Research Note

Bacteriophage use to control *Salmonella* biofilm on surfaces present in chicken slaughterhouses

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ABSTRACT Foodborne diseases represent a major risk to public health worldwide. Pathogenic bacteria can live in the form of biofilm within the food industry, providing a permanent source of contamination. The aim of this study was to evaluate the influence of the types of adhesion surfaces on *Salmonella* biofilm formation at eight different times, and analyze the action time of a bacteriophage *pool* on established biofilms. Most of the samples used were classified as weak biofilm producers, with serovars Enteritidis and Heidelberg showing the highest frequency of biofilm formation. Glass and stainless steel surfaces significantly favored biofilm formation at 60 and 36 h of incubation respectively, but the polyvinyl chloride surface did not favor biofilm production, suggesting that the type of material may interfere with production. The bacteriophage *pool* action period focused on 3 h, but treatment of 9 h on glass surface biofilms was superior to other treatments because it affected the largest number of samples. These results suggests that some surface types and *Salmonella* serotypes may promote biofilm formation and indicate bacteriophages as an alternative to control biofilms. But further studies are required to prove the effectiveness and safety of bacteriophage therapy as an alternative in the antimicrobial control in the processing plants.

Key words: *Salmonella*, biofilm, bacteriophage, chicken meat

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INTRODUCTION

Foodborne disease (**FBD**) represents a major threat to public health worldwide. The salmonellosis is one of the most challenging FBDs and constitutes a major public health problem in most countries. One cause of food contamination is the adhesion of bacteria to processing surfaces, where the presence of organic residues in the environment and refrigerating equipment can provide conditions for the development and persistence of organized communities called biofilms, which may contain pathogenic bacteria and act as a source of cross-contamination (Srey et al., 2013; Bridier et al., 2014).

Most of the pathogenic bacteria associated with FBD are able to adhere to and form biofilms on a variety of materials such as plastic, metal, glass, wood, and foodstuffs and under almost all conditions in food processing plants (Winkelströter et al., 2014). An example of this relationship was represented by a population of bacteria in chicken carcasses able to form biofilms on stainless steel surfaces with different finishes (Arnold and Bailey, 2000).

Common strategies among the several tools used for combating microbial adhesion and biofilm formation are the regular application of cleaning and disinfection procedures in industrial equipment and in the products themselves; but such procedures are not fully effective against biofilms and can induce the selection of resistant phenotypes (Bridier et al., 2014). Once formed, biofilms are extremely difficult to remove, as is the case of *S.* Typhimurium biofilms formed on different surfaces that exhibited resistance to the action of sodium hypochlorite (Scher et al., 2005).

Most conventional strategies of biofilm control are based on chemical attack, but the development of antimicrobial resistance associated with resistance presented by structured microbial communities, together with the current demand for reducing the use of antimicrobials, combine to drive the exploration of new strategies to ensure safe food (Schlisselberg and Yaron,

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2013). Thus new approaches for biofilm control have been studied as potential biological tools including the use of enzymes, bacteriophages, microbial interactions, and metabolic molecules (Srey et al., 2013). Recently, interest has increased in relation to the use of bacteriophages as potential candidates for attacking biofilms (Lu and Collins, 2007).

Bacteriophages, obligate parasites of bacteria, have promising features for controlling FBD-related pathogens, and are considered effective in inhibiting biofilm formation and dispersion of mature biofilms. They present a more natural approach compared to traditional methods used in safety and food preservation (Hosseinidoust et al., 2013). In this context, our study aimed to evaluate the action of a combination of bacteriophages for controlling *Salmonella* spp. biofilm on 3 different surfaces: stainless steel, glass, and polyvinyl chloride (**PVC**), all very common in the food industry, and also to characterize the formation of the biofilm on the surfaces above mentioned.

MATERIALS AND METHODS

Amplification and Selection of Salmonella *spp.*

One hundred and twenty-three *Salmonella* spp. samples were evaluated for their biofilm-forming ability. The samples were isolated from chicken carcasses and poultry farming equipment, belonging to the Laboratory of Ornithopathology, located at FMVZ - UNESP in Botucatu, Sao Paulo. The serotypes *S.* Enteritidis, *S.* Typhimurium, *S.* Heidelberg, *S.* Senftenberg, *S.* Mbandaka, and *S.* Kentucky used in this study were selected for their relevance, according to data on the prevalence of *Salmonella* spp. in FBD outbreaks (Silva and Duarte, 2002). Prior to the experiment, the strains were grown in 5 mL of tryptic soy broth (**TSB**) for 24 hours at 35°C and then diluted in TSB medium at a concentration of 1/20 (1/20 TSB), with a microbiological plate count until obtaining a culture concentration of 10^8 CFU/mL. Aliquots of 250 μL from this dilution of each sample were placed in triplicate in a flat-bottom microplate of 96 wells and incubated for 24 h at 35°C. Then, the contents of the plates were discarded and the plate washed 3 times with wash buffer solution (PBS; pH 7.2) to eliminate non-adhering cells. The remaining cells were fixed by adding 250 μL of 99% methanol for 15 min. The content was discarded again and, after drying the plate, 250 μL of the crystal violet dye 1% was added for 5 minutes. After this period, the excess dye was removed by washing in tap water and then 250 μL of 33% glacial acetic acid was added to the dry plate for 15 minutes for resolubilization of adhering cells. Finally, the reading was performed by using the microplate photometer (Thermoplate TP Reader, Tokai Hit., Japan) with a wavelength of 630 nm.

The samples were classified according to the optical density (**OD**) of the bacterial biofilm in the following categories: non-producing biofilm, weak, moderate, or strong producer of biofilm (Stepanović et al., 2000). The cutoff value for OD (**DOc**) was defined as 3 standard deviations above the average of the negative control, which is the 1/20 TSB not inoculated, spread from the beginning of the test, to correct the absorbance value. Strains were classified according to the following formula:

$$\text{OD} \leq \text{ODc} = \text{not producing biofilm}$$
$$\text{ODc} < \text{OD} \leq (2 \text{ x ODc}) = \text{weak producer of biofilm}$$
$$(2 \text{ x ODc}) < \text{OD} \leq (4 \text{ x ODc}) = \text{moderate producer of biofilm}$$
$$(4 \text{ x ODc}) \leq \text{OD} = \text{strong producer of biofilm}$$

Selection and Amplification of Bacteriophages

The ten samples of bacteriophages used in this study were previously isolated from hospital wastewater and poultry wastewater (Gonçalves et al., 2014). They belong to the Laboratory of Ornitopathology, FMVZ - UNESP in Botucatu, Sao Paulo. Bacteriophages stocks were obtained by incubating with *Salmonella* Enteritidis (Gonçalves et al., 2014). The selected bacteriophages exhibited a clean zone of lysis. For amplification before the experiment, 10 mL of doubly concentrated TSB medium was placed in conical-bottom tubes (Falcon, TPP., Transadingen, Switzerland) with a capacity of 50 mL. Then 1 mL of each bacteriophage and 2 mL of the turbid culture of *Salmonella* spp. were deposited. Each flask was mixed and incubated at 37°C for 18 hours. This culture was centrifuged at 1,096 × *g* for 15 minutes and the supernatant was filtered through a 0.22 μm pore size syringe filter then subjected to the same amplification process again.

Preparation of Analytical Sheets

This study used analytical units of stainless steel, glass, and polyvinyl chloride found in an industrial setting. To represent the surfaces of stainless steel and PVC, circular cuts were made with a laser (1 cm) in stainless steel with cold laminate finish 2B, and square cuts (1 cm^2) with scissors, respectively; for the glass surface we used circular glass coverslips (Glasscyto, MecLab., Jacareí, Brazil) 13 mm in diameter. The PVC and glass units were sterilized by soaking in 5% sodium hypochlorite (v.v.) for 2 hours, and then washing with sterile deionized water. The stainless steel sheets were sterilized by autoclaving at 120°C for 30 min. All materials were placed in Petri dishes and dried in an incubator; then 1% of the chips of each material was incubated in brain heart infusion broth (**BHI**) at 35°C for 24 hours to ensure the sterility of the chips.

Evaluation of Biofilm Formation in Analytical Surfaces (Experiment 1)

The samples of *Salmonella* spp. selected as biofilm producers were incubated in TSB at 37°C for 24 hours and then diluted 1:20 in TSB broth at a concentration of 10^8 CFU/mL, as described in amplification and selection of *Salmonella* spp. Following the methodology of Oliveira et al. (2014), analytical sterile chips were deposited at the bottom of each well of a 24-well cell culture plate (Cell Culture Plate, SPL., Life Sciences, Korea) and (Tissue Culture Test Plate, TPP., Transadingen, Switzerland), with the aid of sterile forceps. Then an aliquot of 600 μL of the dilution was deposited in triplicate in the wells containing the analytical unit. The negative control biofilm was done in triplicate on the plates, using 600 μL of non-inoculated TSB broth at 1/20 dilution. The plates were incubated at 35°C and then read after incubation at the following times: 12, 24, 36, 48, 60, 72, 84, and 96 hours.

After the incubation period, the plates were removed from the incubator and the chips were transferred to new sterile plates with the aid of sterile forceps. On new plates, the chips were washed three times with buffer (PBS) and stained with crystal violet at 1% for 15 minutes. After this period the sheets were washed again with PBS to remove excess of stain, and for resolubilization of the adhering cells 300 μL of 33% glacial acetic acid was added for 15 minutes. A volume of 200 μL was transferred to a flat-bottom 96-well microplate for reading in a TP-Reader. All *Salmonella* samples were cultured individually (triplicate) until each incubation time. The classification of samples as a biofilm formation was carried out according to the criteria described in amplification and selection of *Salmonella* spp.

Selection and Assessment of Bacteriophage Action on Salmonella spp. Biofilms (Experiment 2)

The evaluation of the bacteriophages action for biofilm control was performed from the administration of a combination, or *pool*, of bacteriophages with high lytic activity on biofilm forming *Salmonella* (Gonçalves et al., 2014). Twenty samples of *Salmonella* spp. were selected. They were able to form biofilms at least 3 times at every moment and on every surface, as described in Experiment 1. Each sample was preincubated at 37°C for 24 hours and then seeded in duplicate on plates containing Tryptone soy agar (**TSA**). Then 10 μL of the solution of each one of the ten isolated bacteriophage containing 10^9 PFU/mL was deposited on the plates seeded with *Salmonella* spp., with a total of 5 drops per plate. The Petri dishes were incubated at 37°C for 24 hours and the action of the bacteriophage was assessed by the presence or absence of lysis areas on the *Salmonella* host. From the results, we selected three bacteriophages that had greater lytic action for composition of a *pool* to be administered in *Salmonella* spp. biofilms. For the preparation of the *pool*, the bacteriophage samples were amplified separately and joined together in the ratio of 1:1:1 (volume of suspension), only at the end of each sample amplification process.

The action of the bacteriophage *pool* was evaluated in biofilm formation of shorter and longer times, totaling 8 times with 12 hours interval between them. The plates with the analytical units were prepared and incubated as described in preparation of analytical sheets. After each period of biofilm incubation, 300 μL of the bacteriophage pool with 10^8 PFU/mL was inoculated on the plate containing the biofilm and the analytical surface; then the readings were performed at 3 different times: 3, 6, and 9 hours after administration. In this step, *Salmonella* spp. were incubated in triplicate twice. One triplicate included and another lacked the addition of bacteriophages, with each sample being used as its own positive control. The negative control, 1/20 TSB broth was also used in all incubations.

For visualization of cells adhering to the surfaces of PVC, stainless steel, and glass, analytical chips were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 48 hours, and processed according to the Center's routine Electron Microscopy protocol of Institute of Biosciences - UNESP.

Statistical Analysis

The results obtained on the biofilm formation by *Salmonella* spp. on 3 surfaces were evaluated by the Goodman association test, complemented by multiple comparisons between and within multinomial populations (Goodman, 1965), at the 5% significance level. The data from Experiment 2 are valid, but do not support interferential statistical procedure and therefore will be presented descriptively.

RESULTS

Sample Selection for Biofilm Formation

To evaluate the formation of biofilms on surfaces, we selected samples that displayed a potential for biofilm production. Of the 123 samples available for study, only 58 proved able to form biofilms at least once. The number of biofilm-forming samples of each serotype, as well as their classification as biofilm production, are shown in Table 1.

Experiment 1

Fifty-eight *Salmonella* spp. samples were selected by their ability to form biofilm, as described in evaluation of biofilm formation in analytical surfaces, to evaluate the relation between the type of material composing the adhesion surface and the time required to establish biofilms. The frequency of the samples

Table 1. Selection of *Salmonella* for biofilm-forming ability. Number of forming samples and intensity classification of biofilm production.

	No. positive samples/samples in total (%)		
Sorovar	Weak	Moderate	Strong
Enteritidis	36/63 (57)*	–	–
Typhimurium	3/10 (30)	–	–
Heidelberg	10/14 (71)*	1/14 (7.1)	–
Mbandaka	6/14 (43)	–	–
Kentucky	2/10 (20)	–	–
Senftenberg	1/12 (8)	–	–

*Indicate the serotypes that were significantly different production from the others ($P < 0.05$).

Each sample was tested in triplicate.

forming biofilm was recorded at 8 different times (Figure 1), separated by 12-hour intervals. According to the results of the readings obtained, it was observed that the glass and stainless materials favored ($P < 0.05$) biofilm formation by *Salmonella* spp., but the PVC surface did not favor the production of biofilm. Furthermore, the glass demonstrated an advantage over stainless steel in biofilm formation at most times. The moments of 24 h and 48 h presented the highest frequency of biofilm formation by *Salmonella* spp.

Experiment 2

Initially 10 samples isolated from sewage bacteriophages used in this study were evaluated for their lytic activity against strains of *Salmonella* spp. able to produce biofilm. From the results shown in Table 2, it was possible to select those bacteriophages, namely 4.3, 4.8, and 5.7, that showed lytic action on most bacteria. Bacteriophagic action was observed in most samples, but the serovars Senftenberg and Mbandaka were more resistant than other serotypes.

To evaluate the action of the bacteriophage *pool* against *Salmonella* spp. biofilms on surfaces of stainless steel, glass, and PVC, 20 strains of *Salmonella* spp. were used. Only selected bacterial samples produced biofilm in at least 3 repetitions of the experiment conducted in Experiment 1. To verify the action of the bacteriophage *pool* over formed biofilms, OD-values of each bacteriophage-treated *Salmonella* spp. strain were compared with the values of each bacterial sample as positive control. The 3 h treatment with the bacterio-

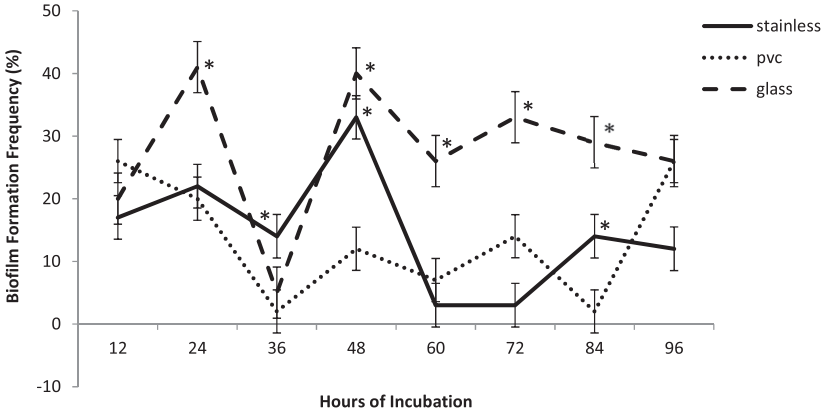

Figure 1. Frequencies of biofilm formation by *Salmonella* spp. on stainless steel, glass, and PVC surfaces. All *Salmonella* samples were cultured individually until each incubation time. The results are the mean of triplicates. ∗Indicates the time where there is statistical difference between the bonding surfaces ($P < 0.05$).

Table 2. Evaluation of lytic activity of bacteriophages against *Salmonella* strains capable of producing biofilm.

	Serotypes – number of positive samples/samples in total (%)						
Bacteriophages identification	Enteritidis	Heidelberg	Kentucky	Mbandaka	Senftenberg	Typhimurium	Total (%)
4.2	14/36 (39)	5/10 (50)	0/2 (0)	1/6 (17)	0/1 (0)	2/3 (67)	38
4.3	14/36 (38)	5/10 (50)	1/2 (50)	1/6 (17)	0/1 (0)	3/3 (100)	41
4.8	22/36 (60)	5/10 (50)	1/2 (50)	0/6 (0)	0/1 (0)	3/3 (100)	53
5.1	8/36 (21)	6/10 (60)	1/2 (50)	0/6 (0)	0/1 (0)	3/3 (100)	31
5.4	6/36 (18)	3/10 (30)	1/2 (50)	1/6 (17)	0/1 (0)	3/3 (100)	24
5.5	16/36 (44)	4/10 (40)	0/2 (0)	0/6 (0)	0/1 (0)	2/3 (67)	38
5.7	15/36 (42)	5/10 (50)	1/2 (50)	1/6 (17)	0/1 (0)	3/3 (100)	43
6.4	6/36 (18)	5/10 (50)	1/2 (50)	0/6 (0)	0/1 (0)	3/3 (100)	25
6.6	14/36 (38)	5/10 (50)	1/2 (50)	0/6 (0)	0/1 (0)	3/3 (100)	39
6.9	16/36 (44)	3/10 (30)	1/2 (50)	0/6 (0)	0/1 (0)	3/3 (100)	39

Amount and percentage (%) of *Salmonella* spp. susceptible to bacteriophagic action.

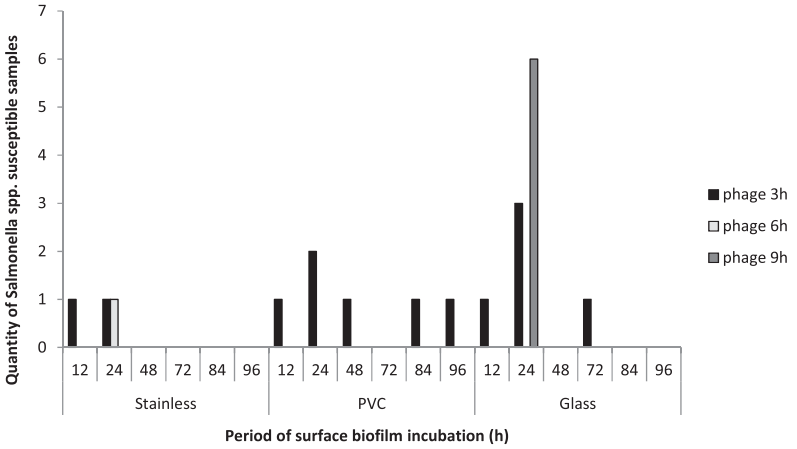

Figure 2. *Salmonella* spp. sample counts, likely the action of the bacteriophage pool in different incubation periods on stainless surfaces, glass, and PVC. The results are the mean of triplicates.

phage *pool* acted upon at least one sample at 12 and 24 hours of biofilm on stainless, at 12, 48, 84, and 96 h on PVC, and at 12 and 72 h on glass. But the same treatment was higher in biofilms at 24 h on PVC and glass, affecting double and triple samples, respectively. However, action at 6 and 9 h of phage treatment was only observed in biofilms at 24 h on the surfaces of stainless steel and glass, affecting one and 6 samples, respectively (Figure 2).

DISCUSSION

Although the food industry has evolved in recent decades, the risk of contamination during processing of food remains high. The presence of biofilm properties responsible for the processing and marketing of food is a major cause of contamination. Characterizing the behavior of microorganisms as to their predilection for some materials, and identifying the time that the bacteria require to become established in the environment, are essential for choosing a safe and efficient approach to ensure the consumer a safe sanitary product.

The present study evaluated whether bacteria of the genus *Salmonella* spp. may preferentially bond to particular surfaces commonly found in the food industry – namely stainless steel, glass, and PVC, by observing the evolution of the formation of these biofilms at 8 specific times – and especially whether the combination of bacteriophages of proven lytic action can eliminate or control the biofilm formation by these pathogens.

Strains of *S.* Enteritidis, *S.* Typhimurium, *S.* Heidelberg, *S.* Mbandaka, *S.* Senftenberg, and *S.* Kentucky were analyzed, totaling 123 samples. It was observed that only 47% of the samples were able to form biofilms on a polystyrene microplate, and that the serotypes Enteritidis and Heidelberg presented a significantly higher number of samples forming biofilm. Among the 58 samples selected for biofilm formation on surfaces, 57 of them were classified as forming weakly, while only one sample of the serovar Heidelberg obtained the moderate classification for its biofilm production capacity. However, other studies have shown *Salmonella* spp. samples with weak, moderate, and strong ratings for biofilm production (Stepanović et al., 2004; Vestby et al., 2009).

The findings obtained in Experiment 1 showed glass and stainless steel materials significantly favoring biofilm formation by *Salmonella* spp. Although both have favored the production of the biofilm, glass presented the highest production frequency at 24, 48, 60, 72, and 84 h of incubation, while the stainless steel showed favorable biofilm formation at 36, 48, and 84 h. Although many studies suggest the production of biofilm by some serotypes of *Salmonella* spp. on glass surfaces, stainless steel, and PVC (Prouty et al., 2002), our results showed no significant biofilm formation on the PVC surface samples. These results do not allow us to account for why there was no biofilm formation in this particular area, but many studies show that such factors as the finish, roughness, hydrophobic interactions, physical and chemical stability, etc., significantly interfere with the adhesion of cells and consequently with biofilm formation (Donlan, 2009; Schlisselberg and Yaron, 2013). From the data obtained in Experiment 1, a selection of samples able to reproduce the same biofilm-formation results as to the surfaces and incubation time for the completion of the second experiment was chosen; but only 34% of these samples showed the same results in three replicates, which suggests an inconsistency in the biofilm production pattern in *Salmonella* spp. used in this study.

Most bacteriophage isolated from sewage environments used in this study (Gonçalves et al., 2014) were able to reduce biofilm-forming strains of *Salmonella* spp., but the serovar Mbandaka was unaffected by 4 out of ten evaluated bacteriophages, while the Senftenberg serovar was not likely to present any bacteriophage action. The resistance presented by the serovar Senftenberg to the bacteriophagic action was also observed by Wall et al. (2010), who used samples of bacteriophages isolated from sewage to reduce the serovars Dublin, Enteritidis, Indiana, Kentucky, Litchfield, Schwarzengrund, Senftenberg, and Tennessee, reported that only

the serotypes Senftenberg and Kentucky showed no susceptibility to the action of the bacteriophage. The use of bacteriophages to control pathogens is becoming more widespread, but high specificity can pose problems regarding their host range. Within each type are subtypes of pathogens that need to be controlled, so that an effective bacteriophage should not have a very narrow or excessively wide range of hosts. One possibility is to overcome these limitations in the production of a bacteriophage cocktail to increase the range of lytic activity of serovars, including *Salmonella*. These have already proven effective in controlling other pathogens, such as *E. coli* O157:H7 in meat (McIntyre et al., 2007).

The application of a bacteriophage *pool* inhibited biofilm formation of *Salmonella* spp. on the 3 surfaces used in this study, concentrating its period of action at 3 hours. Biofilms adhering to the glass were resistant to 6 hours of treatment with the bacteriophage *pool*, but at 9 h of action, bacteriophages had started to reduce the biofilm. The decrease of bacteriophage action on *Salmonella* samples was also observed by Andreatti Filho et al. (2007), who evaluated the ability of bacteriophages isolated from poultry environment and a sewage treatment plant to reduce *S.* Enteritidis in vitro. The authors observed that the bacteriophages reduced recovery of *Salmonella* samples in 1.5 to 5 logs. Similar results were presented by Hosseinidoust et al. (2013), who reported that the combination of two known bacteriophages (PRD1 and P22) was able to control biofilm formation of *S.* Typhimurium, with the resistance decreasing the frequency in 1/100, while the use of bacteriophages PRD1 and P22 separately was able to decrease the biofilm levels only for 12 h and 24 h, respectively. Furthermore, there was a period in which the mixture of bacteriophages is no longer effective in controlling the biofilm, whose action was restored at 72 h of treatment. Among the hypotheses suggested by the authors to explain the absence of action at this time, is the appearance of a momentary resistance where the bacteria have become resistant to favor the selection of bacteriophages able to adapt to this change, generating a selective advantage and increased spectrum of bacteriophage action, and consequently results in the return of action on the biofilm (Comeau and Krisch, 2005). But another explanation of the same event would be the release and accumulation of a bacterial extracellular DNA, released at the time of lysis mediated by bacteriophages, substances that would be responsible for the increase in biofilm levels in the period (Gödeke et al., 2011). It should be also considered that natural biofilms rarely are of a single organism, making the treatment with a single bacteriophage or a *pool* of bacteriophages different in a multiorganism matrix, which normally occurs in a processing plant. The present study shows the importance of a specific approach in relation to the contact surface with the food and the microorganism involved in the contamination, as both are capable of interfering in the permanence of bacteria in industrial environments through the biofilm production. Thus, bacteriophage therapy presents an alternative method for a more specific and thus more secure approach, but its use still requires further study in order to elucidate its interaction with the microorganism and the environment so that it becomes a commercially viable tool.

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