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The Use of Vortex and Ultrasound Techniques for the *in vitro* Removal of *Salmonella* spp. Biofilms

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ABSTRACT

***Background*:** The presence of biofilm is common to all types of surfaces, such as stainless steel. Once formed, biofilms act as a point of constant contamination releasing fragments or planktonic cells of microorganisms, such as *Salmonella* spp., and may impair the microbiological quality of products. The laboratory methods are being used and tested *in vitro* for removal and the subsequent quantification of biofilms, including the use of vortex and ultrasound. The aim of the study was to evaluate the effectiveness of these two laboratory methods for removing *Salmonella* spp. biofilms, *in vitro* cultured on stainless steel surface from the food industry.

***Materials, Methods & Results*:** Three strains were analyzed for biofilm formation by *Salmonella* spp., and they are *S.* Typhimurium ATCC 14028, *S.* Enteritidis ATCC 13076 and *S.* Enteritidis isolated from drag swab on poultry farm and genetically confirmed by Microarray, denominated P106. Coupons of stainless steel AISI 316, with an area of 1 cm² were used. These materials used for the coupons were obtained from the equipments at the cutting room in the poultry slaughterhouse. Biofilms were formed using TSB broth without glucose and incubated at 36°C for 24 h. Six replicates were performed for each microorganism in each removal method. After the biofilm formation, two methods were used in the removal stage, the vortexing, performed for 2 min, and the sonication method, with coupons maintained for 10 min in an ultrasound bath, at a frequency of 40 kHz and potency 81 W. Serial dilutions were made and transferred to PCA agar for quantification in $\log_{10}$CFU.mL^{-1}. The microtopography was performed by scanning electron microscopy (SEM) of surfaces before the removal step. Statistical analysis of the results showed no significant difference between the two removal methods and between the three strains studied ($P > 0.05$).

***Discussion*:** Biofilms can be attached to any surface, biotic or abiotic. This adhesion of bacterias, especially pathogenic, can cause serious hygiene problems and economic losses due to impairment the microbiological quality of the products. Food safety is a concern for consumers and for the food industry, as well as the agencies responsible for public health. Research involving studies with the formation of microbial biofilms has become increasingly present. Laboratory methods are used for removing biofilms *in vitro* and subsequent quantification, including the use of the vortexing and ultrasound. The vortexing acts through tourbillon movements, removing or reducing the adhesion of microorganisms on a surface. Ultrasound destabilizes the structure of the biofilm, dislodging bacterial aggregates through physical, chemical and mechanical results of the cavitation process. These processes are responsible by the cleaning of surfaces with presence of adhered particles. In our study there was no statistical difference between the two removal methods, vortexing and ultrasound. There was also no significant difference between *S.* Typhimurium, *S.* Enteritidis and SE P106, which confirms the similarity between the two methods and among the studied strains. Although there was no statistical difference between the tested methods, the use of ultrasound in moderate frequency (40 kHz) and for 10 min, should be used as standard for detachment of biofilms formed *in vitro*, due to the ease of use and to their hydrodynamic properties that destabilize the structure of the biofilm.

Keywords: *Salmonella*, biofilms, vortex, ultrasound, detachment.

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INTRODUCTION

The presence of biofilm is common to all types of surfaces [16], who suffer wear and tear with the repeated use and increase the possibility of accumulation of soiling and bacteria [10]. Once formed acts as a constant contamination points, releasing fragments or planktonic cells of microorganisms, as *Salmonella* spp., may compromise the microbiological quality of products [8].

Among the materials used in equipments, stainless steel has been the major material of choice due to its resistance to corrosion and oxidation, by having greater durability and ease of cleaning and disinfection process [10,16].

Laboratory methods are been used and tested *in vitro* for removal and subsequent quantification of biofilm, including the use of vortex and ultrasound [12]. The vortex is a method often used in biofilm studies, and acts by swirling movements, through the effect of water flow motion. As a result removes or reduces the adhesion of microorganisms on a surface [15]. Ultrasound, when used in moderate frequency (20 kHz e 40 kHz), detach bacterial aggregates, acting through physical effects, resulting from chemical and mechanical acoustic cavitation [13].

With this aim, it sought to evaluate the efficacy of two laboratory methods, vortex and ultrasound, to remove biofilm of *Salmonella* spp., cultivated *in vitro,* in stainless steel surface from the food industry.

MATERIALS AND METHODS

The assays for comparison of methodologies for biofilms removal were performed in Bacteriology and Mycology Laboratory in the Veterinary Hospital at Faculty of Agronomy and Veterinary Medicine, University of Passo Fundo (FAMV/UPF).

Samples of Salmonella *spp.*

Three strains were analyzed for biofilm formation by *Salmonella* spp., and they are *S.* Typhimurium ATCC 14028, *S.* Enteritidis ATCC 13076 and *S.* Enteritidis isolated from drag swab on poultry farm and genetically confirmed by Microarray by Check&Trace[1] equipment, denominated P106. They were stored in Brain-Heart Infusion broth (BHI)[2] with 20% glicerol[3] frozen at -20°C. Were reactivated utilizing BHI[2] broth, incubated at 36 ± 1°C for 18-24 h, then plated on Xylose-Lysine Deoxycholate Agar (XLD)[2] and incubated at 36 ± 1°C. After 24 h was observed the pattern of colonies to assess whether they were compatible with *Salmonella* spp., and confirmed with biochemical and sorological tests.

Preparation of specimens

Coupons of stainless steel AISI 316 with an area of 1 cm² made in the dimensions of 1 cm X 1 cm and 0.1 cm of thickness were used as specimens. The materials used for the coupons were obtained from the equipments at cuts room in poultry slaughterhouse. The coupons were submitted to the following process: a) manual cleaning with sponge, water and liquid neutral detergent; b) rinse with distilled water; c) immersion in 70% ethanol (v/v) for 1 h at room temperature; d) rinse with distilled water; e) sterilization by autoclaving at 121ºC for 30 min.

Biofilm formation

For the biofilm formation, 12-well, flat-bottomed sterile polystyrene microtiter plates were used[5], into which 2.75 mL of TSB without glucose[4] were placed into each well with the stainless steel coupon and added 250 µL with approximately 10^3 CFU/mL of *S.* Typhimurium ATCC 14028, *S.* Enteritidis ATCC 13076 and *S.* Enteritidis SE106, and incubated at 36ºC for 24 h. All tests were conducted with six replicates for every microorganism individually and for the single removal method. The coupon was removed with sterile tweezers and immersed in 5 mL of peptone water (AP)[1] at 0.1% for 1 min to remove planktonic cells. After was transferred to another tube with 10 mL of AP[1] at 0.1% to removal of sessile cells.

Techniques for biofilm removal

After the biofilm formation, two methods were used in the removal step, the vortexing, performed for 2 min [15], and sonication method [16], with coupons maintained for 10 min in an ultrasound bath, at a frequency of 40 kHz and potency 81 W. Serial dilutions were made and transferred to PCA agar[1] for quantification with drop plate. To this end, the plate was divided into sectors and inoculated five drops of 10 µL of each dilution, incubated at 36ºC for 24 h. To determine the result was applied the formula: $CFU.cm^{-2} = (V_D/V_A).Av.D/A$, as follow: V_D: diluent volume used for rinse (mL), V_A: volume of the aliquot used in the plating (mL), Av: average of the counting obtained on the plates (CFU), D: dilution used for counting, A: coupon area (cm^2), and expressed in $\log_{10}.CFU.cm^{-2}$ [4,9].

Scanning Electronic Microscopy

The microtopography of the surfaces was performed by scanning electronic microscopy (SEM) at the

Electron Microscopy Center (CME) da UFRGS [18]. The coupons were immersed in 10 mL of PBS (KH_2PO_4, 0.31 M, pH 7.2) for 1 min, for removal planktonic cells, and fixed with glutaraldehyde 2.5%[3]. After, they were rinsed with 0.2 M phosphate buffer. Dehydration was carried out using acetone at 30%, 50%, 70%, 90% and 100%. After complete dehydration, the samples were placed in a critical point drying, and following on *stubs* for metallization.

Statistical analysis

The results obtained were examined by analysis of variance (ANOVA). The comparison of means was performed with Tukey test at 5% probability ($P > 0.05$).

RESULTS

All the three strains of *Salmonella* spp. formed biofilm after 24 h incubation in stainless steel, as can be seen in Figure 1. After removal and quantification, there was no significant difference ($P > 0.05$) between *S*. Typhimurium, *S.* Enteritidis and SE P106. Either, there was no significant difference between the two removal methods, vortexing and sonication by ultrasound ($P > 0.05$), according Table 1, confirming the similarity between the two methods and among the strains studied.

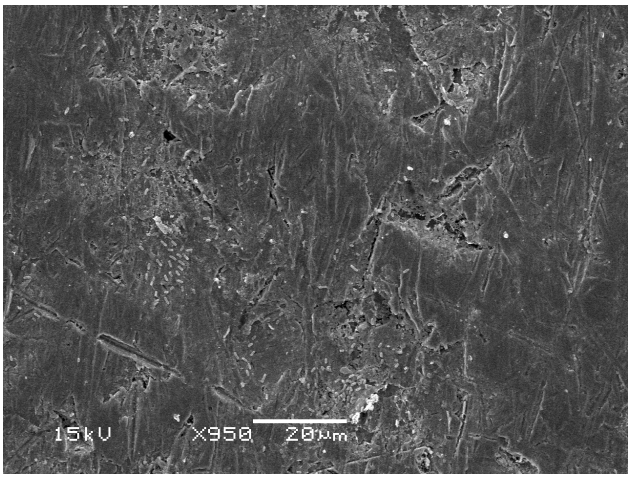


Figure 1. Stainless steel surface with biofilm formation by *S.* Enteritidis ATCC 13076.

Table 1. Vortexing and ultrasound methods in removing biofilms *in vitro* formed by *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* Enteritidis P106.

Sample	Vortexing (log_{10} CFU/cm²)	Ultrasound (log_{10} CFU/cm²)
S. Typhimurium	7,0264 Aa	7,6297 Aa
S. Enteritidis	7,2717 Aa	7,6831 Aa
SE P106	7,1681 Aa	7,2092 Aa

Means followed by the same capital letters in the lines and small letters in the same column, do not differ in the Tukey test ($P > 0.05$).

DISCUSSION

Biofilms are communities consisting of sessile cells, mono or multispecies [7]. They may be in any surface, biotic or abiotic, and are surrounded by a matrix of organic polymers, strongly adhered through of protein or polysaccharide filaments, known as extracellular polymeric substances (EPS) [3,5]. Adherence these bacteria, particularly the pathogenic, may cause serious hygienic problems and economic losses due to impairment of microbiological quality [2].

Although the use of food processing techniques and advanced hygienization in industry, the increasing number and severity of foodborne diseases around the world have increased considerably, worrying the consumers, as well as the agencies responsible for public health, in relation to quality of food [14].

Research with microbial biofilm formation it becomes ever more present, and different laboratory methods may be used for removing biofilms *in vitro* to subsequent quantification, including the vortex and ultrasound [12].

The vortexing is a methodology used in biofilm studies, and acts through typical tourbillon movements, by the effect of the water flow motion and as a result removes or reduces the adherence of microorganisms on a surface [15]. Despite the similarity between methods, ultrasound features cavitation effect, that is the formation of cavities or bubbles in the liquid medium, generating some gas, which may cause structural or functional changes in the cells due to disruption of molecular bonds [6]. Scherba *et al.* [17] demonstrated that a hydrodynamic property of the ultrasound destabilizes the biofilm structure at frequency

of 26 kHz, and the higher the intensity and the time of exposure, is greater the breaking of biofilm.

The sonication with ultrasound bath is able to inactivate the bacteria when used at high frequencies (512 kHz and 850 kHz). However, when used in moderate frequency (between 20 kHz and 40 kHz), acts dislodging bacterial aggregates through physical, chemical and mechanical effects of acoustic cavitation [13].

During this process, cavitation bubbles will produce enough energy to mechanical weaken bacteria or cells. This may happen through numerous processes, such as change in the bacterial surface induced by cavitation; pressure gradient, resulting from the collapse of gas bubbles colliding with the surface of biofilm; shearing force inside the biofilm; and chemical attack due to radical formation, such as H and OH^-, during the cavitation, weakening the biofilm [13]. These processes are responsible for cleaning of surfaces with presence of adhered particles. Similar results are cited by Lindsay & Holy [12], who studied the biofilm removal of *Pseudomonas fluorescens* in stainless steel surface, by three different methods, including the use of vortexing and ultrasound, and no significant differences was found.

Ultrasonic equipments have been used to estimate microbial contamination of surfaces. However, these must be small enough and removable, so that they can be immersed in diluent and put on the ultrasonic device, where the energy generated will result in release of the microorganisms [11]. Regarding the removal of biofilms, the ease of use of the ultrasound bath when compared to the vortexing can also be emphasized.

Ali *et al*. [1] evaluated the biofilms removal in ultrasound bath at different times, 5, 10, and 15 min, and observed that there was no statistical difference between the sonication times. Thus, they selected 10 min for use in all the tests, since the recovery of cells were consistent and elevated, and this time reduce the possibility of cell damage caused by prolonged sonication.

Based on this assumption, the ultrasound, when employed in frequency and time proper, becomes an excellent method for detachment of biofilms *in vitro*, mainly by destabilizing the biofilm properties and the usability.

CONCLUSION

Although there was no statistical difference between the tested methods, the use of ultrasound in moderate frequency (40 kHz) for 10 min should be indicated as the standard method for detachment of biofilms formed *in vitro*, due to its ease of use and hydrodynamic properties that destabilize the biofilm's structure.

MANUFACTURERS

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