academicJournals

Vol. 9(27), pp. 1706-1715, 8 July, 2015
DOI: 10.5897/AJMR2015.7498
Article Number: 8AF3B9A54170
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Resistance to disinfectants and antibiotics of *Pseudomonas* spp. and *Listeria* spp. biofilms on polystyrene and stainless steel

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Received 27 March, 2015; Accepted 6 July, 2015

The capacity of *Pseudomonas* spp. and *Listeria* spp. isolates in forming polystyrene and stainless steel biofilms was assessed and their resistance to disinfectants and antibiotics agents was verified. Isolates originated from chicken and buffalo meat cuts in abattoirs and retail outlets in the southern region of the state of Rio Grande do Sul, Brazil. Isolates which formed stainless steel biofilm were tested with regard to the activities of the disinfectant agents organic chlorine and ammonium quaternary. Isolates of *L. monocytogenes* formed polystyrene and stainless steel biofilm. Further, 32 and 72% of *Pseudomonas* spp. isolates respectively formed polystyrene and stainless steel biofilm. The disinfectant agent ammonium quaternary was more efficient than organic chlorine in the decrease of biofilms on stainless steel surfaces for *Listeria* isolates. Multi-resistance to antibiotics was high for *Listeria* spp. (94.7%) and *Pseudomonas* spp (84%). From these results, isolates from chicken and buffalo meat cuts were developers of biofilm on polystyrene and stainless steel, and resistants' to antibiotics, putting at risk consumers´ health.

Key words: Bacterial adhesion, ammonium quaternary, organic chlorine, chicken meat, buffalo meat.

INTRODUCTION

Increase in consumer demands with regard to the hygiene and sanitary conditions of meat has made producers focus on improvement in microbiological quality and food safety. Meat products are frequently

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associated with occurrences of food transmitted diseases (FTD) since meat is one of the best medium for the development of bacteria. Bacteria which develop in meat may be connected to deterioration processes or even to the transmission of diseases (Doulgeraki et al., 2012).

Bacteria of the genera *Pseudomonas* and *Listeria* may multiply and survive in fridge temperatures and may develop in cold rooms or throughout the cold chain where meat is normally stored (Jay, 2005; Todd and Notermans, 2011). *Pseudomonas* spp. are particularly deteriorating bacteria and are in the main the cause of the meat´s sensorial alterations, with a consequent decrease in shelf life (Arslan et al., 2011). Within the context of pathogenic bacteria, *Listeria monocytogenes* causes listeriosis, a serious disease with high lethality rates in risk groups (20-30%) (Lecuit and Leclercq, 2012; EFSA, 2012).

Food industries, especially meat industries, have to face several problems related to cleaning processes and sanitization of utensils and equipments. These problems are often related to the inefficiency of hygiene products and of hygiene processes in the killing or inactivating of microorganisms from the environment, with the subsequent transformation of the sites into focuses of crossed contamination. The above is due to the formation of bacterial biofilms on the equipments and in the production chain.

The formation of biofilms is enhanced in such an environment; it is actually caused by the accumulation of organic and inorganic material used by microorganisms for their fixation on the surface and the subsequent development of biofilms where communities of bacteria establish themselves and resist for long periods (Uhitil et al., 2004; Oliveira et al., 2010).

The term biofilm was created to describe the sessile form of microbial life, characterized by adhesion of microorganisms to biotic or abiotic surfaces, with consequent production of extracellular polymeric substances (Nikolaev et al., 2007; Steenackers et al., 2012). In fact, food industries, especially the processing section, are greatly impaired by biofilms which adhere to various types of surfaces especially stainless steel equipments and utensils (Marques et al., 2007; Sofos and Geornaras, 2010). Further, these bacteria are more resistant to antimicrobial activities and to disinfectant agents, causing deterioration and loss of quality in food and the dissemination of pathogens (Stepanovic et al., 2004; Hamanaka et al., 2012).

Bacterial cells in biofilms may be up to one thousand times more resistant to antibiotics than in their planktonic condition (Ouyang et al., 2012; Sharma et al., 2014). During the last decades, antimicrobial resistance, especially the multi-resistant ones, has been considered a major public health issue worldwide. The excessive and inadequate use of antibiotics may trigger the emergence of resistant bacteria favoring the dissemination of antimicrobial resistant genes in the environment (Filiousis et al., 2009; Domenech et al., 2015).

The relevance of in-depth studies on pathogenic bacteria, with special mention of *Pseudomonas* spp. and *Listeria* spp. mainly derived from animal-derived food, such as chicken and buffalo meat, should be underscored. Further, the formation process of biofilms of these bacteria in the food industry should be understood, coupled to their resistance to antibiotics and disinfectant. Preventive and corrective attitudes throughout the food chain to warrant consumers´ health will be adopted.

Current assay aimed at assessing the capacity of *Pseudomonas* spp. and *Listeria* spp. originating from chicken and buffalo meat cuts in abattoirs and retail outlets in the southern region of the state of Rio Grande do Sul, Brazil. In the formation of biofilms on polystyrene and stainless steel objects. Considering it biofilm-forming bacteria show greater resistance to antibiotics, like drugs or industrial disinfectant; the resistance to disinfectant agents used in the food industry, and to antibiotics commonly employed in people and animals also will be evaluated.

MATERIALS AND METHODS

Bacterial isolates

Current assay employed 69 bacterial isolates. Among the isolates from buffalo meat, there were fourteen isolates identified as *Listeria* species (1 isolate of *L. innocua*; 1 isolate of *L. rocourtiae* and 12 isolates of *L. grayi*) and twenty-five 25 isolates identified as *Pseudomonas* genus. Although not all species of *Listeria* used in this study are pathogenic to man as *L. monocytogenes*, some are pathogenic to animals and all have similar characteristics and the presence of a species, among chosen in this study, may indicate of the possible presence of *L. monocytogenes*. Considering the character of deterioration of the genus *Pseudomonas* in meat, and his capacity to biofilm forming, the genus identification was sufficient for selecting the isolated. They all came from a buffalo abattoir in the southern region of the state of Rio Grande do Sul, Brazil, borrowed from the bacterial bank of the Laboratory of Inspection of Animal derived Products of the Universidade Federal de Pelotas (UFPel). In the case of isolates from chicken meat, five were *L. monocytogenes* and 25 *Pseudomonas* spp. derived from the carcasses and meat cuts of chickens from a fowl abattoir and from the retail market in the southern region of Rio Grande do Sul, Brazil. Table 1 shows the isolates´ origin.

The species of *Listeria* spp. isolated from buffalo meat were confirmed in a previous study with PCR molecular tests with specific primers (data not shown), whereas the species of isolates from chicken meat were confirmed by serological tests undertaken at the Osvaldo Cruz Institution (FIOCRUZ). The genus *Pseudomonas* spp. was confirmed by biochemical phenotype tests. All isolates were frozen in a Brain and Heart Infusion Broth (BHI, Acumedia®) supplemented with glycerol (25%) till use. *Listeria* spp. isolates were recovered in Tryptone Soy Broth supplemented with 0.6% yeast extract (TSB-YE, Acumedia®) whereas *Pseudomonas* spp. isolates were recovered in a BHI broth.

Evaluation of the biofilms on polystyrene

Bacteria isolates were assessed according to their capacity for biofilm formation on polystyrene microplates following method by

Table 1. Origin of *Pseudomonas* spp. and *Listeria* spp. isolates from chicken and buffalo meat, in southern Brazil.

Isolates (n)	Origin
L. monocytogenes (3)	Chicken carcass from processing
L. monocytogenes(2)	Chicken cut from retail outlet
L. rocourtiae(1)	Buffalo carcass from processing
L. innocua(1)	Buffalo meat cut vacuum packed
L. grayi(8)	Buffalo meat cut vacuum packed
L. grayi(4)	Buffalo carcass from processing
Pseudomonas spp (14)	Chicken carcass from processing
Pseudomonas spp (11)	Chicken cut from retail outlet
Pseudomonas spp (16)	Buffalo meat cut vacuum packed
Pseudomonas spp (9)	Buffalo carcass from processing

Stepanovic et al. (2007), with modifications. Isolates were cultivated in Tryptone Soy agar (TSA, Acumedia®) at 37°C for 18 h (h) and later the bacterial concentration of the suspension was standardized by McFarland scale at 0.5, corresponding to 8 Log of Colony Forming Units per milliliter (CFU/mL). Using exactly the same volumes used with success by Stepanovic a 20 µL aliquot of the standardized suspension was distributed on microplate wells with BHI broth (180 µL, this concentration was diluted 10x on microplate) and incubated at 35°C for 24 h. Negative control comprised 200 µL of BHI broth without inoculum, whereas positive control comprised 180 µL of BHI broth and 20 µL of standardized suspension with *Staphylococcus epidermidis* (ATCC 25923) which was previously tested and classified as biofilm former. After biofilm formation, were realized modifications in relationship at protocols used by Stepanovic et al. (2007), whereas the maximum volume of each well is 200 µl the plates were washed three times with 200 µL of a sterile saline solution (NaCl 0.9%, pH adjusted to 7.0) to remove all non-adherent cells to the plate. Microplates were inverted on absorbing paper for drying. The samples were then fixed in 150 µL methanol (CH_3OH) for 20 min. After this span of time, the methanol was disposed of and the plates were kept upside down during 18 h. Adherent cells were stained with 150 µL violet crystal (0.5%) for 15 min. The stain was then removed under running water and, after drying for 3 min, 150 µL ethanol (CH_3CH_2OH) (95%) were added. Plates were kept at rest for 30 min and biofilms were counted. The optic density (OD) of the bacterial biofilm was quantified by a microplate reader (ThermoPlate®) at 450 nm.

Readings were interpreted following Stepanovic et al. (2007). Mean OD of the samples and of negative control was calculated first; then cut rate (ODc) was calculated as follows:

DOc = [average of OD negative control + (3 x standard deviation of negative control)]. Final OD rate of tested samples (DOf) was given by ODf = (mean of OD of each sample – DOc).

Samples were divided in categories, as follows:

ODf≤ODc = no biofilm former;
ODc<ODf≤2xODc = weak biofilm former;
2xODc<ODf≤4xODc = moderate biofilm former;
4xODc<ODf = Strong biofilm former.

Assessment of biofilm formation on stainless steel surface

The capacity of biofilm formation on stainless steel surfaces by bacterial isolates was assessed according to method by Rossoni and Gaylarde (2000), with modifications. Stainless steel specimens (AISI 316) measuring 7 cm x 2 cm x 0.1 cm were used. The specimens were immersed in a neutral detergent solution for 1 h; scrubbed manually with a sponge; rinsed with distilled water; sprayed with alcohol 70% and dried at 60°C. They were autoclaved at 121°C for 15 min after sterilization.

Overnight culture were prepared by seeding bacterial isolates separately in 2 mL of BHI broth and incubated at 37°C for 24 h. One milliliter of each culture was added in 40 mL of peptone water 0.85% (Silveira, 2010). Inoculum concentration added to the suspension was standardized with McFarland scale so that the bacterial concentration in 40 mL of peptone water 0.85 % would contain approximately 10^7 CFU/mL. Sterile stainless steel specimens were immersed in the bacterial suspension for 24 h at 25°C.

After immersion, the specimens were washed with 1 mL sterile distilled water to remove all weakly adhering cells. They were then scrubbed by moist swabs and immersed in test tubes with a saline solution 0.1% and homogenized in a tube shaker (Phoenix Luferco®) for 3 min (Asséré et al., 2008). Serial decimal dilutions up to 10^{-5} were performed for each sample and a 10 µL aliquot of each was seeded in TSA medium (Acumedia®) in drops (Silva et al., 2007). Plates were incubated at 37°C for 24 h for CFU counts. The microorganism *Staphylococcus epidermidis* (ATCC 25923) was the positive control. Biofilm formation on stainless steel specimens was taken into account when counts indicated a number higher than or equal to adhered 10^3 CFU/cm^2, following Wirtanen et al. (1996).

Assessing biofilm removal with disinfectant agents

A modified method by Rossoni and Gaylarde (2000) was employed to assess the removal capacity of biofilm on stainless steel plates with the disinfectant agents organic chlorine and ammonium quaternary at a concentration of 200 parts per million (ppm). Disinfectant agents and their concentration were used due to their wide use in hygiene processes in the food industry.

Induction to biofilm formation on stainless steel specimens was as described above. After the biofilm formation and the last washing, the specimens were immersed separately in flasks with organic chlorine and ammonium quaternary for 10 min. When contact time occurred, the specimens were removed from the disinfectant solution and placed in contact during 3 seconds (s) with a Tween 2% solution to neutralize the ammonium quaternary action. Each specimen was rubbed with moist swabs, followed by immersion in test tubes with a saline solution 0.1% and homogenized with a tube shaker (Phoenix Luferco®) for 3 min. Serial decimal dilutions were done for each sample; 10 µL of the suspensions were seeded in Agar TSA by drops (Silva et al., 2007); and plates were incubated at 37°C for 24 h for CFU/cm² counts. Control comprised a specimen of the material with the biofilm immersed in peptone water 0.1%, but not in contact with the disinfectant agent.

The removal of the biofilm from the stainless steel specimens was considered to have occurred when counts were less than or equal to 10^2 CFU/cm² (APHA, 1992). In this case, statistics tests were realized (analysis of variance and Tukey test at 5%).

Susceptibility to antibiotics

The susceptibility of isolates to antibiotics was tested by the disk-diffusion method following protocol by the Clinical and Laboratory Standards Institute – CLSI (CLSI 2005a). Specific antimicrobial tests were undertaken for Gram positive microorganisms in *Listeria* spp. isolates: cefepime 10 µg; rifampicin 30 µg; chloramphenicol 30 µg; vancomycin 30 µg; tetracycline 30 µg; gentamicin 10 µg;

Table 2. Classification of biofilm formers of *Listeria* spp. and*Pseudomonas* spp. isolates retrieved from chicken and buffalo meat in southern Brazil.

Isolates (n[1])	Classification about capacity biofilm forming			
	Strong	Moderate	Weak	Non-forming
L. monocytogenes[2] (5)	0	0	5	0
L. grayi[2] (12)	0	3	4	5
L. innocua[3] (1)	0	0	1	0
L. rocourtiae[3] (1)	0	0	1	0
Pseudomonas spp.[2] (25)	0	1	9	15
Pseudomonas spp.[3] (25)	0	1	5	19

[1]number of isolates; [2]originate in chicken meat; [3]originate in buffalo meat.

oxacillin 1 µg; penicillin 10 U; erythromycin 15 µg; clindamycin 2 µg; ciprofloxacin 5 µg; trimethoprim-sulfamethoxazole 25 µg. In the case of *Pseudomonas* spp. isolates, specific antibiotics for microorganisms Gram negative were tested: gentamicin 10 µg; amikacin 30 µg; trimethoprim-sulfamethoxazole 25 µg; ciprofloxacin 5 µg; meropenem 10 µg; ampicillin 10 µg; cefalotin 30 µg; cefuroxim 30 µg; amixylin 20 µg + clavulanate 10 µg; cefoxitin 30 µg; cefepime 30 µg; ceftazidime 30 µg.Standard cultures at 0.5 concentration in McFarland scale were seeded with a sterile swab in Agar Muller-Hinton (Himedia®) and disks (Multidisco, Laborclin®) impregnated with the above mentioned antibiotics were applied under the surface of the medium. After incubation at 35°C for 24 h, inhibition haloes were measured and interpreted, following CLSI (2005b).

RESULTS AND DISCUSSION

The capacity of forming biofilm on polystyrene microplate

Table 2 shows results on the classification of isolates with regard to the formation of biofilms on polystyrene plates, following Stepanovic et al. (2007). Further, 73.7 and 32% were biofilm formers, respectively for isolates *Listeria* spp. and *Pseudomonas* spp. All isolates of *L. monocytogenes* were classified weak biofilm formers. Three *L. grayi* isolates were classified moderate and four were weak biofilm formers (Table 2).

The adhesion of *Listeria* spp. to the surfaces is greatly facilitated due to its flagella, especially in the initial phases of the biofilm formation (van Houdt and Michiels, 2010). The high number of biofilm-forming *Listeria* spp. from chicken and buffalo carcasses in the processing demonstrate lack of hygiene in handling, in the sanitization of equipments and utensils and even in the conservation of the product.

Several studies have reported high biofilm formation capacity of *L. monocytogenes* on polystyrene material (Rodrigues et al., 2010; Kadam et al., 2013) and thus reveal that the material is propitious to colonization by *L. monocytogenes* biofilms.

One isolate from chicken meat and another from buffalo meat out of the evaluated 50 *Pseudomonas* spp. isolates were classified as moderate biofilm formers. Nine isolates from chicken meat and 5 from buffalo meat were classified as weak biofilm formers. The above results were corroborated by Ghadaksaz et al. (2015) who registered that 47.1% of the clinical isolates of *P. aeruginosa* were biofilm former on polystyrene. The low adhesion of *Pseudomonas* spp. isolates on polystyrene in current analysis occurred because *Pseudomonas* spp. is a hydrophobic bacterium and tends to adhere on hydrophobic surfaces rather than on hydrophilic ones (Freitas et al., 2010).

Results in current study bring great health concern since the biofilm-forming pathogenic bacteria, such as the *L. monocytogenes*, and the deterioration-causing ones, such as *Pseudomonas* spp., are a serious challenge for the food industry since they may cause crossed contamination of products, with subsequent disease transmission and decrease in shelf life (Maia et al., 2009; Giaouris et al., 2014).

The capacity of forming biofilm on stainless steel

All *Listeria* spp. isolates and 72% of *Pseudomonas* spp. in current study formed biofilms on stainless steel specimens. Further, 48 and 96% of *Pseudomonas* spp. isolates respectively retrieved from buffalo and chicken meat formed biofilm on stainless steel. Even if the number of adhered bacterial cells were less than 10^3 CFU/cm², there would still be a great risk of microbiological contamination due to microbial concentration (Wirtanen et al., 1996; Oliveira et al., 2010).

Other researchers have shown that, similar to current analysis, bacteria of the genus *Listeria* have a great ability in adhering to and forming biofilms on the surfaces of stainless steel. The bacterium proves to be a potential risk for the food industry (Moltz et al., 2005; Silva et al., 2008; Berrang et al.,2010; Oliveira et al., 2010; Bonsaglia et al., 2014).

Biofilm formation by *Pseudomonas* spp. has already been reported in previous studies. Vanhaecke et al. (1990) registered that *P. aeruginosa* isolates adhered

and formed biofilms on stainless steel surfaces within a 30-second contact. Hood and Zottola (1997) showed the formation ability of *P. fluorescens* biofilm on stainless steel with different culture media. Rossoni and Gaylarde (2000) and Rosado et al. (2006) also demonstrated the capacity of *P. fluorescens* in forming biofilms on the surface of the same material. When previously-mentioned research works performed in different places, geographically distant one from the other, and results in current study are taken into account, it may be surmised that, regardless of its origin, *Pseudomonas* spp. is capable of forming biofilms on stainless steel surfaces. The surface adhesion of *Pseudomanas* spp. may be due to flagella, since these structures give mobility to the bacterium and make it approach the substratum on the surface and, consequently, its adherence (O'Toole and Kolter, 1998).

Results obtained and the use of stainless steel in equipments and on surfaces in food processing demonstrate that *L. monocytogenes* and *Pseudomonas* spp. may contaminate food that contact the surfaces if adequate hygiene methods, coupled to adequate disinfectant agents, are not used in the food processing industries.

Biofilm removal by sanitization

Nineteen (19) biofilm formers of isolates of *Listeria* spp. and 36 isolates of *Pseudomonas* spp. on stainless steel specimens evaluated in current analysis were assessed for the removal of biofilm by organic chlorine and ammonium quaternary, two common disinfectant agents usually employed in the food industry (Table 3) (Brazil, 1988).

The disinfectant agents should remove pathogenic bacteria and reduce the number of deterioration-causing microorganisms to reasonable levels. For example, 2 CFU/cm² of mesophilic aerobic microorganisms for stainless steel surfaces at the end of the hygienization process (APHA, 1992). Taking into consideration APHA standards, 36.8% of *Listeria* spp. isolates and 77.7% of *Pseudomonas* spp. isolates adhered on stainless steel were reduced by organic chlorine. Ammonium quaternary was efficient in removing all *Listeria* spp. and 91.6% of *Pseudomonas* spp. on the surface evaluated (Table 3). Ammonium quaternary was more efficient than organic chlorine in case of isolates of *Listeria* ($p = 0.000119$), but for *Pseudomonas* isolates no significant differences between this sanitizers ($p=0.238358$). Disinfectant agents made from ammonium quaternary have a wide spectrum of activities. In fact, they change their permeability by stimulating glycolysis when in contact with the cell membrane of microorganisms and cause cell exhaustion (Andrade et al., 1996).

Studies that evaluate disinfectant agents in the killing or inactivating of *Listeria* spp. Biofilms have already been performed. However, only rare reports are extant with regard to isolated of meat cuts and to meat processing industries in south Brazil. Aarnisalo et al. (2007) and Somers and Wong (2004) showed that chlorine-based disinfectant agents were more efficient than ammonium quaternary ones in the elimination of *L. monocytogenes* adherent to stainless steel. On the other hand, Pan et al. (2006) also analyzed biofilm formation on stainless steel chips and reported the resistance of *L. monocytogenes* isolates to chlorine and ammonium quaternary. Parikh et al. (2009) assessed the efficiency of three disinfectant agents (lactic acid, sodium hypochloride and ammonium quaternary) inbiofilms composed of *L.* monocytogenes and reported that all disinfectant agents were efficacious in biofilm decrease. Ammonium quaternary was the most efficient against the developed biofilms.

Several studies analyzed disinfectant agents in the killing or inactivating of *Pseudomonas* spp. biofilms. Taylor et al. (1999) showed that the treatment of *P. aeruginosa* with chlorine-based disinfectant agent caused a decrease in biofilm within the space of 5 minutes. Wirtanen et al. (2001) reported that chlorine-based disinfectant agent was efficient in the killing or inactivating of *Pseudomonas* spp. biofilm from stainless steel surfaces, although tension-active based sanitizers were efficacious in biofilm elimination. *Pseudomonas* spp. are important bacteria in the food industry since they cause the deterioration of food products and may form biofilms in food processing equipments, albeit with great difficulty in their killing or inactivating due to their resistance to sanitizers (Zhu et al., 2014).

Susceptibility to antibiotics

The first *L. monocytogenes*strain resistant to antibiotics was isolated in 1988. Resistant strains were thenceforth detected in food, on surfaces where food is handled and in clinical samples (Gomézet al., 2014). In current study, isolates of the genus *Listeria*are highly resistant to penicillin (94.7%), followed by clindamycin (84.2%), oxacillin (73.7%) and cefepime (57.9%). Table 4 shows resistance of *Listeria* spp. isolates against 12 antibiotics that may be used in the treatment of listeriosis (Jay 2005; Arsalanet al., 2011; Allen et al., 2014; Goméz et al., 2014).

Several researchers have detected high resistance levels to penicillin in *L. monocytogenes* strains (Harakeh et al., 2009; Fallah et al., 2012), even though concern is greater when *L. monocytogenes* isolates are resistant to important antibiotics in the treatment of listeriosis. Ampicillin or penicillin with gentamicin is the first choice for the treatment of listeriosis (Charpentier et al., 1999; Conter et al., 2009).

Similar to results in current analysis, the resistance to clindamycin was also reported by Kovacevicet al. (2013),

Table 3. Efficiency of the disinfectant agents, organic chlorine and ammonium quaternary, in the removal of biofilms formed by *Listeria* spp. And *Pseudomonas* spp. retrieved from chicken and buffalo meat, in southern Brazil, on stainless steel specimens.

***Listeria* isolates**	**Bacterial cells adhered on stainless steel (CFU/cm²)**	**Organic chlorine (CFU/cm²)**	**Ammonium quaternary (CFU/cm²)**
L. monocytogenes	$2.7x10^4$	$2.1x10^3$	-
L. monocytogenes	$5.7x10^5$	-	-
L. monocytogenes	$1.2x10^4$	$1x10^2$	-
L. monocytogenes	$1x10^6$	-	-
L. monocytogenes	$5.1x10^6$	$8.5x10^4$	-
L. innocua	$4.2x10^2$	-	-
L. rocourtiae	$5.1x10^5$	$2.3x10^4$	-
L. grayi	$6.4x10^6$	$1.2x10^5$	-
L. grayi	$2.1x10^5$	$1.9x10^4$	-
L. grayi	$3.8x10^5$	$1.2x10^4$	-
L. grayi	$6.4x10^4$	$5.9x10^4$	-
L. grayi	$1.9x10^6$	-	-
L. grayi	$2.1x10^5$	$1x10^5$	-
L. grayi	$8.1x10^5$	$2.7x10^4$	-
L. grayi	$4.2x10^5$	-	-
L. grayi	$3.8x10^4$	-	-
L. grayi	$1.2x10^5$	$5.1x10^4$	-
L. grayi	$2.3x10^4$	$6.4x10^3$	-
L. grayi	$1.9x10^4$	$8.5x10^3$	-
Pseudomonas spp.	$1.4x10^5$	-	-
Pseudomonas spp.	$4.8x10^5$	-	-
Pseudomonas spp.	$1.4x10^5$	$6.4x10^2$	-
Pseudomonas spp.	$2.5x10^7$	$4.2x10^3$	-
Pseudomonas spp.	$2.1x10^5$	-	-
Pseudomonas spp.	$2.1x10^6$	-	-
Pseudomonas spp.	$1.6x10^6$	-	-
Pseudomonas spp.	$2.3x10^5$	$2.1x10^2$	-
Pseudomonas spp.	$3.8x10^5$	-	-
Pseudomonas spp.	$1.4x10^6$	$2x10^2$	-
Pseudomonas spp.	$1.2x10^7$	-	-
Pseudomonas spp.	$3.8x10^3$	-	$2x10^3$
Pseudomonas spp.	$6.4x10^6$	-	-
Pseudomonas spp.	$4.5x10^6$	$2.1x10^2$	-
Pseudomonas spp.	$6.4x10^5$	-	-
Pseudomonas spp.	$1.2\ x10^6$	-	-
Pseudomonas spp.	$4.8\ x10^6$	-	-
Pseudomonas spp.	$2.3\ x10^6$	-	-
Pseudomonas spp.	$4.8x10^6$	-	-
Pseudomonas spp.	$3.6x10^4$	-	-
Pseudomonas spp.	$1.6\ x10^6$	-	-
Pseudomonas spp.	$2.1x10^5$	-	-
Pseudomonas spp.	$2.9\ x10^6$	-	-
Pseudomonas spp.	$1.6\ x10^6$	-	-
Pseudomonas spp.	$1.6x10^4$	-	-
Pseudomonas spp.	$8.3x10^4$	-	$8.5x10^3$
Pseudomonas spp.	$8.3x10^5$	-	-
Pseudomonas spp.	$3.8x10^5$	-	-

Table 3. Contd.

Listeria isolates	Bacterial cells adhered on stainless steel (CFU/cm²)	Organic chlorine (CFU/cm²)	Ammonium quaternary (CFU/cm²)
Pseudomonas spp.	$8.2x10^4$	-	-
Pseudomonas spp.	$3.8x10^5$	-	-
Pseudomonas spp.	$1.4x10^4$	-	-
Pseudomonas spp.	$5.3x10^4$	$2.1x10^3$	-
Pseudomonas spp.	$5.3 x10^4$	-	-
Pseudomonas spp.	$3.4 x10^4$	-	-
Pseudomonas spp.	$1.4x10^5$	$2.1x10^3$	-
Pseudomonas spp.	$1.4x10^5$	$3.8x10^3$	$1.7x10^3$

-: Bacterial absence.

Table 4. Resistance to antibiotics of *Listeria* spp. isolated retrieved from chicken and buffalo meat, in south Brazil.

Isolate (n)	Number of isolates of the *Listeria* spp. species											
	CPM (R/I)	RIF (R/I)	CLO (R/I)	VAN (R/I)	TET (R/I)	GEN (R/I)	OXA (R/I)	PEN (R/I)	ERI (R/I)	CLI (R/I)	CIP (R/I)	SUT (R/I)
Chicken												
L. monocytogenes (5)	1/2	-	-	-	-	1/0	3/1	4/0	0/3	3/1	1/0	-
Buffalo												
L. grayi (12)	8/3	0/1	-	-	2/3	0/6	10/1	12/0	2/4	11/1	0/1	-
L. innocua (1)	1/0	0/1	-	1/0	1/0	-	0/1	1/0	0/1	1/0	-	-
L. rocourtiae (1)	1/0	-	-	-	1/0	0/1	1/0	1/0	0/1	1/0	-	-
Total	11/5	0/2	0	0	4/3	1/7	14/3	18	2/9	16/2	1/1	0

CPM: cefepime 10 µg; RFI: rifampicin 30 µg; CLO: chloramphenicol 30 µg; VAN: vancomycin 30 µg; TET: tetracycline 30 µg; GEN: gentamicin 10 µg; OXA: oxacillin 1 µg; PEN: penicillin 10 U; ERI: erythromycin 15 µg; CLI: clindamycin 2 µg; CIP: ciprofloxacin 5 µg; SUT: trimethoprim-sulfamethoxazole25 µg; (R/I), where R = Resistance, and I = Intermediary resistance.

where 33% of *Listeria* spp., derived from fish, meat and processing factories, were resistant to clindamycin. Gómez et al. (2014) also registered clindamycin-resistant isolates, 35% *L. monocytogenes* and 46.2% *L. innocua*, retrieved from meat products and from the processing environment. According to Harakehet al. (2009), resistance of *L. monocytogenes* to penicillin and clindamycin may have been caused by drug excess in veterinary medicine.

All isolates tested in current analysis are sensitive to chloramphenicol and only one was resistant to gentamicin and ciprofloxacin. Similar results were reported in studies by Doménech et al. (2015) in which all *L. monocytogenes* isolates from ready-made food were sensitive to the three antibiotics. Gómez et al. (2014) also detected sensitivity to chloramphenicol in all *L. monocytogenes* isolates and in 99.2% of *L. innocua*. Kovacevicet al. (2013) reported sensitivity in all *Listeria* spp. isolates to gentamicin. The high sensitivity of isolates to gentamicin may be due to the fact that it is neither an antimicrobial agent usually used in veterinary therapy nor a growth enhancer in beef cattle (Harakehet al., 2009).

Sensitiveness to trimethoprim-sulfamethoxazole and vancomycin occurred in all isolates in current study. Yan et al. (2010) reported few *L. monocytogenes* isolates retrieved from food which were resistant to trimethoprim-sulfamethoxazole (Sulfazotrim) and vancomycin. However, Kovacevic et al. (2013) and Korsak et al. (2012) reported all isolates as sensitive to vancomycin. Doménech et al. (2015) registered that all *L. monocytogenes* isolates derived from pork sausages were resistant to trimethoprim-sulfamethoxazole. These results are highly relevant since theantimicrobial agent ranks second in the treatment for listeriosis, especially in patients allergic to penicillin (Pesaventoet al., 2010). According to Harakeh et al. (2009), vancomycin is the last ranking in treatment for infections with listeriosis in humans.

In general terms, *L. monocytogenes*, retrieved from chicken meat on the retail market, was the only isolate sensitive to all the antibiotics under analysis, although

Table 5. Resistance of *Pseudomonas* spp. isolates retrieved from chicken and buffalo meat in southern Brazil, to antibiotics.

Isolate (n)	Number of *Pseudomonas* spp. isolates											
	GEM (R/I)	AMI (R/I)	SUT (R/I)	CIP (R/I)	MER (R/I)	AMP (R/I)	CFL (R/I)	CRX (R/I)	AMC (R/I)	CFO (R/I)	CPM (R/I)	CAZ (R/I)
Chicken												
Pseudomonas spp. (50)	0/1	-	-	-	25/0	1/5	0/13	13/7	10/11	13/8	-	-
Buffalo												
Pseudomonas spp. (50)	-	-	1/2	0/2	24/1	6/1	1/4	12/2	3/15	11/0	-	-
Total	0/1	0	1/2	0/2	49/1	7/6	1/13	25/9	13/26	24/8	0	0

GEM: Gentamicin 10 µg; AMI: amikacin 30 µg; SUT: sulfazotrim 25 µg; CIP: cipofloxacin 5 µg; MER: meropenem 10 µg; AMP: ampicillin 10 µg; CFL: cefalotin 30 µg; CRX: cefuroxime 30 µg; AMC: amixillin+clavulanate 30 µg; CFO: cefoxitin 30 µg; CPM: cefepime 30 µg; CAZ: ceftazidime 30 µg; (R/I) where R = Resistance; I = Intermediary resistance.

21% of isolates tested were resistant to two antibiotics and 73.7% were resistant to three to five antibiotics. Isolates resistant to two or more antibiotics, totally 94.7%, were classified as multi-resistant. In fact, multi-resistance is not restricted to these isolates in southern Brazil since several studies have detected *Listeria* spp. isolates, multi-resistant to antibiotics, as a worldwide issue (Conter et al., 2009; Yan et al., 2010; Pesavento et al., 2010; Fallah et al., 2012; Goméz et al., 2014). *Listeria* spp. multi-resistant isolates against antibiotics usually used in the treatment of human listeriosis are a grave issue in public health due to a more difficult therapy especially for people in risk groups, involving elderly people, children, pregnant women and immunocompromised people (Goméz et al., 2014).

Table 5 shows the susceptibility of *Pseudomonas* spp. isolates to the 12 antibiotics tested, used for treatment of infections mainly caused by *P. aeruginosa* (Tassios et al., 1998; Jeukens et al., 2014). The highest resistance rate occurred for meropenem, with all isolates derived from chicken meat and 96 % from buffalo meat. There was no resistance in chicken and fish isolates to antibiotics among the *P. aeruginosas* strains belonging to the carbapenemclass (imipenemand meropenem), evaluated by Maia et al. (2009). In fact, they are used for multi-resistant isolates. Results in current analysis are grave since meropenem is an effective antimicrobial agent in the treatment of infections caused by Gram negative bacteria (Gales et al., 2002).

In the case of multi-resistance, 92% of isolates retrieved from chicken meat and 76% of isolates retrieved from buffalo meat were resistant to more than two antibiotics. Multi-resistant increase to antibiotics in Gram negative bacteria and specifically in *P. aeruginosa* indicate a reduced availability of effective agents for treatments in infections caused by this bacterium. Resistance increase to antibiotics and the potential for global dissemination of resistance genes to pathogen bacteria have become a world health issue for human and veterinarian medicine (Arslan et al., 2011; Sharma et al., 2014). The excessive use of antibiotics in veterinary medicine may be related to pathogens derived from the food chain resistant to antibiotics used by humans (Wang et al., 2007). It is highly important in the context of resistance and multi-resistance to anti-microbial agents to control and monitor the correct employment of these antibiotics in the treatment of people and in veterinary medicine to decrease the transmission of resistance in the food chain.

The testing was performed considering the hypothesis that biofilm-forming bacteria show greater resistance to antimicrobial agents, like drugs, antibiotics or industrial disinfectant. All isolates, who underwent removal test by sanitizers, have formed biofilm on stainless steel. However, not all isolates were resistant antibiotic. Among the *Listeria* isolates, seven *L. gray* which were resistant to organic chlorine were also resistant to two types of antibiotics; One *L. innocua* was resistant the organic chlorine and also to two types of antibiotics; One *L. roucotiae* which was resistant to organic chlorine was also to five types of antibiotics. Among the three *L. monocytogenes*, which were resistant to organic chlorine, two of these were resistant to three types of antibiotics and one was sensible to all antibiotics. In the case of *Pseudomonas* spp isolates, nine isolates were resistant to organic chlorine; eight of these were also resistant at least to two antibiotics. Three isolates, which were resistant to the ammonium quaternary, were also resistant to two types of antibiotics. In this study, it not possible establish a clear relationship positive or negative between the antibiotics and disinfectant resistances verified.

Conclusions

Results demonstrate the importance of control of microbial biofilms in the meat industry since current analysis revealed that isolates of *Listeria* spp. and *Pseudomonas* spp. Derived from chicken and buffalo

meat were capable of forming biofilms on polystyrene and stainless steel specimens.

The activities of the two disinfectant agents, organic chlorine and ammonium quaternary, were efficient in removing biofilms of *Listeria* spp. and *Pseudomonas* spp. on stainless steel specimens. The second agent was more efficient for *Listeria* spp. So that biofilm risk may be minimized, it is important that the food industry employs control strategies, such as efficient hygiene process that comprises correctly all the stages of cleaning and disinfectant, with recommended products and at the best concentrations for the elimination of microorganisms.

This study identified multi-resistance and resistance to antibiotics in several *Listeria* spp. and *Pseudomonas* spp. isolates.

Conflict of interest

The authors have not declared any conflict of interests.

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