ULTRASOUND USE FOR *LISTERIA MONOCYTOGENES* ATTACHED CELLS REMOVAL FROM INDUSTRIAL BRINE INJECTION NEEDLES

D. HAMANN[1], K.F. TONKIEL[1], A. MATTHIENSEN[2], J. ZENI[1], E. VALDUGA[1], N. PAROUL[1], C. STEFFENS*[1], G. TONIAZZO[1] and R. CANSIAN[1]

[1]Department of Food Engineering, Universidade Regional Integrada do Alto Uruguai e das Missões, Av. Sete de Setembro, 1621, Erechim-RS 99709-910, Brazil

[2]Empresa Brasileira de Pesquisa Agropecuária, Embrapa Suínos e Aves. BR 153 km 110, Vila Tamanduá, Concórdia-SC, 89700-000, Brazil

*Corresponding author: Tel.: +55 54 35209000, Fax: +55 54 35209090

E-mail address: claristeffens@yahoo.com.br

ABSTRACT

This work aimed at studying methods for *Listeria monocytogenes* attached cells removal from food industry equipment. In order to verify possible contamination points, a tracking was carried out in food products and equipment surfaces (brine injection needles) by swab. Brine injection needles were defined as a study due to their higher contamination potential. *L. monocytogenes* adhesion after 6 h contact with needles was observed. Minimum bactericidal concentration (MBC) of peracetic acid on *L. monocytogenes* attached cells was 0.06 and 0.24% with 6 and 24 h contact with bacteria, respectively. 75% power (700 W) and 14 min exposure ultrasound treatment showed a 3.25 Log CFU microbial load reduction in injection needles. The combination of ultrasound and peracetic acid showed an MBC reduction from 0.24 to 0.03%, hence, it may be recommended for meat industry application.

Keywords: *L. monocytogenes*, biofilms, sanitizer, ultrasound, food industry

1. INTRODUCTION

Brine application with or without seasoning (marination), is a process used to improve the physical and sensory characteristics of meat. This process involves immersing the meat in a brine solution to increase its fluid retention, to improve its sensory attributes, color, texture, flavor, protein addition, water binding capabilities, as well as meat safety (RUST and KNIPE, 2014). The application can be performed in different ways, either by immersion, injection, or massage (dynamic). Curing brine injection into marinated products or into pieces of meat is performed using injection needles. The needles shapes varies depending on the product to be injected.

However, the cleaning and sanitization of the injection system, especially the brine injection needles, is a hard process due to the difficulty for disassembling and carry out the complete sanitization and/or immersion. The industrial scale injection system cleaning is done by manual and/or mechanical scrubbing with alkaline detergent and subsequently, peracetic acid for sanitization. Scrubbing removes organic matter, while sanitization removes microorganisms.

The injection needles mounted onto the equipment, having their internal diameter reduced when only sanitized by the pressure drag system. This presents a potential for biofilm formation (or cell adhesion), due to the difficulty in total organic material removal, as sanitization does not cover the entire internal surface of the needle.

Biofilms are characterized by increased biomass accumulation of microorganisms and extracellular materials on a solid surface, detrimental to both human health and industrial processes (HORI and MATSUMOTO, 2010; BAN and KANG, 2015). BRANDA *et al.* (2005) defined biofilm as a structured bacterial cells comunity embedded in an exopolysaccharides matrix produced by the adhered cells themselves on either an abiotic or biotic surface. The cells presented greater resistance to antimicrobial agents and detergents when biofilm is formed. This resistance could be partially related to the passage through the extracellular matrix due to cells phenotypic changes in the biofilm structure and on the resistance mechanism generated by the three-dimensional structure.

Studies show that washing and sanitizing used by the food industry do not always guarantee complete biofilms elimination (BANACH *et al.*, 2015; MALHEIRO and SIMÕES, 2017). Adhered cells are highly resistant to acid disinfection, as they also show tolerance/resistance to disinfectants' lethal concentrations due to exopolysaccharides production that protects against chemical agents (CARPENTIER and CERF, 1993; MYSZKA and CZACZYK, 2011).

Bacterial adherence to stainless steel, glass, and polypropylene surfaces is a potential source of contamination, which may lead to disease transmission (CHAVANT *et al.*, 2007). One of the most serious food-borne diseases is listeriosis, caused by *L. monocytogenes*, due to its sequelae degree and high lethality index (20 to 30%) (BAN and KANG, 2015). *L. monocytogenes* may persist in industrial equipment and installations due to its high adhesion potential and biofilm forming capacity at low temperatures (CARPENTIER and CERF, 2011; BELTRAME *et al.*, 2016).

Among the sanitizers used in the food industry against food-borne pathogens, peracetic acid is excellent for Gram-positive and Gram-negative bacteria, filamentous fungi and yeasts, viruses, and bacterial spores. In addition, it attacks the proteins' cell wall and migrates into the cell, breaking internal components (MCDONNELL and RUSSELL, 1999). However, in order to be effective, it requires direct contact with the microorganism for a period of time, which varies depending on the sanitizer concentration.

Ultrasound is another widely investigated method, responsible for thinning bacteria cell walls, making them more susceptible to rupture and inactivation. Ultrasound can be combined with other treatments in the food industry, such as heat and chemicals to

inactivate bacteria (BETTS *et al.*, 2014). In addition, it has been reported that this process could be effective in biofilm removal (SREY *et al.*, 2013).
Ultrasound presents a cavitation effect, which is cavities or bubbles formed in the liquid medium, resulting in a certain amount of gas that can lead to structural or functional changes on cells due to molecular bonds disruption (JOSÉ and VANETTI, 2012). The energy generated by this process releases microorganisms from biofilms (JAY, 2005). Ultrasound hydrodynamic properties destabilize the biofilm structure, and higher power and longer exposure time were associated with improved bacteria elimination in biofilms (SCHERBA *et al.*, 1991).
Due to *L. monocytogenes* inherent risks in the food industry, it is necessary to identify and map out the most favorable sites for microbial adherence and provide effective methods for contaminant cells and biofilms elimination, avoiding cross contamination. In this sense, this work aims at tracking microbial contamination spots in a large meat product plant and evaluate the effects of using ultrasound alone and its combination with peracetic acid in the *L. monocytogenes* removal, previously adhered to brine injection needles under industrial conditions.

2. MATERIALS AND METHODS

2.1. Tracking contamination spots in a meat production plant

The study was carried out in a slaughterhouse located in Southern Brazil, which industrializes pork meat products, inspected by the Brazilian Inspection Body. The plant has a wide range of products such as seasoned and cold cuts, as well as fresh and smoked sausages.
Microorganisms presence in processed products was evaluated before freezing in order to map out possible microbial contamination spots in the plant. Analyses for *Salmonella* sp. and *L. monocytogenes* were performed after product handling in the following sectors: (A) cutting and (B) seasoning "traditional technique, used to tenderize and improve the meat flavor and succulence, similar to marinated products use".
After data evaluation for each product and sector, a surface swab analysis of the highly contaminated site was performed. The analyzed spots were drain/gutter, kick/mat, internal equipment, wall/floor, pallet/container, and roof.
Analyses were carried out on a weekly basis between January and September, in products and different surfaces, aiming at being indicative of production conditions.

2.2. *L. monocytogenes* adhesion on brine injection needles

L. monocytogenes (ATCC 7644) adhesion in brine injection needles was evaluated after identifying the main contamination spots.
For bacterial adhesion experiments, hypodermic needles were used (120 mm length x 3.0 mm external thickness x 1 mm internal thickness), which are commonly used in the industry for brine application by injection process. For this study, used needles were employed (worn out due to usage), aiming for the most favorable conditions for biofilms formation or cell adhesion.
The needles were washed with running water, then scrubbed with liquid neutral detergent, rinsed with running water, followed by distilled water and disinfected in distilled boiling water immersion (100°C), for 5 min.
After sanitization and disinfection, the needles were immersed in a Luria Bertani - LB broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) previously inoculated with 0.1

mL *L. monocytogenes* pre-inoculum (~ 1x10^{8} CFU/mL) and incubated at 35-37°C for 3, 6, 12, and 24 h contact with the needles to allow bacterial adhesion. After incubation, the needles were removed from the LB broth and rinsed with deionized water for 15 s, followed by three washes with sterile water (vortex 20 s). Bacterial adhesion was evaluated by external swab on the needle and standard counting in LB agar plates in accordance with Barbosa *et al.*, (2016) and Beltrame *et al.*, (2016).

2.3. *L. monocytogenes* removal on brine injection needles by ultrasound

An ultrasound probe (20-kHz QSonica, CT., USA) was used to evaluate the effects of *L. monocytogenes* removal on brine injection needles at different times (0, 3.5, and 14 min), with 30 s intervals in ice bath, at different powers (60, 75, and 90%/700 W). The times were chosen due to equipment limitation. Ultrasound application schematic representation on injection needles are shown in Fig. 1. The brine injection needles were previously subjected to adhesion (described above), ultrasound treatment was applied and then, the ultrasound-removed liquid (0.1% peptone water) was plated in LB agar medium.

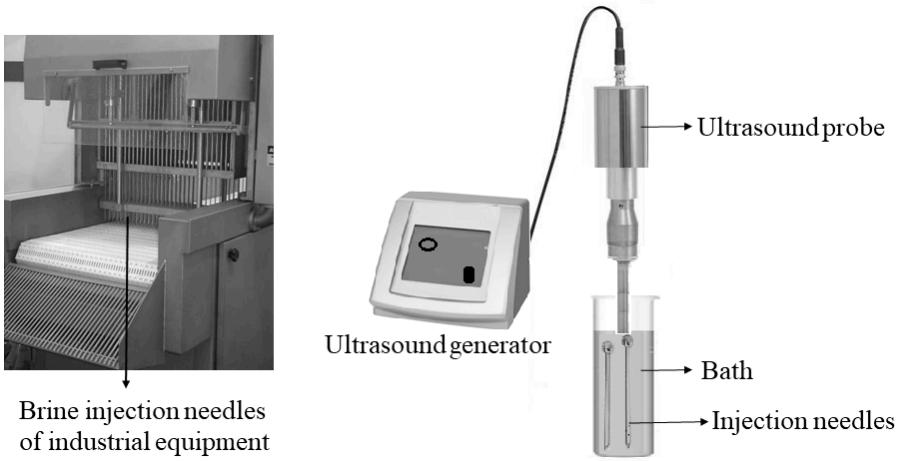

Figure 1. Representations of ultrasound treatment on brine injection needles.

Dilutions were performed in peptone water for surface plating on LB agar-containing plates, incubated at 35-37°C for 24 h, followed by colony counting. All analyses were performed in triplicate. The microbial counting was expressed in Log CFU, in relation to time 0 (control).

2.4. Minimal bacterial concentration determination

Assays were performed using peracetic acid in order to evaluate the MBC and all experiments are performed in triplicate. The needles were incubated with test microorganism for 6 and 24 h growth to simulate the times used in industrial sanitization. After incubation, three rinses with deionized water were performed, and then they were immersed in test tubes containing 30 mL chlorinated alkaline detergent solution (Easyfoam/Diversey) and/or a combination with acid detergent (Kalyclean 244) for 10 min, as specified by the manufacturer.
After detergent application, the needles were immersed in peracetic acid sanitizer (17% - ECOPER Quimica) at different concentrations (0.03, 0.06; 0.12, 0.24, and 0.30%). After

10 min contact with the sanitizer (as per manufacturer's recommendation), the needles were submitted to external swab and inoculated on LB agar (LB and agar 15 g/L), and incubated at 35-37°C for 24 h, followed by colony counting.
MBC was evaluated on needles after *L. monocytogenes* adhesion for 6 and 24 h, then rinsed with sterile water to remove unbound cells and sprinkled inside and outside with a 10 mL sanitizing solution. The industrial peracetic acid-PA sanitizer (17% - ECOPER Quimica) was evaluated at different concentrations (0.015, 0.03, 0.06, 0.12, 0.24, and 0.30 %) for application to previously incubated needles for 6 and 24 h. After 10 min contact with the needles, they were internally sprinkled with a 10 mL peptone water solution (peptone 10.0 g/L, hydrogen phosphate 9.0 g/L, NaCl 5.0 g/L, phosphate potassium 1.5 g/L) and 3% Tween-80 for residual peracetic acid inactivation. The liquid was plated by immersion in LB agar (LB and agar 15 g/L), incubated at 35-37°C for 24 h, followed by colony counting.

2.5. Effect of ultrasound and peracetic acid combination

The effect of ultrasound and peracetic acid combination on MBC reduction with sanitizer were evaluated through the best result for *L. monocytogenes* removal using ultrasonic method combined with different peracetic acid concentrations (0.03, 0.06, 0.12, 0.24, and 0.30%).

2.6. Microbiological analysis

L. monocytogenes: Presence/absence analyses in 25 g sample were carried out according to ISO 11290-1 (1996).
Salmonella sp.: The analysis was performed using VIDAS equipment, according to methodology described by AOAC (2016).

2.7. Statistical analysis

The ultrasound effect results were subjected to Tukey test at a 5% significance level for comparison between the means. All statistical analyses were performed using software SPSS Student version.

3. RESULTS AND DISCUSSION

3.1. Plant Contamination Tracking

The seasoned meat products of the studied industry were submitted to brine injection, both in the early and intermediate stages. In addition, the equipment was used to temper an array of products. The injection system was formed by needles block with an extremely small diameter. Thus, the injection step turns into a microbiological control point to optimize both hygiene and sanitation, due to possible cross-contamination inside the industrial plant.
L. monocytogenes presence may indicate a hygiene system failure that should be evaluated in order to identify the contamination source (HENRIQUES *et al.*, 2016). This reinforces the importance of an effective diagnosis to evaluate sanitation system by microbiological tests.
Table 1 shows results of L. *monocytogenes* and *Salmonella* sp. presence in different handling Sectors (A and B) of the industrial plant.

Table 1. Results in percentage of the presence of *L. monocytogenes* and *Salmonella* sp. in different sectors (A and B) of the industry, during the period between January and September.

Sectors	Presence of *L. monocytogenes* (%)	Presence of *Salmonella* sp. (%)
A (n=450 analysis)	0	3
B (n=315 analysis)	47	8

The products handled in sector B presented *L. monocytogenes*, contamination, while no contamination was observed in sector A. The *Salmonella* sp. contamination was higher in sector A, but with low indexes in relation to sector B. Considering the higher *L. monocytogenes* incidence in sector B, that site was choosen to continue the evaluations for that bacterium.

In order to track *L. monocytogenes*, contamination points in sector B, swab analyses were performed on surfaces in contact with the product. The points were mapped out and divided into the following categories; drain/gutter, kick/mat, internal equipment, wall/floor, pallet/container, and roof. The swab results after operational hygiene showed no presence of this bacterium at the different points analyzed.

Sector B processed food products were analyzed in order to confirm *L. monocytogenes* contamination. The results demonstrated contamination only in the products after brine injection process, since there was no detected contamination before injection in any products. In order to track the possible contamination points, the brine solution was subsequently evaluated, which showed no *L. monocytogenes* presence

Since all swab-tested injection equipment, points showed no contamination, the possible contamination may be in the needles, as the swab analysis was not performed due to their reduced internal diameter. Therefore, this point was chosen to study biofilm formation and removal, since there is a lack of brine injection needles microbial contamination studies, which are difficult to sanitize due to their shape.

3.2. *L. monocytogenes* adhesion on brine injection needles

The results presented in Fig. 2 demonstrated *L. monocytogenes* adhesion on brine injection needles. An initial fast growth can be observed up to 12 h, tending to stabilize in 18 and 24 h.

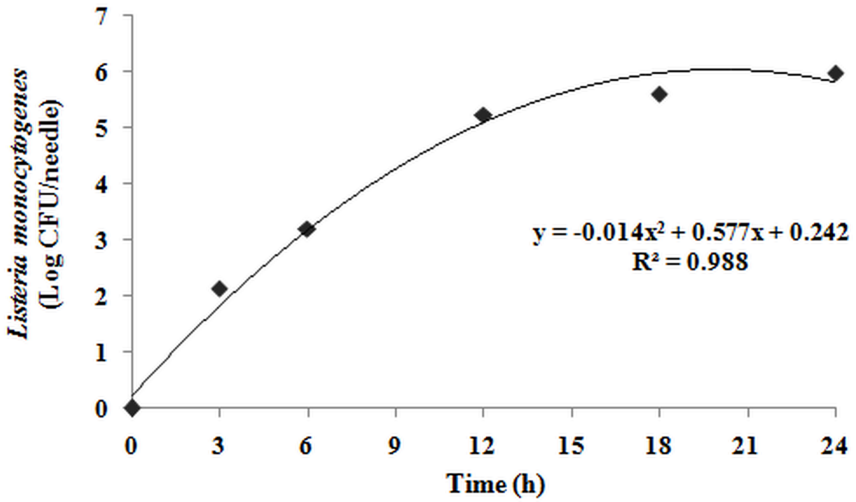

Figure 2. *L. monocytogenes* adhesion on brine injection needles.

Biofilm formation can occur in a short period of time and in relation to counting (bacterial cells adhesion) (BARBOSA *et al*., 2016). According to BARBOSA *et al.* (2016) and BELTRAME *et al.* (2016) a 3.0 log CFU or $1x10^3$ CFU adhering cell counting per square inch may be identified as biofilm formation. In this way, according to the above mentioned authors' criteria, *L. monocytogenes* attached cells occurred after 6 h contact with injection needles.

3.3. *L. monocytogenes* removal adhered to injection needles using ultrasound

Table 2 shows the *L. monocytogenes* counting reduction adhered to brine injection needles at different contact times (0, 3.5, and 14 min) and in different ultrasound powers (60, 75, and 90%/700 W), at 20 KHz.

Table 2. Reduction of the *L. monocytogenes* count (Log CFU/cm^2) as a function of contact time and ultrasound power. Results in Log CFU/cm^2.

Ultrasound power (20 KHz/700W)	Contact time (min)	*L. monocytogenes* initial count (CFU)	Reduction (Log CFU)	Reduction (%)
60%	0 (control)	$1.4x10^{6\ ab}±6.1x10^3$	-	0
60%	3.5	$9.3x10^{4\ bc}±8.3x10^2$	1.18	93.36
60%	14.0	$2.0x10^{4\ c}±8.9x10^2$	1.84	98.57
75%	0 (control)	$1.4x10^{7\ a}±8.6x10^3$	-	0
75%	3.5	$2.0x10^{5\ b}±3.1x10^3$	1.85	98.57
75%	14	$9.7x10^{3\ cd}±2.4x10^1$	3.25	99.93
90%	0 (control)	$1.3x10^{5\ b}±4.4x10^2$	-	0
90%	3.5	$4.3x10^{3\ d}±4.9x10^1$	1.48	96.69
90%	14.0	$1.8x10^{3\ d}±1.9x10^1$	1.86	98.61

Means (± standard deviations) followed by same letters on the column, represents no significant difference at 5% level (Tukey test).

It was observed that the increased exposure time caused a reduction in the microbial counting of 1.18 to 1.84 Log UFC with 60% power, 1.85 to 3.25 Log UFC with 75%, and 1.48 to 1.73 Log UFC with 90% ultrasound power (Table 2). This reduction could be explained by the microbial death mechanism generated by cell membrane wear, heating, and free radicals production (BRONDUM *et al.*, 1998; BUTZ and TAUSCHER, 2002).
Although at the highest power (90%), the initial microbial counting was lower than the one at 60% power, the Log UFC reduction after 14 min sonication was similar. However 3.5 min high-power sonication resulted in a higher reduction than the 60% power, indicating that the contact time and power influenced the microbial destruction of the attached cells. PIVASENA *et al.*, (2003) reported that exposure/contact time and ultrasonic waves amplitude affect microbial inactivation effectiveness.
In food processing, high-power ultrasound is able to cause cavitation, consequently causing microbial inactivation and elimination. The ultrasound waves create cavitation bubbles that pass through the solution creating a negative pressure, hence breaking both the cell wall and membrane structures (BILEK and TURANTAS, 2013).
The highest reduction (3.25 Log CFU) among treatments was observed at 75% power. This may be justified, since, according to ERRIU *et al.* (2014) potency and contact time may be effective for biofilm removal or may be a formidable bacterial viability enhancer.

Ultrasound effectiveness in biofilm removal is dependent on the bacteria being treated (PIVASENA *et al.* 2003). *L. monocytogenes* could be inactivated by ultrasound combined with other treatments. FERRANTE *et al.* (2007) observed that high-intensity ultrasound combined with mild heat treatment and natural antimicrobials was effective for *L. monocytogenes* inactivation in orange juice.

3.4. Peracetic acid MBC of peracetic acid on *L. monocytogenes*

The efficacy of different peracetic acid concentrations (0.03; 0.06; 0.12, 0.24, and 0.30%) was assessed by establishing the MBC. Such evaluation was performed to simulate industrial conditions of sanitizer application under pressure on the injection needles for 10 min until rinsing (due to needles disassembling difficulty). The MBC of peracetic acid was 0.24% (v/v) and 0.06% (v/v), respectively for 24 h and 6 h incubation with *L. monocytogenes* (Fig. 3).

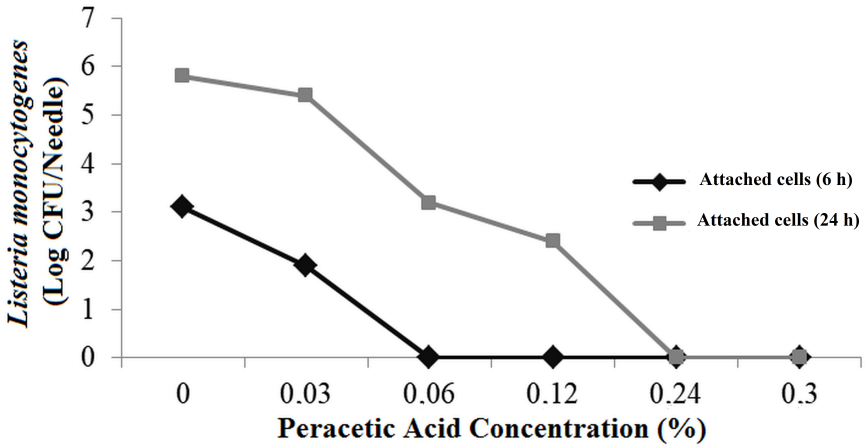

Figure 3. Bactericidal action of peracetic acid on *L. monocytogenes* on brine injection needles.

The data found in this work may be compared to the one found by POIMENIDOU *et al.* (2016), who found a MBC of peracetic acid on *L. monocytogenes* in food processing plants ranging from 115 to 2713 ppm (0.0115 to 0.2713%). The authors also observed higher MBC for longer incubation times. Therefore, incubation time is directly related to MBC, supporting a possible biofilm formation and increase resistance to sanitizers.
BELESSI *et al.* (2011) studying *L. monocytogenes* biofilm resistance under food processing conditions found that the number of surviving bacteria decreased as the contact time at 2% peracetic acid increased.

3.5. Effect of ultrasound and peracetic acid combination

MBC for the combination of ultrasound (14 min to 75%- 20 kHz) with peracetic acid concentrations (0.03; 0.06; 0.12, 0.24, and 0.30%) on *L. monocytogenes* removal from brine injection needles with (24 h contact) was evaluated. Ultrasound use at a 75% power for 14 min showed a 3.25 log CFU reduction, however; without total reduction, it was not possible to establish an ultrasound minimum bactericidal power/time. On the other hand, the combined method reduced the MBC of peracetic acid from 0.24% to 0.03% (Fig. 4),

demonstrating the combined method effectiveness with a low peracetic acid concentrations.

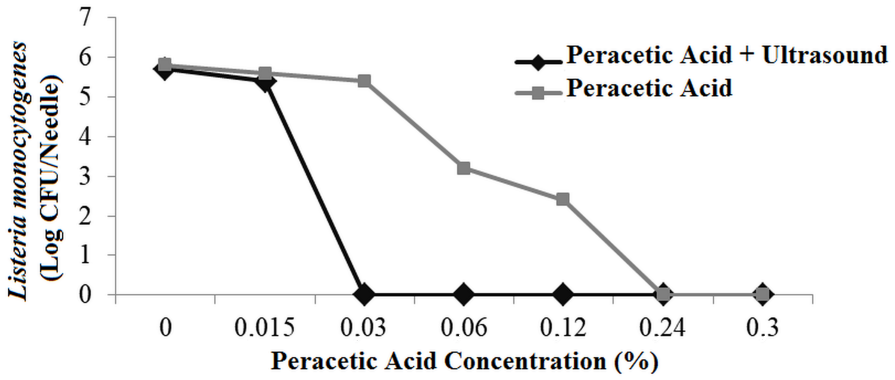

Figure 4. Effect of combined ultrasound and peracetic acid methods in the *L. monocytogenes* removal on brine injection needles.

The ultrasound process was studied as a "green", non-chemical technology in the industrial plant to improve meat quality and safety. Based on the *L. monocytogenes* removal results combining ultrasound and peracetic acid methods, this sanitization process could be suitablefor the food industry.

As mentioned in a number of literature studies, the combined method was effective in many quality criteria processes, such as tenderness, changes in proteins functional properties, shelf life improvement, microorganisms inactivation in meat and its products (TURANTAS *et al.*, 2015), knives sanitization used in the meat industrial plants (BRASIL *et al.*, 2017), raw salmon fillets (MIKS-KRAJNIK *et al.*, 2017), and fresh-cut bell pepper (LUO and OH, 2016). The combination of ultrasound and peracetic acid could potentially increase the microorganism inactivation rate (D value reduction) using the ultrasound cavitation process, either partially removing attached cells or facilitating sanitization.

4. CONCLUSIONS

It was observed a greater contamination with *L. monocytogenes* in relation to *Salmonella* sp. during the tracking. The tracking in the sector B indicated the brine injection needles as the source of contamination. The *L. monocytogenes* on the needles indicated cells adhesion after 6 hours of contact. The peracetic acid showed a MBC of 0.06 and 0.24% for attached cells with 6 and 24 h of contact, respectively. The application of 75% (700W) of the ultrasound power and 14 min of exposure time in the needles provided a microbial reduction of 3.25 Log CFU. The combined use of ultrasound (75%- 700W, 14 min) with peracetic acid showed a reduction of MBC from 0.24% to 0.03%. The results indicate that the combined use of ultrasound and sanitizer may increase food safety, removing the *L. monocytogenes*, and/or serve as a specific treatment when this bacterium occurs in the industry.

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