, the ability of *Salmonella* to form biofilm along the food chain difficult its eradication. Also, ineffective cleaning and disinfection measures favors biofilm formation. The widespread use of biocides along the food chain has led to the emergence of resistant *Salmonella* strains. Therefore, it is necessary to look for alternatives to biocides to eradicate *Salmonella* biofilms. In this chapter we evaluate the use of bacteriocins and bacteriophages and their derivatives as a new alternative to eliminate *Salmonella* biofilms along the food chain.

Keywords: *Salmonella*, biofilms, control, bacteriocins, bacteriophages

1. Introduction

Salmonella genus is composed only by two species, *S. enterica* and *S. bongori* and more than 2600 different serotypes. *S. bongori* is composed of about 20 different serotypes and strains of this species are rarely isolated. Most of the serotypes belong to *S. enterica.* This species is subdivided in six different subspecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (VI) (**Table 1**) [1]. The subspecies *enterica* attracts most of the attention of researchers as it is responsible for more than 99% of *Salmonella* infections in humans. Although the other *S. enterica* subspecies can also cause infections in humans, these infections tend to occur mainly in people with a very weakened immune system. The non-*enterica* subspecies of *Salmonella enterica* are usually isolated mainly from cold-blooded animals such as reptiles [2].

Species	Subspecies	Number of serotypes
S. enterica		2637
	S. enterica subspecies *enterica*	1586
	S. enterica subspecies *salamae*	522
	S. enterica subspecies *arizonae*	102
	S. enterica subspecies *diarizonae*	338
	S. enterica subspecies *houtenae*	76
	S. enterica subspecies *indica*	13
S. bongori		22

Table 1.
Number of serotypes present in each Salmonella *species and subspecies.*

Salmonella is important because it is one of the world's leading food-borne pathogens. In the European Union (EU), *Salmonella* is the second food-borne pathogen in number of human infections only behind the genus *Campylobacter*. In the year 2018, *Salmonella* was responsible of 91,857 human cases of salmonellosis and 119 deaths in the EU. Most infections are due to the consumption of food contaminated with *Salmonella* [3]. Thus, this pathogen can be isolated from different type of animals and their food derived products as bovine, porcine, ovine, fish or seafood [4–6]. But the largest number of human infections are related to the consumption of poultry products, especially meat and eggs as well as derived products [3]. As a consequence, the EU has developed legislation for member states to implement national control plans for *salmonella* in poultry production [7, 8]. The objective of this legislation is to reduce annually the prevalence of *Salmonella* in different types of farms including breeder farms, layer farms and broiler farms. Furthermore, this legislation also establishes that those serotypes that are of major epidemiological importance will be subject to special surveillance. For example, in broiler flocks *S.* Typhimurium and *S.* Enteritidis are subjected to this control. The ultimate goal of the European Union is for the combined prevalence of *S.* Typhimurium and *S.* Enteritidis to be less than 1% [9]. This due to these two serotypes are responsible of more than the 70% of human infections in the EU [3]. Cleaning and disinfection processes are of great importance to reduce the prevalence of *Salmonella* in the food chain. The implementation of inadequate control measures may result in *Salmonella* being able to resist in the food chain environment and contaminate different batches of food [10]. One of these bacterial resistance mechanisms is the formation of biofilms. For decades, biocidal substances such as quaternary ammoniums have been used to eliminate the presence of biofilms in the food industry [11]. However, the presence of multidrug-resistant strains is increasing [12]. This is a major concern as it may hinder the removal of biofilms from the food chain. Therefore, the development of alternative substances to combat food pathogen biofilms is necessary [13]. A brief description of *Salmonella* biofilms and the use of natural alternatives such as bacteriocins and bacteriophages to combat biofilms will be given throughout this chapter.

2. *Salmonella* biofilms

2.1 Basic concepts on biofilms

Costerton et al. [14] were the first researchers in stablish the term biofilm in paper published in *Scientific American* in 1978. They propose that most bacteria in

aquatic ecosystems growth attached to surfaces in a closed self-produced matrix. Researchers also postulates that sessile cells (biofilm) differ from the planktonic cells (floating). It is important to note that the authors include the reference to aquatic environment because it was the first place where bacterial biofilms were observed. But, at present it is known that biofilms are the predominant style of life of bacterial in environment and its related with 80% of bacterial infections. Actually, biofilm is defined as a community of bacterial cells enclosed in a self-produced polymeric matrix and adhered to biotic (plant surfaces, epithelial cells, gallstones) or abiotic surfaces (plastic, rubber, glass, stainless steel). Biofilms have a great importance in the food production chain and human health because cells enclosed in this matrix are extremely difficult to eradicate because are more resistant to environmental stressors as antibiotics, disinfectants, host immune system [15–18].

There are four different steps of biofilm formation: 1) bacterial attachment, 2) microcolony formation, 3) bacterial maturation and 4) dispersion (**Figure 1**). The initial adhesion of bacterial cells is highly influenced by surface properties (roughness, hydrophobic interactions), environmental changes and bacterial regulation. Biofilm maturation and architecture is regulated by the signals of bacteria cells that compose biofilm and its stability depends on the accumulation of specific proteins, eDNA and polysaccharides. The presence of disruptive factors as proteases and nucleases and other enzymes activates biofilm dispersion. Factors as quorum sensing play an important role in this last step which function is the colonization of new niches [19].

2.2 Biofilm formation steps

2.2.1 Adhesion

Salmonella cells adhesion can be active or passive according the motility of bacteria or gravitational transport of planktonic cells. Both surfaces of bacterial cells and substrate surface highly influence the initial cell attachment. At this point bacterial cells have small quantities of extracellular polymeric substance (EPS) and maintain independent movement from other bacterial cells. Adhesion is reversible during this phase and cells do not present the morphological changes associated with biofilm cells and they can return to its planktonic state [16].

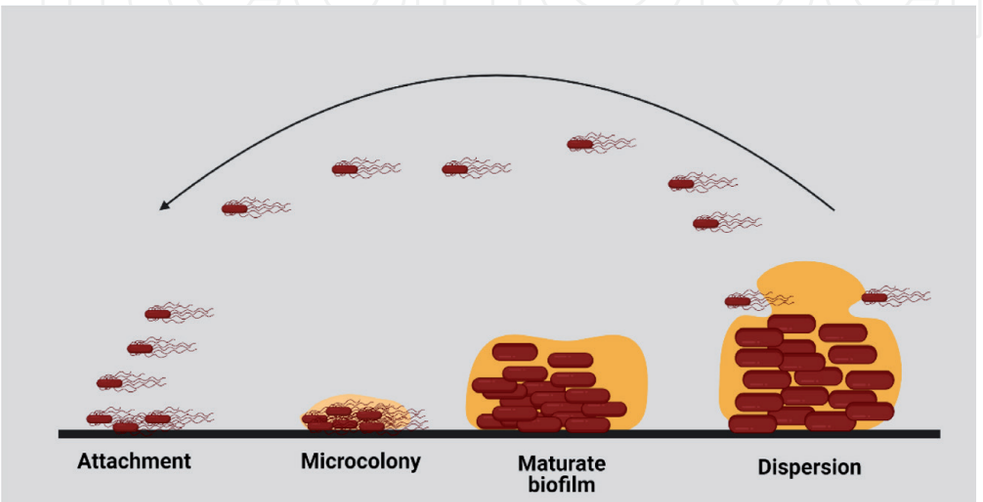

Figure 1.
Steps involved in Salmonella *biofilm formation. Created with biorender.com.*

2.2.2 Irreversible adhesion

The change from a weak interaction to a strong interaction between surface and bacterial cells is responsible to the switch from a reversible adhesion to an irreversible adhesion step. This change can happen in minutes and the production of EPS is key. The secretion of this polymeric substance by bacterial cells enhances the cell-surface interaction being necessary shear forces or chemical substances to break the adhesion [16, 20].

2.2.3 Microcolony formation

The formation of biofilm microcolony results from the accumulation of bacteria growth and the production and association with EPS. As a result, the bond between bacteria and substrate increases and protect bacteria from different environmental stressors. The cell-to-cell communication mechanism play an important role in this step of biofilm formation by regulating the expression of biofilm related genes. This results in an increased EPS production and caption of planktonic cells [21].

2.2.4 Maturation

The small microcolonies formed join to form the mature biofilm and its characteristic three-dimensional structure. The production of EPS and union between cells permits that mechanical pressure do not detach the biofilm from the surface. There are three different parts in mature biofilm. The bottom layer is a biofilm that forms a network structure that did not completely covers the surface that supports the biofilm. The intermediate layer is composed by a compact basement membrane. Finally, in the outer layer are located the planktonic cells [16].

2.2.5 Dispersion

The last step of biofilm formation is dispersion. In this phase the biofilm cells revert to their planktonic form. There are different factors that influences biofilm dispersion including external disturbance, starvation, endogenous enzymes, the release of EPS or surface binding proteins. This is an important step for the colonization of new niches by bacterial cells [22].

2.3 Structural components of *Salmonella* biofilms

Salmonella biofilm matrix is composed by proteins and exopolysaccharides among other things. There are two main proteins related with biofilms. Curli, an amyloid fimbria, and BapA protein. In the other hand, cellulose and colonic acid are the main exopolysaccharides of biofilm matrix. Also the type I fimbriae, Lpf and Pef are important in the initial steps of biofilm formation. Other components as fatty acids and lipopolysaccharides have also a role in biofilm formation.

Curli fimbriae is the most important protein involved in biofilm formation. Also is related to other processes as colonization, persistence, motility and invasion. This is a highly aggregative, unbranched, amyloid-like protein that promote cell-to-cell interactions through surfaces interactions and forms a complex with cellulose and O-capsule antigen. Other protein involved in biofilm formation is fimbriae type I. This protein is necessary for adhesion and biofilm formation in enterocytes. The protein BapA has an important role in bacterial aggregation and biofilm formation in air-liquid interface through homophilic interaction between bacterial cells [23–26].

Cellulose is the main polysaccharide involved in *Salmonella* biofilm formation. It is necessary for biofilm maturation phase in different surfaces, and it is inversely correlated with virulence as its production is suppressed in *Salmonella* enterocyte colonization phase. Another exopolysaccharide is the lipid bound O-antigenic capsule, with importance in resistance to desiccation and environmental persistence. This exopolysaccharide has demonstrated a role in biofilm formation in gallstones and plants but lower importance in adhesion to abiotic surfaces as glass or plastic. In other hand, cholinic acid is important for three-dimensional structure formation in enterocytes but not in abiotic surfaces, gallstones or alfalfa seeds. Therefore, some polysaccharides are only important for some types of biofilm formation [27–33].

Flagella, which are basic for cell movement and swarming in *Salmonella* also play a role in biofilm formation. In the initial step of reversible and irreversible adhesion, motility is important. Also, motility is necessary for 3D biofilm structure and the dispersion phase. But in other steps of biofilm formation the expression of flagella is inhibited. There is switch mechanism system that causes a reduction of flagella function and increased the expression of cellulose, resulting in the inhibition of flagellar rotation. This demonstrates the ambivalent role of flagella in biofilm formation. Fatty acids have also a role in *Salmonella* biofilm formation, especially in hydrophilic surface such as glass but not in hydrophobic surfaces as gallstones [34–36].

2.4 Genetic control of *Salmonella* biofilms

The change from a planktonic to a biofilm cell lifestyle needs some physiological changes. This switch is controlled by a complex genetic machinery that regulates the production of substances that conform the biofilm extracellular matrix, bacterial metabolism and the response to environmental signals. The transition between planktonic to biofilm cells and the expression of specific biofilm matrix-associated components is the master regulator of biofilm formation CsgD. It forms part of the operon that control the synthesis of curli fimbriae and acts as a transcriptional activator of the quorum sensing LuxR family. CsgD expression respond to different environmental signals as nutrient concentration, temperature, growth phase, oxygen tension, osmolarity, membrane integrity, tryptophan, and indole. CsgD positively regulates cellulose biosynthesis in *Salmonella* through direct stimulation of adrA transcription. AdrA synthetize c-di-GMP, a signaling molecule, that also activates the cellulose synthase BcsA, resulting in increased production of cellulose. Although it is the most important, there are other enzymes involved in cellulose synthesis [37–40].

RpoS and Crl are other important regulators of *Salmonella* biofilm formation regulating the expression of several components. Gene *rpoS* encodes a sigma factor called σS that regulates genes involved in stress response and stationary phase. It has been observed that almost the 25% of genes regulated with this sigma factor are overexpressed in biofilm cells of *S.* Typhimurium. For example, RpoS increases the expression of *csgD* and biofilm formation in environments with limited iron availability and regulate the expression of *adrA* in some steps of biofilm formation and is involved in the expression of genes related with motility. In other hand, the transcriptional regulator Crl protein regulates the activity of σS. RpoS and Crl have an effect in each other and their concentration are negatively correlated. Crl is necessary for maximal expression of *csgB, csgD* or *bcsA* and increased the expression of other genes related to RpoS. It is also remarkable that its effect are higher at 28°C than at 37°C. This indicates that this transcriptional regulator acts as a temperature sensor of *Salmonella* biofilm formation [41–44].

The bacterial messenger molecule c-di-GMP regulates several biological functions as virulence, motility, cell cycle regulation, differentiation, and biofilm formation. This molecule promotes *Salmonella* biofilm formation by regulating the production of some important components of biofilm matrix as cellulose and curli fimbriae. The c-di-GMP has a positive feedback on *csgD* expression. Thus, high levels of c-di-GMP increased the levels of CsgD, this increased the levels of AdrA and therefore c-di-GMP and cellulose synthesis [45, 46].

Other regulatory system implicated in motility and biofilm formation is the two-component system BarA/SirA. This system is modulated by factors as external pH, metabolic end products (formate, acetate), short chain fatty acids or bile salts. SirA modulates the *Salmonella* Csr system, an important regulator of motility, virulence, carbon storage, secondary metabolism and biofilm formation. CsrA control the change between sessile cells and motility, mainly activating motility. SirA activate the transcription of small RNAs CsrB and CsrC that inhibits CsrA activity and motility related genes. This increases type I fimbriae production and therefore biofilm formation [47, 48].

2.5 Quorum sensing

Another mechanism implicated in biofilm formation is Quorum sensing (QS). This is a cell-to-cell communication mechanism used by bacteria to adapt to environmental changes and implant a common bacterial strategy to respond to environmental stressors. QS is implicated in responsive defense against eukaryotic host cells, nutrient access, growth restriction environments, survive in hostile environments as well as cell differentiation to other form of life as biofilm cells. This communication is based in small molecules called autoinducers and that diffuse through bacterial membranes. Autoinducers are secreted at a basal level during bacterial growth. The concentration of this molecules increases with the growth of bacterial population until reach a threshold level and modulate the expression of QS target genes (**Figure 2**) [49, 50].

Gram-negative bacteria QS is divide into three categories: (i) N-acyl homoserine lactones (AHLs) called AI-1; (ii) furanosyl borate diester derived from the recycling of S-adenosyl-homocysteine to homocysteine called Autoinducer II (AI-2) for interspecies and intraspecies communication; and (iii) Autoinducer (AI-3) related to the recognition of host catecholamines epinephrine and norepinephrine. In the

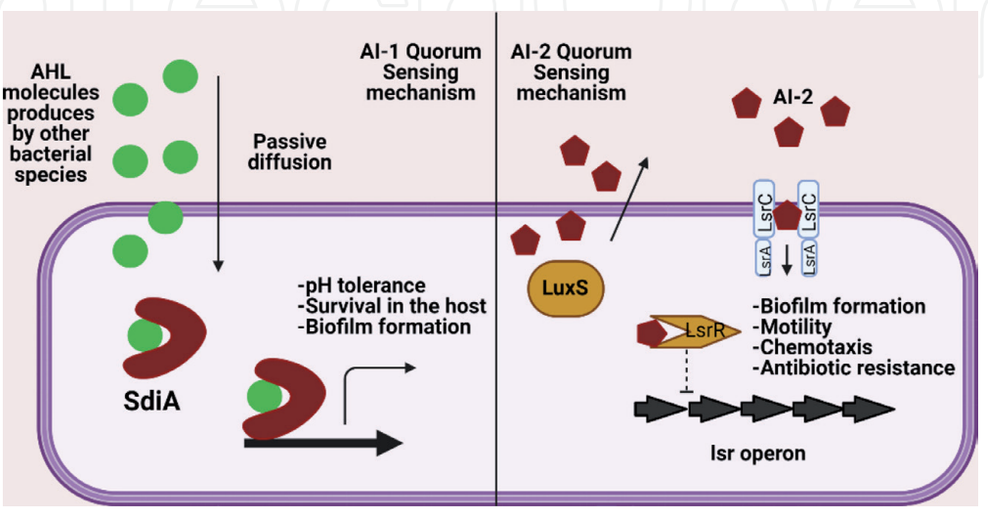

Figure 2.
Schematic representation of quorum sensing mechanisms AI-1 and AI-2 in Salmonella. *Created with biorender.com.*

case of *Salmonella*, only encode the receptor for AHLs but do not produce AI-1 molecules. But *Salmonella* can recognize AHLs produced by other bacterial genera as *Pseudomonas aeruginosa* or *Yersinia enterocolitica*. QS is basic in the formation of healthy biofilms and have a role in every stage. Genes regulated by the AI-1 receptor SdiA promote *Salmonella* cell adhesion and the production of extracellular proteins that compose biofilm matrix. Thus, *Salmonella* can response to the presence of AHLs molecules produced by other bacteria and increase biofilm formation. In the same way, AI-2 LuxS also can increase the expression of motility and biofilm related genes. Therefore QS is key a component of biofilm formation regulation [51–53].

3. Biofilms in the food industry

Nowadays, it is totally accepted that most bacteria grow in biofilm in the environment. Biofilms can have beneficial effects. For example, biofilm formation by *Lactobacillus* and *Lactococcus* results in more efficient fermentation processes and in the case of human health protect against the adhesion of pathogenic bacteria in the gut. But biofilm formation by undesirable bacteria, as food-borne pathogens, has a negative impact on food industry. Also, bacteria growing in biofilm can cause deterioration in the machinery as corrosion, efficiency reduction in heat transfer or clogging filters [54, 55].

Biofilms are a persistent source of contamination in the food industry. This cause hygiene and economic issues due to the spoilage of different food product batches with bacteria that persist in biofilms [56]. This is especially important in today's globalized world where food is globally distributed. Also, in the last years consumers demand fresh and minimally processed food products. Hygiene measures must therefore be strict to avoid contamination of food products. The presence of food-borne pathogen biofilms in the food processing environment can result in large number of food batches contaminated and outbreaks worldwide [57]. A good example was the salmonellosis outbreak caused by contamination of different batches of infant formula manufactured in a single facture and causing an outbreak that affected different countries around the world. Poor cleaning and disinfection procedures of food industry surfaces results in the presence of food residues that in the presence of humidity favors the development of bacterial biofilms as *Salmonella.* Cross-contamination occurs when food contact with surfaces with bacterial biofilms or also through aerosols from contaminated equipment. Until now, there is limited information of the real presence of *Salmonella* biofilms in the food processing environment. But *in vitro* studies have demonstrated that *Salmonella* can attach to different material commonly present in the food industry as plastic, glass, or stainless steel [57, 58].

Biofilm formation is influences but different factors as bacterial genus, species and even strains. But surface have a high influence on the ability of bacteria to adhere and form biofilm [59, 60]. Different type of material as stainless steel, glass, rubber, polystyrene and polyurethane, Teflon, nitrile and rarely wood are present in the food industry [61–63]. Physical properties have influence on biofilm formation, especially surface tension. Bacterial adhesion is favor by moist, energy free surfaces. Bacterial cells have better adherence to hydrophilic surfaces in comparison to hydrophobic surfaces. Surface roughness also influence cell adherence [57, 64]. In this sense, polished stainless steel showed less bacterial adherence than unpolished stainless steel [65]. Also, a study that compared stainless steel, glass and wood found that this latter surface favor biofilm formation because its porosity and ability to hold organic matter [66]. But also, surface influences biofilm formation in food industry. In this sense, welds, joints, corners or equipment design could enhance

initial bacterial cell adherence [67]. But the presence of organic molecules on food industry surfaces is one of the major factors that influences biofilm formation. The presence of a layer of molecules as milk or meat proteins, EPS produced by other bacteria, favor the initial adhesion of bacterial cells. Diverse studies have observed that the presence of chicken juice macromolecules in stainless steel surfaces favor the initial adhesion of *C. jejuni* or *S.* Typhimurium. However, in some occasion macromolecules have the opposite effect. In this sense, an study observed that milk proteins reduced the initial adhesion of *L. monocytogenes* [68–70].

In the food production chain, there are different environmental conditions that can modulate *Salmonella* biofilm formation ability through modulation of initial adherence. Nutrient availability is one of these environmental conditions to which bacteria have to adapt. Under specific conditions, *Salmonella* has to persist under limited nutrient availability [71]. Biofilm formation is one strategy used for *Salmonella* cells to survive under this environmental stress conditions [72]. *In vitro* studies have demonstrated that *Salmonella* enhance a biofilm under limited nutrient conditions. These studies used common laboratory media as Tryptic Soy Broth or peptone water. These studies are a first approximation of the possible behavior of *Salmonella* under nutrient-limited conditions [71]. Temperature is another factor that changes through the food production chain. Several studies have demonstrated that *Salmonella* strains showed different biofilm formation amount under different temperatures tested. Interestingly, temperatures below 37°C and specially temperatures of 20°C favored *Salmonella* biofilm formation. The pH also influences *Salmonella* biofilm formation. A study that evaluated a total of 60 *S. enterica* strains under different pH, NaCl concentrations and temperature concluded that pH was the environmental factor that most influenced biofilm formation in *S. enterica* strains tested. This is probably due to the different ability of strains to adapt to acidic pH through an acid tolerance response mechanism [60, 73]. In the same way, another study found that weak acidic pHs (6) increased initial adhesion to stainless steel surfaces in comparison to neutral pHs. But curiously, acidic pHs reduced the number of cells present in mature biofilms due among other things to a lower presence of biofilm matrix components as polysaccharides and proteins [74]. Gene expression showed that acidic pH caused changes in the expression of virulence and biofilm related genes [75]. The environmental conditions under biofilms are formatted also influences its resistance to disinfectants. In this sense, biofilms formed under refrigeration temperatures showed higher sensitivity to disinfectants than those produced at 25°C under nutrient restriction as well as biofilm formed under acidic pH. In the other hand, mature biofilm are more resistant to substances such as quaternary ammonium compounds, peroxyacetic acid or organic acids. This is probably due a higher presence of matrix compounds as cellulose and curli fimbriae [76].

Although monospecies biofilm studies are interesting to understand the mechanism involved in biofilm formation under different environmental conditions of a specific bacteria, in nature biofilms are commonly composed by bacteria of different species and genera. These different bacteria communicate with each other through diverse mechanism as quorum sensing stablishing synergistic interactions that increase the resistance of biofilm to stressful environments. Also, genetic exchanges between different bacteria can occur in the biofilm environment [77]. This is specially interesting when resistance genes are transmitted. Dual biofilm studies are the first step to study multi-species biofilms. In this kind of studies, the biofilm formation ability of each bacterial group is studied individually, and then conjunct studies are carried out to determine the synergic mechanism stablished between the different groups [78]. In this sense, a study observed that *Salmonella* and *E. coli* mixed biofilms are more sensitive to disinfectants that biofilm of only

one species [79]. In other hand, *S.* Enteritidis and *P. aeruginosa* mixed biofilms are more resistant to chlorine treatments [80]. In the same way, it was observed that mixed biofilms of *S.* Typhimurium and cultivable lettuce microorganism increased resistance to cold oxygen plasma treatments [81]. These studies provide a first clue of mixed biofilms. These studies are a first approach to multi-species studies. But undoubtedly the study of biofilms composed of hundreds of different bacterial genera will provide valuable information to fully understand how biofilms behave in nature. Such studies supported by genomics, metabolomics and high-resolution imaging will be the trend of the coming years in this field of microbiology.

4. Bacteriocins

4.1 Briefly definition and characteristics

Bacteriocins are defined as a group of ribosomally produced antimicrobial peptides synthesized by both Gram-positive and Gram-negative bacteria. These molecules are characterized by its ability to act against closely related bacteria (narrow spectrum) or a diverse group of bacteria (broad spectrum) [82]. Bacteriocins can be dived in two general groups: Class I composed by peptides with post translational modifications and Class II composed by unmodified peptides. The production of bacteriocins is considered as a competition mechanism that allows bacteria to kill other bacteria that can compete with it for a certain niche or for nutrients. This suggests that many bacterial groups produce at least one bacteriocin, which means that there are still many bacteriocins to be discovered [83, 84]. Bacteriocins have a great antimicrobial capacity against their targets at nanomolar concentrations and exerts its activity by membrane permeabilization [85].

In recent years these molecules have received much interest in general and in particular their application in the food chain. The main reason is the search for alternatives to antibiotics due to the emergence of antimicrobial resistance [86]. While the use of antibiotics to treat enteric pathogens can cause harm to commensal bacteria in the intestinal microbiota, narrow-spectrum bacteriocins can be used in such a way that only the target bacteria are affected by the treatment [86]. On the other hand, the bacteriocins can be used as modular of the intestinal microbiota. For example, they can be used to establish a microbiota that favors the fattening of the chickens and therefore as natural substitutes for antibiotics as growth promoters [87]. In addition, today consumers are demanding food products where the use of chemicals is reduced to a minimum, and natural alternatives such as bacteriocins would be welcomed. Finally, another advantage is that the bacteriocins can be used directly or bacteriocin-producing probiotic cultures can be used resulting in the production of these molecules in situ. This would eliminate the process of production and purification of bacteriocins making their application more economical. But bacteriocins can be also useful to inhibit and eradicate biofilm biofilms in the food production chain (**Figure 3**).

4.2 Applied studies on *Salmonella* biofilms

One of the first studies in this field, two concentrations of enterocin AS-48 (25 and 50 mg/L) produced by *Enterococcus* were tested in combation with antibiotics and biocides against four *Salmonella* strains [88]. Concentrations of 25 mg/L of bacteriocin in combination with antimicrobials highly inhibited the growth of *Salmonella.* This bacteriocin also have effects on sessile biofilm cells. Preformed biofilms were treated with different combinations of bacteriocin and antimicrobials. Enterocin AS-48 at 50 mg/L had a synergic effect in combination with some

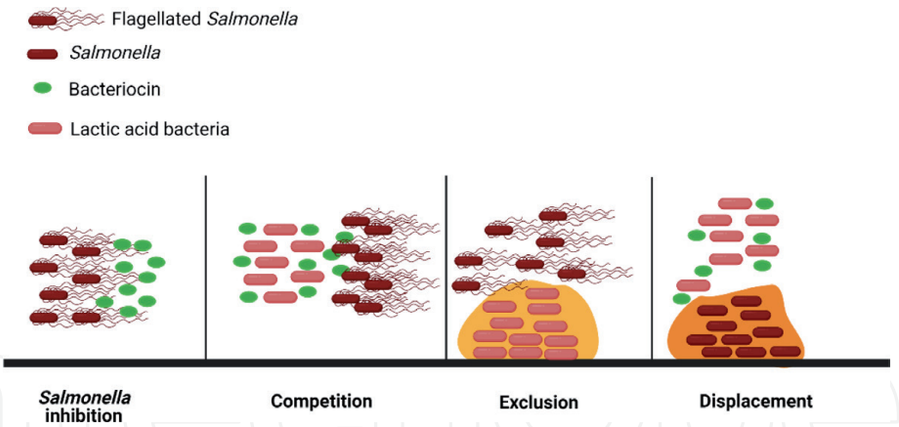

Figure 3.
Mode of action of bacteriocin and bacteriocing-producing bacteria to inhibit and/or eradicate Salmonella *biofilms.*

biocides. But the results differ between strain tested. In another study, Bag and Chattopadhyay [89] tested the antibiofilm activity of nisin alone or in combination with essential oil components. *S.* Typhimurium preformed biofilms were treated with MIC doses of nisin alone or in combination with ρ-coumaric acid. MIC doses of nisin only reduced in 20% biofilm formation. However, in combination with ρ-coumaric acid were reduced in almost 80%. This study demonstrated that nisin by itself have a low antibiofilm activity. Kim et al. [90] tested the crude bacteriocin DF01 derived from *Lactobacillus brevis* DF01 against *S.* Typhimurium biofilms. The incubation of this pathogen with bacteriocin DF01 reduced *S.* Typhimurium biofilm formation in almost 47%. However, the treatment of preformed biofilms with bacteriocin did not reduce biofilm mass. Therefore, the main action of DF01 bacteriocin is interfere in the biofilm formation process. In a similar study, Seo and Kang [91] evaluated the antibiofilm effect of bacteriocins purified from *Pediococcus acidilactici* K10 and HW01 in *S.* Typhimurium biofilm formed in stainless steel and chicken meat. Crystal violet staining method and fluorescence microscopy showed that those two bacteriocins reduces *S.* Typhimurium biofilm formation. In contrast to previous studies, this work demonstrates the ability of bacteriocins to also reduce the formation of biofilms in the food matrix itself.

In addition, instead of bacteriocins, the bacteriocin-producing bacteria themselves can also be used as alternative way to reduce *Salmonella* biofilm formation through competition, exclusion and displacement [92]. Das et al. observed that *L. plantarum* KSBT 56 isolated from Indian traditional food reduces in 2 log CFU/mL the cells present in *S.* Enteritidis biofilms [93]. Gómez et al. [94] used potential probiotic lactic acid bacteria (LAB) to inhibit the formation of food-borne pathogens biofilms. In this study they evaluated both bacteriocinogenic (sakacine and nisin producer strains) and non-bacteriocinogenic *Lactobacillus* and *Lactococcus* strains against *S.* Typhimurium. The researchers preformed biofilms of LAB and after formation added a culture of *S.* Typhimurium. Preformed biofilms of LAB significantly reduced the attachment and biofilm formation of *Salmonella* in comparison to control. However, it is important to note that this reduction was not influenced by the production of bacteriocins. In another interesting study, the adhesion of food-borne pathogens as *S.* Typhimurium to wood commonly used in traditional cheese production in Sicilia was evaluated. The results showed that indigenous milk LAB highly adhere to wood surfaces while in samples artificially contaminated with *S.* Typhimurium, no adherence of this food-borne pathogen was observed. The researchers propose that biofilms formed by LAB in wood surfaces have a protective effect in biofilm formation by food-borne pathogens [95].

5. Bacteriophages and derived protein endolysin

5.1 Briefly definition and characteristics

Bacteriophages are viruses that infects bacterial cells with a high specificity. The life cycle of bacteriophages can be classified in two general categories: the lytic cycle (virulent) and the lysogenic cycle (temperate phage). In the lytic cycle the infection process starts with the irreversible attachment of the phage tail proteins to a receptor of the bacterial cell surface (protein or lipopolysaccharides). The ability of the bacteriophages to recognize and attach to molecules of the bacterial cell surface defines its host range. Once the phage DNA is in the host cell, specific enzymes are synthetized to drive host cell to the production of proteins necessary for the generation of new phage particles and cell lysis enzymes. At the end of the phage cycle, cell lysis, release of progeny phage and infection of neighboring susceptible cells occurs. Temperate phages combine its capacity to carried out the lytic cycle with the ability to persist as a prophage in the genome of the host cell and replicate with them. Diverse environmental signal can result in the prophage entering in the lytic cycle [96]. The use of temperate phages in medical and food applications is avoided because can cause transduction of genetic material between bacteria including virulence genes. In addition, due to its cycle, they do not kill all the bacteria that infect [97].

Lytic phages are those chosen for being used in phage therapy because they can replicate exponentially on bacterial culture and can eliminate multidrug resistant bacteria [86]. Based on their activity spectrum can be defined as monovalent phages when they are specific to one type of bacterial species and polyvalent phages when they are able to attack two or more bacterial species. But normally phages have a narrow host range, strains specific in most cases, and therefore cocktails composed by two or more phages are normally used to broaden the antimicrobial spectrum and reduce phage resistance [98].

Although bacteriophages have been known for over a century, the development of antibiotics resulted in their use not being explored in the Western world. However, the global problem of antimicrobial resistance and the need to seek alternatives has resulted in bacteriophages being brought back into the spotlight. Its applications in the food chain are very wide. They can be used for the treatment of bacterial diseases of production animals, for the disinfection of facilities and the elimination of biofilms or they can be added to food or packaging to inhibit the growth of food pathogens [86, 97]. In fact, there are different commercially available bacteriophage solutions to be applied to food or food processing facilities. Some examples are ListShield™, SalmoFresh™ and EcoShield PX™ commercialized by Intralytix or PhageGuard Listex and PhageGuard S commercialized by PhageGuard.

The bacteriophages synthetized at the end of the phage multiplication cycle peptidoglycan hydrolases commonly called endolysin. Its function is to lyse the host bacterial cell by directly target bonds in the bacterial cell wall peptidoglycan structure. This result in the degradation of the rigid murein layer and the release of newly assembled bacteriophage virions [99]. While endolysins can act as exolysins in the Gram-positive bacterial peptidoglycan layer, they cannot degrade the bacterial outer membrane of Gran-negative bacterial cells. Therefore, the outer membrane can prevent the access and the effect of endolysins [100]. For that reason, it is necessary to combine endolysins with other treatments for the lysis of Gram-negative bacteria. The combination of endolysin with outer membrane disruptors in one the main options for the application of enzymes in Gram-negative bacteria. Gram-positive phages endolysins have a modular structured formed by a cell-wall-binding domain that specifically recognizes the cell wall-associated

ligand molecules and an enzymatically active domain that cleaves the peptidoglycan structure. Although gram-negative bacteriophage endolysins may also have this structure, they usually have a globular structure that only possesses a an enzymatically active domain [101, 102].One of the main advantages of the use of endolysins is that a very small amount of purified enzyme is enough to lyse in minutes or even seconds a dense suspension of bacterial cells. This in combination with their substrate specificity makes them have great potential for application in food science [103]. Endolysins are considered to be safe and also have some advantages compared to the use of bacteriophages because do not create gene transduction issues and therefore not contribute to the emerging problem of antimicrobial resistant bacteria [104]. Its applications in the food industry are very wide. They can be added directly to food, can be part of bioactive packaging or can even be used to remove biofilms in the food industry environment Furthermore, due to their specificity, they can be applied directly to treat intestinal infections in farm animals without causing alterations in the intestinal microbiota [103].

5.2 Applied studies on *Salmonella* biofilms

Tiwari et al. [105] tested a specific *S.* Enteritidis virulent phage called SE2 against planktonic and biofilm cells of an antimicrobial resistant *S.* Enteritidis strain. The phage showed a high bacteriolytic effect. This phage reduced in 4.2 log UFC/mL the count of *S.* Enteritidis after incubation of 4 h at 37°C and 2.5 log UFC/mL after incubation at 4°C. These results demonstrate that this phage can also be used effectively at refrigeration temperature. Also, biofilm studies showed that treatments with phage SE2 concentrations of 10^{11} PFU/mL reduced in 97% viable cells present in biofilms formed in glass. Also this phage showed that could maintain its activity at different ranges of pH and temperature. It has been also proposed that phage predation could increase biofilm formation by bacteria in some specific conditions. Hosseinidoust et al. [106] carried out and study to evaluate this theory in different pathogens including *S.* Typhimurium and to determinate if the increase of biofilm formation is due to the development of phage resistance or to non-evolutionary mechanism as spatial refuge. The results indicate that phage resistance was the mechanism implicated in increased biofilm formation in *P. aeruginosa.* However, in the case of *S.* Typhimurium it was due to non-evolutionary mechanisms [106]. Karaca et al. [107] evaluated the effect of phage P22 in *S.* Typhimurium biofilm formation in polystyrene and stainless-steel surfaces. The authors evaluated both the incubation of phage particles with *Salmonella* in biofilm studies and the treatment of preformed biofilms. *S.* Typhimurium biofilm formation was significantly reduced at high phage titer ($\leq 10^6$ PFU/mL). Also, all phage titers were effective against biofilm formation in 24 h incubation period but only higher phage titers were effective in 48–72 h incubation time. In addition, the ability to reduce biofilm formation was lower in polystyrene than in stainless steel. In the other hand, phage treatment was not effective in eradicating pre-formed *Salmonella* biofilms. This is probably due to the presence of extracellular matrix components that prevent bacteriophages from binding to specific receptors on the bacterial surface. In this sense, Yüksel et al. [108] combined phage P22 with EDTA and nisin to improve the antibiofilm activity of phage. The combination of the three inhibit in 93% *S.* Typhimurium biofilm formation at low phage titer concentrations but only reduced 70% mature biofilms. Therefore, the combination of phages with other antimicrobial substances could enhance antibiofilm activity. But it is still difficult to reduce biofilm in mature stages, when high quantities of extracellular matrix substances are present.

Garcia et al. [109] tested a cocktail of lytic bacteriophages biofilm to eradicate biofilms formed by different *Salmonella* serotypes in different surfaces (stainless

steel, glass, and polyvinyl chloride) at short and long incubation times. Preformed biofilms were treated with 10^8 PFU/mL during 3, 6 and 9 h. The results were not very promising and had a lot of variation between different surfaces and *salmonella* serotypes. In the same way, Gong et al. [110] tested different phage concentration (10^4–10^8 PFU/mL) against hard *Salmonella* biofilms formed in microtiter plates. Phages were selected based in its range activity against the different *Salmonella* serotypes included in the study. The reduction of biofilm formation was of 90% when *Salmonella* was incubated in combination with phages and 66% in preformed biofilms. Milho et al. [111] tested the phage PVP-SE2 against *S.* Enteritidis biofilms formed in food contact surfaces polystyrene and stainless steel. This phage caused reductions of 2 to 5 log CFU cm^2 at room temperature of 24 h and 48 h old *Salmonella* biofilms, showing its efficacy to control *S.* Enteritidis biofilms. Also, it was observed that this phage inhibited the growth of *S.* Enteritidis in poultry skin, even in freezing phage-pretreated poultry skin. The same research group evaluated the antibiofilm effect of phages in *E. coli* and *S. Enteritidis* dual-species biofilms [112]. The results of this study showed that phages were more effective to eradicate mono-species biofilms than dual-species biofilms. It is important to consider this when designing products that include phages to eradicate biofilms as biofilms in the food industry are often composed of various bacterial species. Kosznik-Kwasnicka et al. [113] evaluated three phages vB_SenM-1, vB_SenM-2, and vB_SenS-3 with lytic activity against different *Salmonella* serotypes. The phages were able to reduce biofilm cells and biomass in different strains tested and under different temperatures. This is important as there are different temperatures in the food chain and this study would indicate that phage treatment could be used over a wide temperature range. In the same way, Esmael et al. [114] tested to *S.* Typhimurium lytic phages against 72 h-old biofilms formed in microtiter plates. Concentrations of 8 log10 PFU/mL reduced more than three times biofilm formation. However, most of the studies conducted so far focus on specific *Salmonella* serotypes. One of the main characteristics of phages is their specificity. Thus, phages usually show activity against specific species, serotypes or even strains. This leads to a number of studies evaluating phage cocktails. Even so, it is difficult to find a phage cocktail effective against all *Salmonella* serotypes. This is one of the main problems to be solved with the use of phages in the food industry.

Using a food model, Sadekuzzaman et al. [115] evaluated the efficacy of 2 h bacteriophage treatment against *Salmonella* biofilms formed in lettuce surface. Although effective, phage treatment only reduced 1.0 log CFU/cm the count of *Salmonella*. Another alternative is the use of the active parts of the phages, for example the phage-encoded proteins. Altought some of the functions performed by proteins can be also performed by the phage itself, the use of proteins can have advantages in consumer acceptance and in terms of regulation. In this sense. Zhang et al. [116] tested endolysin LysSTG2 against *S.* Typhimurium biofilms. One hour treatment with 100 μg/mL of this endolysin, reduces 72 h biofilm in 13%. However, the combination of this endolysin with slightly acidic hypochlorous water containing 40 mg/L available chlorine reduces *S.* Typhimurium biofilm cells in 99%. Therefore, the combination of endolysin with other antimicrobial substances is a potential alternative against *Salmonella* biofilms.

6. Conclusion

Salmonella biofilm formation in the food production chain is a major public health problem. Mechanisms regulating biofilm formation in *Salmonella* are complex and is regulated by a wide range of environmental factors. The ability of

Salmonella to form biofilm in a wide temperature or pH range as well as in other stressful situations poses a major problem for its eradication. Also of concern is the increase of *Salmonella* strains with resistance to multiple biocides. Both bacteriocins and bacteriophages are a potential alternative to eliminate *Salmonella* biofilms. In addition, they can be combined synergistically with traditional antimicrobials, thus reducing the amount of antimicrobials used. One of the main limiting factors in its application is its range of activity. Normally bacteriocins and bacteriophages present a narrow spectrum of activity. They are therefore very useful for use against a specific pathogen. But in order to have a broad spectrum of activity to prevent different bacterial groups in the food chain, formulations combining a cocktail of bacteriocins and phages are needed. Studies evaluating such products as an alternative to traditional biocides are still limited, but future research and the use of recombinant technologies will make it possible to obtain products with high efficacy against *Salmonella* biofilms.

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Conflict of interest

The authors declare no conflict of interest.

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