Contamination sources, serogroups, biofilm-forming ability and biocide resistance of *Listeria monocytogenes* persistent in tilapia-processing facilities

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Accepted: 31 August 2017 / Published online: 22 September 2017
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Abstract The major contamination sources, serogroups, biofilm-forming ability and biocide resistance of *Listeria monocytogenes* persistent in tilapia-processing facilities were assessed. Twenty-five processing-control points were examined twice in two factories, including whole tilapias, frozen fillets, water and food-contact surfaces. *L. monocytogenes* were detected in 4 and 20% of points of Factory A and B respectively, but at low concentrations. Contamination was due to inadequate handling of tilapias in the slaughter room of Factory A and to the application of ineffective sanitizing procedures in Factory B. Seven strains were characterized by RAPD-PCR using primers HLWL85, OPM-01 and DAF4. Genotypic similarity allowed tracing the contamination source of tilapia fillets in Factory B and detecting a prevalent strain in Brazilian tilapia-processing facilities. The serogroup II (including the serotype 1/2c) was the most frequently found, followed by serogroup I (1/2a) and III (1/2b), whereas the serotype 4b was not detected. All strains showed high biofilm-forming ability on stainless steel and polystyrene, but biofilm formation was positively correlated with the type of origin surface. Biofilms were highly resistant to peracetic acid and sodium hypochlorite, being required doses higher than those recommended by manufacturers to be eradicated. Peracetic acid was more effective than sodium hypochlorite, but the use of disinfectants with similar mechanisms of action increases the risk of cross-resistance. Case-by-case

approaches are thus recommended to determine the sources and degree of contamination present in each factory, which would allow applying precise responses to control the persistence of bacterial pathogens such as *L. monocytogenes*.

Keywords *Listeria monocytogenes* · Tilapia-processing facilities · RAPD · Serogroup · Biofilm formation · Biocide resistance

Introduction

Oreochromis niloticus tilapias are one of the major fish species exported due to their high international demand (FAO 2016), being freshwater aquacultures of the South-east of Brazil a large production region (IBGE 2015). Good Manufacturing Practices (GMPs), Good Hygienic Practices (GHPs) and Hazard Analysis and Critical Control Point (HACCP) systems are often implemented to guarantee the quality and safety of products. Nevertheless, novel trends in food production such as minimal processing, mass production and globalization have introduced new factors and conditions that can promote the growth of bacterial pathogens (Abee and Wouters 1999; Havelaar et al. 2010). Chlorine-based biocides and peroxides are commonly applied in the food industry to control the growth of bacterial pathogens (Stanga 2010). But hygienic procedures frequently use sub-lethal doses of disinfectants and unsuitable exposure times that can cause the emergence of antimicrobial resistances and a long-term presence (even persistence) of some pathogens (Langsrud et al. 2003).

Listeria monocytogenes is a foodborne pathogen capable to cause in humans from febrile gastroenteritis to fatal bacteraemia and meningitis. Moreover, listeriosis

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outbreaks reported in Europe (EFSA 2016) and USA (Cartwright et al. 2013) showed high rates of mortality, but few of them have been associated to the consumption of fishery products. Nevertheless, *L. monocytogenes* serotypes 1/2a, 1/2b, 1/2c and 4b, involved in most cases of human listeriosis, have been frequently found in both fishery products and fish-contact surfaces (Abdollahzadeh et al. 2016; Chen et al. 2016; Jamali et al. 2015; Kramarenko et al. 2013; Wu et al. 2015). The prevalence of *L. monocytogenes* in fish-related environments seems to be correlated with the degree of human activity, being higher in freshwater fish farms, fish slaughterhouses and fish smokehouses than in freshwater streams (Hansen et al. 2006). In addition, the ability of *L. monocytogenes* to form biofilms can considerably enhance the stress tolerance (e.g. exposure to disinfectants) and, thus, its persistence in food-related environments (Carpentier and Cerf 2011; Ferreira et al. 2014).

Therefore, the present study aimed to determine the major contamination sources, serogroups, biofilm-forming ability and resistance to sodium hypochlorite and peracetic acid of *Listeria monocytogenes* strains persistent in tilapia-processing plants.

Materials and methods

Description of the tilapia-processing factories

Two tilapia-processing plants (Factory A and B) situated in the State of São Paulo (Southeast of Brazil), but distanced in 400 km approximately from each other, were examined. Tilapia-processing facilities are located at 1–3 km from the freshwater fish farms. Plants are divided in six sections: reception and slaughter room, desquamation section, filleting section, inspection section, packaging section and freeze storage warehouses. GMPs and GHPs were implemented in both factories, obtaining HACCP certification. Factory A processed up to 40 t/day of tilapia, whereas Factory B manages approximately 4.2 t/day.

Sampling

Twenty-five processing-control points were inspected twice in Factory A (Table 1) and B (Table 2) at approximately 2-month interval, following methods described in APHA (2013). Briefly, freshly caught whole *Oreochromis niloticus* tilapias from aquaculture were aseptically placed in sterile bags. Samples of contact surfaces and gloves were collected in areas of 100 cm² by using two sterile swabs moistened with neutralizing broth (10 g/L of triptone (Neogen Corporation, Brazil), 5 g/L yeast extract (Becton–Dickinson, São Paulo, Brazil), 2.4 g/L sodium bisulphite

and 1 g/L sodium thioglycolate (Sigma Aldrich, Brazil), 1 g/L histidine and 0.7 g/L soy lecithin (Dinâmica Química Contemporânea Ltda., Brazil), 5 g/L sodium chloride, 1 g/L sodium thiosulfate and 5 mL/L polysorbate 80 (Labsynth, Brazil), adjusted at pH 7.6 ± 0.2), which were subsequently immersed in 10 mL of peptone (10 g/L triptone (Neogen Corporation, Brazil) and 5 g/L sodium chloride (Labsynth)). Water samples of 1 L were aseptically placed in sterile bottles. Samples of water and gloves were taken during processing of tilapias, whereas surface samples were collected after daily cleaning and sanitizing to assess the validity of procedures applied. All these samples, as well as packaged frozen tilapia fillets, were placed into isothermal containers and immediately shipped to the laboratory under chilled conditions.

Isolation, identification and serotyping of *Listeria monocytogenes*

Samples of 25 g of tilapia were mixed with 225 mL of peptone in auto-sealable bags. Water samples were vacuum filtered through a 0.45 µm paper filter (F. Maia Indústria e Comércio Ltda., Brazil) and transferred to auto-sealable bags containing 10 mL of peptone. All samples, including those from gloves and food-contact surfaces collected in tubes, were then maintained for 1 h at room temperature to revitalize cells damaged. Tilapia and water samples were subsequently homogenized for 1 min in a stomacher masticator (ITR Instrumentos para Laboratórios Ltda., Brazil), whereas samples in tubes were vigorously vortexed for 1 min to resuspend cells adhered to swabs. After homogenization, 3 mL of each sample were inoculated twice in 3 M Petrifilm Environmental *Listeria* plates (3 M, Brazil), following manufacturer's instructions. Plates were incubated for 28 h at 37 °C. Afterwards, typical colonies of *Listeria* spp. (red-violet colonies) were counted to calculate the number of CFU/g (tilapias), CFU/cm² (surfaces) and CFU/L (water samples). Between one and five typical colonies from each contaminated point were selected for the confirmatory tests. Stock cultures of suspected isolates were maintained in 50% glycerol (w/w) at –80 °C.

Presumptive *L. monocytogenes* isolates were confirmed and serotyped, optimizing the method proposed by Doumith et al. (2004). Bacterial DNA was extracted from 24 h cultures in Brain Heart Infusion broth (Neogen Corporation) by using an InstaGene™ Matrix kit (Bio-Rad Ltda., USA). DNA was quantified by assuming that an absorbance value at 260 nm of 0.100 corresponds to 5 µg/mL of DNA. A multiplex PCR detecting both the genus-specific *prfA* gene and the four major serogroups of *L. monocytogenes* was performed for each isolate. Primer pairs designed by Doumith et al. (2004) were used (Table 3). Each PCR mixture contained 200 ng DNA, 0.4 µM of primer pair *prfA*,

Table 1 Processing-control points examined in Factory A

Section	Point	Sample	Surface	n	<i>Listeria</i> spp.	<i>Listeria</i> <i>monocytogenes</i>
General	A1	Freshly caught whole tilapias ^a	Biotic	10		
Reception and slaughter	A2	Cutting boards ^b	Polyethylene	4		
	A3	Helix ^b	Stainless steel	4		
	A4	Glove ^b	Cotton	5		
	A5	Disposable glove ^b	Nitrile	10	0.18 ± 0.01	0.18 ± 0.01
	A6	Water ^c	Water	4		
	A7	Desquamation machine IS069 (Baader ehf, Iceland) ^b	Stainless steel	4		
Desquamation	A8	Disposable glove ^b	Nitrile	10		
	A9	Cutting boards ^b	Polyethylene	4		
	A10	Carrier ramps ^b	Polyethylene	4		
Fileting	A11	Skinner machine 9000S (Townsend M/C Inc., USA) ^b	Stainless steel	4		
	A12	Disposable glove ^b	Nitrile	10		
	A13	Glove ^b	Stainless steel	5		
	A14	Water ^c	Water	4		
	A15	Cutting boards ^b	Polyethylene	4		
	A16	Carrier ramps ^b	Polyethylene	4		
Inspection	A17	Disposable glove ^b	Nitrile	10		
	A18	Water ^c	Water	4		
	A19	Balance 2096-H/1 (Toledo Ltda., Brazil) ^b	Stainless steel	4		
Packaging	A20	Packaging machine R105 (MULTIVAC, Brazil) ^b	Stainless steel	4		
	A21	Disposable glove ^b	Nitrile	10		
	A22	Knives ^b	Stainless steel	10		
General	A23	Crates ^b	Polyethylene	6		
	A24	Ice ^c	Water	4		
	A25	Frozen tilapia fillets ^a	Biotic	10		
Freeze storage warehouse						

^a Results expressed as Log CFU/g of product^b Results expressed as Log CFU/cm²^c Results expressed as Log CFU/L

1 µM of primer pairs *lmo0737*, *ORF2819* and *ORF2110*, 1.5 µM of primer pair *lmo1118* (Thermo Fisher Scientific, Brazil), 1 × Green GoTaq Flexi Buffer added with 2 mM MgCl₂, 0.2 mM dNTPs, 2 U GoTaq Hot Start Polymerase (Promega Corporation, USA), and sterile Milli-Q water up to a final volume of 50 µL. *Listeria monocytogenes* ATCC 15313 purchased from the Spanish Type Culture Collection (CECT) was used as positive control. PCR was performed in a thermocycler C1000TM (BioRad Ltda., USA) following the conditions proposed by Doumith et al. (2004). PCR products were subjected to electrophoresis on 1.5% agarose

gel containing ethidium bromide for 2 h at 75 V and 100 mAmp. A DNA ladder of 100–1517 bp (100 bp DNA ladder, New England Biolabs, Brazil) was included as a molecular size marker. Gels were visualized in a Gel Doc XR+ system (Bio-Rad) using the ImageLabTM software (Bio-Rad Ltda.).

Genotyping of *Listeria monocytogenes*

Listeria monocytogenes isolates were genotyped by RAPD-PCR. DNA was extracted and quantified as

Table 2 Processing-control points and number of samples (n) examined in Factory B

Section	Point	Sample	Surface	n	<i>Listeria</i> spp.	<i>Listeria monocytogenes</i>
General	B1	Freshly caught whole tilapias ^a	Biotic	10		
Reception and slaughter	B2	Bleeding tank ^b	Ceramic	4	0.54 ± 0.25	0.42 ± 0.13
	B3	Water ^c	Water	4		
Desquamation	B4	Access ramp ^b	Stainless steel	4		
	B5	Desquamation machine EG 100 (Engemac, Brazil) ^b	Stainless steel	4		
	B6	Catch tray ^b	Stainless steel	4		
Fileting	B7	Water ^c	Water	4		
	B8	Cutting boards ^b	Polyethylene	4		
	B9	Ramp ^b	Stainless steel	4		
	B10	Table ^b	Stainless steel	4		
	B11	Skinner machine 460VL (Cretel N.V., Belgium) ^b	Stainless steel	4		
Inspection	B12	Gloves ^b	Cotton	5		
	B13	Disposable gloves ^b	Vinyl	10		
	B14	Water ^c	Water	4		
	B15	Cutting boards ^b	Polyethylene	4	1.32 ± 0.37	0.84 ± 0.10
	B16	Table ^b	Stainless steel	4		
	B17	Disposable gloves ^b	Vinyl	10		
Packaging	B18	Water ^c	Water	4		
	B19	Trays ^b	Stainless steel	4	0.77 ± 0.33	0.46 ± 0.03
	B20	Balance 2096-H/1 (Toledo Ltda., Brazil) ^b	Stainless steel	4		
General	B21	Disposable gloves ^b	Vinyl	10		
	B22	Knives ^b	Stainless steel	10		
	B23	Crates ^b	Polyethylene	6	0.05 ± 0.01	0.05 ± 0.01
	B24	Ice ^c	Water	4		
Freeze storage warehouse	B25	Frozen tilapia fillets ^a	Biotic	10	2.20 ± 0.63	1.50 ± 0.01

^a Results expressed as Log CFU/g of product^b Results expressed as Log CFU/cm²^c Results expressed as Log CFU/L

aforementioned. Two independent cultures of each isolate were used to verify the reproducibility of banding patterns. Primers DAF4 (5'-CGGCAGCGCC-3') (Wiedmann-al-Ahmad et al. 1994), HLWL85 (5'-ACAACTGCTC-3') (Wernars et al. 1996) and OPM-01 (5'-GTTGGTGGCT-3') (Lawrence et al. 1993) were used in single reactions for each isolate. Each PCR mixture contained 200 ng DNA, 4 μM primer (Thermo Fisher Scientific), 1 × Green GoTaq Flexi Buffer added with 2.5 mM MgCl₂, 0.2 mM

dNTPs, 2 U GoTaq Hot Start Polymerase (Promega Corporation) and sterile Milli-Q water up to 50 μL. *L. monocytogenes* ATCC 15313 was also genotyped to assess possible similarities with the isolates. RAPD-PCR was performed following conditions proposed by Vogel et al. (2001) for primers HLWL85 and OPM-01. Meanwhile, conditions for primer DAF4 were optimized as follows: an initial denaturing of 3 min at 94 °C, followed by 40 cycles of 50 s at 94 °C, 50 s at 35 °C and 90 s at 72 °C, with a

Table 3 Nucleotide sequences of primers designed by Doumith et al. (2004) used for the confirmation and serotyping of *L. monocytogenes*

Gen	Primer	Sequence (5' → 3')	Size (bp) of amplicons	Serotype	Serogroup
<i>lmo0737</i>	lmo0737-F	AGGGCTTCAAGGACTTACCC	691	1/2a, 1/2c, 3a, 3c	I
	lmo0737-R	ACGATTTCTGCTTGCCATTC			
<i>lmo1118</i>	lmo1118-F	AGGGGTCTTAAATCCTGGAA	906	1/2c, 3c	II
	lmo1118-R	CGGCTTGTTCCGCATACTTA			
<i>ORF2819</i>	ORF2819-F	AGCAAAATGCCAAAACTCGT	471	1/2b, 3b, 4b, 4d, 4e	III
	ORF2819-R	CATCACTAAAGCCTCCCATTTG			
<i>ORF2110</i>	ORF2110-F	AGTGGACAATTGATTGGTGAA	597	4b, 4d, 4e	IV
	ORF2110-R	CATCCATCCCTTACTTTGGAC			
<i>prs</i>	prs-F	GCTGAAGAGATTGCGAAAGAAG	370	All <i>Listeria</i> spp.	
	prs-R	CAAAGAAACCTTGGATTGCGG			

final extension of 10 min at 72 °C. Amplicons were separated and visualized as previously described.

The exponential relationship between molecular size and mobility in each gel ($r > 0.99$) was used to determine the molecular size of DNA bands. Differences in at least one band resulted in unlike RAPD patterns. Variances in band intensity and bands too weak to be reproduced were not considered. RAPD patterns were coded with a binary value (0 or 1) denoting absence or presence of each band. Similarity analysis assessing the Dice coefficients (Struelens et al. 1996) was performed by IBM SPSS 15.0, whereas cluster analysis by UPGMA (Sneath and Sokal 1973) and dendrograms were executed with StatistiXL 1.11.

Quantification of biofilm formation

Stainless steel coupons (AISI 304, 2B finish) (Botam Oxicorte, Brazil) of 1 cm² (and 0.8 mm thickness) and polystyrene microtiter plates (Kasvi, Brazil) with 24 flat-bottomed wells of 1.93 cm² each were used as experimental surfaces. Coupons were previously soaked in 2 M NaOH to remove residues, rinsed several times with distilled water and dry-heat sterilized for 1 h at 170 °C. Coupons were placed into wells of sterile 24-well flat-bottom microplates.

The number of cells adhered to these surfaces was quantified in triplicate after 5, 24 and 48 h for each strain. Overnight cultures were adjusted to an optical density of 0.100 ± 0.01 at 700 nm with phosphate buffer saline (PBS, composed by 7.6 g/L NaCl, 0.2 g/L KCl, 0.245 g/L Na₂HPO₄ and 0.71 g/L K₂HPO₄ (Labsynth)), corresponding to 10^8 CFU/mL approximately. Adjusted cultures were then serially diluted in tryptic soy broth (TSB) (Kasvi), and aliquots of 700 µL (containing approximately 7×10^5 CFU) were added into each well. Inoculum size was checked by plating on tryptic soy agar (TSA) (Kasvi). A

negative control with no inoculum was included in all assays. Microplates were incubated at 25 °C under static conditions until analysis. After each incubation time, non-adhered cells were discarded and surfaces washed with 1 mL of PBS. Biofilm cells were collected by thoroughly rubbing the surfaces with two sterile swabs (Cremer Diagnostica), which were immersed in 10 mL of peptone. Cells were then resuspended by vortexing for 1 min and aliquots of 0.1 mL of appropriate dilutions in peptone were spread in triplicate on TSA plates, which were incubated for 24 h at 37 °C. The number of CFU/cm² of each strain adhered to stainless steel and polystyrene surfaces was then calculated. Assays were repeated twice using independent bacterial cultures.

Biocide resistance assays

Sodium hypochlorite and 39% (v/v) peracetic acid solution (Sigma-Aldrich, Brazil) were tested against *L. monocytogenes* strains with the highest biofilm-forming ability on each surface. Disinfectants were diluted in ultrapure water to working concentrations (10, 25, 50, 75, 100, 500, 1000, 2000, 3000 and 4000 mg/L) prior to each assay.

The minimal inhibitory concentration (MIC) of disinfectants for planktonic cells was assessed following an optimized method based in Mann and Markham (1998). Aliquots of 75 µL of adjusted cultures (containing 7.5×10^4 CFU approximately) were exposed to 75 µL of each disinfectant concentration in sterile 96-well U-bottom microplates (Kasvi). Each concentration of disinfectant were tested in triplicate in two independent experiments. Positive controls with no disinfectant, negative controls with no inoculum and blanks with medium only were also included. After 24 h at 37 °C, wells were stained with 10 µL of 0.01% (w/v) resazurin sodium salt solution (Sigma-Aldrich). Colour change from blue to pink indicating the presence of viable cells in cultures was checked

after 2 h of incubation. Visually undetectable growth was also checked by plating 0.1 mL of cultures on TSA.

Both factories apply sanitizing treatments once a day, so the logarithmic reduction of viable cells (LR) caused by disinfectants was determined in 24-h-old biofilms. After biofilm formation, non-adhered cells were removed by washing surfaces with 1 mL of PBS. Biofilms were then exposed to 0.5 mL of disinfectant for 30 min. Concentrations of 100, 500, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750 and 3000 mg/L of each disinfectant were tested in triplicate in two independent assays. Biofilms exposed to sterile water were also included as positive controls. Treatments were neutralized with 2.5 mL of neutralizing broth for 10 min at room temperature. Surviving biofilm cells were then collected and quantified as aforementioned. Counts were also added up with the number of viable biofilm cells washed out during neutralization, which were quantified in TSA plates spread with 0.1 mL of appropriate dilutions of neutralizing broth after 24 h at 37 °C. LR caused by each disinfectant concentration was calculated as the difference between the logarithm of the total number of viable cells in non-disinfectant-exposed biofilms and the logarithm of the number of surviving viable cells in disinfectant-exposed biofilms.

Statistical analysis

Statistical analysis were performed with IBM SPSS 19.0. Significance of the data was assessed using a one-way ANOVA. Homogeneity of variances was examined by a post hoc least significant difference (LSD) test. Otherwise, a Dunnett's T3 test was performed. An independent-samples student's *t* test was also done to compare variables in pairs. Bivariate correlations were analysed using the Pearson correlation coefficient. Statistical significance was accepted at a confidence level greater than 95% ($P < 0.05$).

Results

Contamination level in tilapia-processing plants

Typical colonies on 3 M Petrifilm Environmental *Listeria* plates were detected in 6 out of 50 processing control points, being affected the same points in both samplings. Forty colonies were picked up, isolated and definitely confirmed as *Listeria* spp. by genus-specific *prs* PCR. Genes related to *L. monocytogenes* serogroups were found in eighteen isolates, being present in all contaminated points. Three *L. monocytogenes* were isolated in Factory A (all from A5), whereas fifteen isolates were obtained in five points of Factory B (B2, B15, B19, B23 and B25).

Contamination level in Factory A (4% of points) was therefore lower than in Factory B (20%).

Concentrations of *L. monocytogenes* lower than 100 CFU/cm² (or g of product) were detected in both tilapia-processing plants (Tables 1 and 2). Failures in handling procedures applied in the reception and slaughter room were the major responsible of contamination in Factory A. Meanwhile, ineffective sanitizing procedures applied in Factory B led to the persistence of *L. monocytogenes* in several tilapia-contact surfaces, allowing the cross-contamination with tilapia fillets. Other *Listeria* spp. (i.e. isolates only carrying *prs* gene) were also present in four points of Factory B (B2, B15, B19 and B25), but at lower concentrations than *L. monocytogenes*.

RAPD patterns and similarity of *L. monocytogenes* isolates

RAPD-typing of *L. monocytogenes* isolates generated six, nine and seven banding patterns with primers DAF4, HLWL85 and OPM-01, respectively (Fig. 1). Primer DAF4 amplified 18 bands with 195–1644 bp, HLWL85 produced 20 bands with 176–1540 bp, and OPM-01 amplified 21 bands with 157–1673 bp. Amplification of DNA from different cultures of each isolate showed a good reproducibility of patterns. The discriminatory power of the analysis was increased from 0.889 to 0.978 by the combination of patterns obtained for each primer. Nine global patterns were thus created, being named by a three-digit code that indicates the pattern number obtained with primers DAF4, HLWL85 and OPM-01, respectively (Fig. 2). Cluster analyses based on similarity measurements between global patterns did not showed any relationship between RAPD patterns and origin surfaces, probably due to the purity of clusters. In fact, only patterns 6.9.7 and 6.7.7 were clustered at a relative genetic similarity of 0.70. This lack of relationship was confirmed by the validity assessment of cluster analyses, which rendered hierarchical F-measures of 0.846 for global banding patterns, as well as high values of F-measure, precision and recall.

Handler gloves of the reception and slaughter room of Factory A (A5) were contaminated by two different strains (L2 and L3). Meanwhile, six strains (L2, L4, L5, L6, L7 and L8) were present in Factory B. Three strains (L4, L5 and L6) were detected in the bleeding tanks (B2), whereas the other affected points were colonized by only one strain. Frozen tilapia fillets (B25) were contaminated by strain L7 during their processing on the polyethylene cutting boards of the inspection room (B15). Interestingly, strain L2 was isolated in both factories despite they are 400 km distanced, suggesting a possible prevalence of this strain in Brazilian tilapia-processing facilities. All isolated strains

Fig. 1 Agarose gels showing RAPD patterns of *L. monocytogenes* for primers DAF4 (a), HLWL85 (b) and OPM-01 (c). Lane 1: DNA molecular weight marker (100 bp DNA ladder, 100–1517 bp, New England Biolabs)

Fig. 2 Dendrogram from cluster analysis of global banding patterns obtained with primers DAF4, HLWL85 and OPM-01 for *L. monocytogenes* ATCC 15313 and isolates from tilapia-processing plants, indicating the serogroup and the origin of each strain

showed low similarity with ATCC 15313, originally from a rabbit sample.

Listeria monocytogenes serogroups found in tilapia-processing plants

A high diversity of *L. monocytogenes* serogroups were found in both tilapia-processing facilities (Fig. 2). Serogroup II was the most frequently detected in tilapia-processing plants, followed by serogroups I and III, whereas none isolate belonged to serogroup IV of *L. monocytogenes* ATCC 15313. Strains belonged to serogroups I (L2) and III (L3) were detected in handler gloves of the reception and slaughter room of Factory A (A5). Isolates of L2 from B19 were also gathered in serogroup I, which definitely confirmed the presence of this strain in both tilapia-processing factories. Bleeding tanks of Factory B (B2) were contaminated by strains of serogroups I (L6), II (L4) and III (L5). Strain L7 isolated in B15 and B25 were classified in the serogroup III, whereas L8 from B23 belonged to serogroup II. Cluster analysis did not discriminate any large cluster comprising strains of the same serogroup. In addition, no relationship between serogroups and origin surfaces was found.

Biofilm-forming ability of *L. monocytogenes*

The biofilm-forming ability of *L. monocytogenes* strains from tilapia-processing facilities was compared with that of ATCC 15313 (L1), used in enteric researches. All strains increased significantly ($P < 0.01$) the number of adhered

cells on polystyrene and stainless steel for 48 h at 25 °C (Fig. 3). Interestingly, biofilm formation on stainless steel was positively correlated with metallic origin surfaces, as well as biofilm formation on polystyrene was positively correlated with plastic origin surfaces (nitrile and polyethylene) (Table 4). Moreover, a relationship between biofilm-forming ability on stainless steel and serogroups was found, indicating that strains of serogroup I showed a higher biofilm formation on stainless steel than those from serogroup II, followed by strains from serogroups III and IV. However, no relationship was found between serogroups and biofilm formation on polystyrene.

Strain L2 showed the highest biofilm-forming ability on stainless steel, but moderate on polystyrene, which could explain the higher concentrations detected on B19 (i.e. stainless steel trays) than on A5 (i.e. nitrile gloves). Meanwhile, strains L7 and L8 showed the highest biofilm-forming ability on polystyrene. However, L7 reached higher concentrations on polyethylene surfaces than L8 (Table 2), suggesting that L7 have a higher tolerance to environmental stresses present in Factory B. Strain L3 from nitrile gloves (A5) also had remarkable biofilm-forming ability on polystyrene, but low on stainless steel. In contrast, strains from bleeding tanks made of ceramic (L4, L5 and L6) and ATCC 15313 (L1) showed moderate biofilm formation on both experimental surfaces. Therefore, L2 and L7 were selected to simulate worst-case scenarios on stainless steel and polystyrene surfaces under conditions present in tilapia-processing facilities (i.e. 25 °C and frequency of sanitizing of 24 h).

Fig. 3 Biofilm-forming ability of *L. monocytogenes* strains on stainless steel and polystyrene after 5 h (a), 24 h (b) and 48 h (c) at 25 °C. Letters indicates significant ($P < 0.05$) differences between strains in each surface

Table 4 Correlations between biofilm formation of *L. monocytogenes* on stainless steel and polystyrene at 25 °C and the type of origin surface (metallic or plastic) and serogroups (I, II, III and IV), expressed as r values

Parameter	Stainless steel			Polystyrene		
	5 h	24 h	48 h	5 h	24 h	48 h
Metallic origin surface	0.896 ^a	0.925 ^a	0.961 ^a	−0.321	−0.307	−0.274
Plastic origin surface	−0.275	−0.269	−0.275	0.955 ^a	0.951 ^a	0.952 ^a
Serogroup	−0.593 ^a	−0.572 ^a	−0.515 ^b	−0.186	−0.180	−0.092

An r value of zero indicates no correlation, whereas a value of 1 or −1 indicates a perfect positive or negative correlation

^a $P < 0.01$

^b $P < 0.05$

Biocide resistance of *L. monocytogenes*

The resistance of planktonic cells and 24-h-old biofilms of strains L2 and L7 to sodium hypochlorite and peracetic acid was assessed. As expected, *L. monocytogenes* biofilms showed higher biocide resistance than planktonic cells. Peracetic acid was ten-fold more effective against planktonic cells of both strains (MIC = 100 mg/L) than sodium hypochlorite (MIC = 1000 mg/L). LR caused by peracetic acid in biofilms formed by S8 and S10 was also significantly ($P < 0.01$) higher than that of sodium hypochlorite for all concentrations tested (Fig. 4). In fact, doses of 2750 mg/L of peracetic acid were able to eradicate completely biofilms formed on experimental surfaces. The effect of both disinfectants against biofilms was positively correlated with the increase of concentrations, but peracetic acid showed a higher effect ($r = 0.991$ for L2, $r = 0.970$ for L7, with $P < 0.01$) than sodium hypochlorite ($r = 0.897$ for L2, $r = 0.902$ for L7, with $P < 0.01$). No significant differences were observed between biocide resistance of biofilms formed by L2 on stainless steel and L7 on polystyrene for each disinfectant.

Discussion

Aquaculture of *Oreochromis niloticus* tilapias are highly extended worldwide due to the high international demand, being Brazil one of the major producers and exporters (FAO 2016). Contamination level of cultivation water and

tilapia fillets produced in Brazil was previously investigated (Bartolomeu et al. 2011; Boari et al. 2008; Carbonera et al. 2011; Junior et al. 2014), but limited to those food-borne pathogens included in the Brazilian legislation in force (Brazil 2001), being *L. monocytogenes* excluded. However, the incidence of *L. monocytogenes* in fish products is a cause of concern to the fishery industry, particularly in raw fish and ready-to-eat fish products (Abdollahzadeh et al. 2016; Chen et al. 2016; Cruz et al. 2008; Kramarenko et al. 2013; Shimojima et al. 2016). Policies about the control of *L. monocytogenes* in ready-to-eat fish products have been implemented in several countries (Jami et al. 2014), achieving promising results. Therefore, the major contamination sources and factors involved in the persistence of *L. monocytogenes* in tilapia-processing facilities were investigated in this study to provide relevant data that allow optimize accurately the manufacturing and sanitizing procedures and, consequently, increase the safety of tilapia products produced in Brazil.

Low concentrations of *L. monocytogenes* were detected in both tilapia-processing factories, but important differences in the number and type of contamination sources were observed in each case. Sanitizing procedures applied in Factory A were effective to control *L. monocytogenes* along the tilapia-processing chain. However, the frequency of changing gloves in the reception and slaughter room seemed to be not in concordance with the daily workload (40 t/day of tilapia processed), generating the accumulation of *L. monocytogenes*, which were possibly present in tilapia

Fig. 4 Logarithmic reduction of viable cells (LR) caused by 30 min of exposure to disinfectants in 24-h-old biofilms formed at 25 °C by *L. monocytogenes* strains L2 and L7 on stainless steel (a) and polystyrene (b), respectively

gills (Jami et al. 2014). In contrast, *L. monocytogenes* persisted in several tilapia-contact surfaces of Factory B, mainly due to the use of unsuitable sanitizing procedures (i.e., low efficacy of disinfectants, improper frequency of application, short exposure times or, possibly, the application of sub-lethal doses), allowing the cross-contamination with tilapia fillets. Persistence of *L. monocytogenes* was also reported in facilities processing other fish species, but different sources and degree of contamination were observed (Ferreira et al. 2014). Case-by-case approaches are thus recommended to apply precise control strategies that avoid (or at least reduce) the emergence of antimicrobial resistances, which increase the potential persistence of bacterial pathogens in food-related environments. Moreover, the presence and reestablishment of *L. monocytogenes* in fish-processing facilities should be continuously monitored to guarantee the safety of products.

Several methods are currently available to detect and identify rapidly *L. monocytogenes* in food-processing facilities (Välimaa et al. 2015). In this study, RAPD-PCR allowed characterize efficiently the diversity of *L. monocytogenes* strains in each factory, trace the contamination source of tilapia fillets in Factory B, and detect a possibly prevalent strain in Brazilian tilapia-processing facilities (L2), but further factories should be examined. Similarly, a prevalent *L. monocytogenes* strain was identified by RAPD in different fish slaughterhouses and smokehouses of Denmark and Faeroe Islands (Vogel et al. 2001; Wulff et al. 2006). However, the reproducibility of RAPD must be confirmed by using DNA extracts of each isolate from different cultures. Combination of banding patterns generated by high-yield primers is also recommended to increase the discriminatory power of RAPD.

Most of listeriosis cases are caused by the serotypes 1/2a, 1/2b, 1/2c and 4b (Cartwright et al. 2013; EFSA 2016), but their distribution in food-related environments is highly variable. Serotypes 1/2a and 1/2b seemed to be the most commonly found in *L. monocytogenes* isolated from fishery products and fish-processing factories (Abdollahzadeh et al. 2016; Chen et al. 2016; Jamali et al. 2015; Kramarenko et al. 2013; Shimojima et al. 2016; Wu et al. 2015). However, the serogroup II (including the serotype 1/2c) was the most frequently detected in this study, followed by serogroup I (1/2a) and III (1/2b), whereas the serotype 4b was not detected in both tilapia-processing facilities. In contrast, a higher occurrence of *L. monocytogenes* serotypes 4b, 1/2a and 1/2b than 1/2c was reported in 5953 food samples collected from 12 Brazilian states (including São Paulo) since 1990–2012, but no fish sample was examined (Vallim et al. 2015).

The persistence of *L. monocytogenes* in food-related environments mainly depends on the biofilm-forming ability, which increases the stress tolerance in comparison

with planktonic cells (Carpentier and Cerf 2011; Ferreira et al. 2014). Strains from tilapia-processing facilities showed a noteworthy biofilm-forming ability on stainless steel and polystyrene, demonstrating that these materials commonly found as food-contact surfaces can serve as reservoirs of *L. monocytogenes*. Moreover, biofilm cells seemed to be highly adapted to the type of origin surface (i.e., biofilm-forming ability on polystyrene and stainless steel was higher in strains isolated from plastic and metallic surfaces, respectively). Several studies also reported a high biofilm-forming ability of *L. monocytogenes* on polypropylene, nylon, Teflon, polyester floor sealant and glass surfaces (Blackman et al. 1996; Chae et al. 2006; Saá-Ibusquiza et al. 2012). Furthermore, the presence of scratches in food-contact surfaces can improve the formation of *L. monocytogenes* biofilms (Verran et al. 2010), which would explain the persistence of this pathogen in Factory B. However, no clear relationship between *L. monocytogenes* serogroups and biofilm-forming ability was observed in the present study, being only correlated data from stainless steel. The consistence of this relationship also varied greatly between previous studies, as shown in Ferreira et al. (2014).

A high resistance to peracetic acid and sodium hypochlorite, two disinfectants widely used in the food industry, was showed by *L. monocytogenes* strains with the highest biofilm-forming ability. In fact, biofilms resisted doses considerably higher than that recommended by manufacturers for peracetic acid (50–350 mg/L) and sodium hypochlorite (50–800 mg/L) (Gaulin et al. 2011). This high biocide resistance of *L. monocytogenes* strains was probably caused by the application of short exposure times and sub-lethal doses of disinfectants in tilapia-processing facilities (Langsrud et al. 2003). *L. monocytogenes* strains were more susceptible to peracetic acid than to sodium hypochlorite, which was in concordance with previous studies on food-related environments (Baert et al. 2009; Beltrame et al. 2015; Walter et al. 2009). Although both disinfectants are non-specific powerful oxidizing agents (Kitis 2004; Russell 2003), sodium hypochlorite have a limited diffusion in the biofilm, which reduces its effectiveness drastically (De-Beer et al. 1994). The use of disinfectants with similar mechanisms of action increases the risk of cross-resistance, particularly in biofilms (Chapman, 2003). Therefore, the use of novel working-safe, environmentally-friendly and cost-effective biocides and the development of innovative sanitizing procedures are recommended to avoid, or at least reduce, the risk of biofilm formation and antimicrobial resistance.

Conclusion

Listeria monocytogenes was found in both tilapia-processing facilities, including some final products. The serogroup II (including the serotype 1/2c) was recurrently found, followed by serogroup I (1/2a) and III (1/2b), whereas the serotype 4b was not detected. The high biofilm-forming ability and resistance to peracetic acid and sodium hypochlorite, two disinfectants widely used in the food industry, could explain the persistence of *L. monocytogenes* in several points of the tilapia-processing facilities. Handling and sanitizing procedures should be particularly revised to be in concordance with the daily workload of each factory and to increase the effectiveness against persistent *Listeria monocytogenes*, respectively. Case-by-case approaches are recommended to determine the sources and degree of contamination present in food-processing factories, which would allow applying precise responses to control the persistence of bacterial pathogens such as *L. monocytogenes*. Moreover, the presence and reestablishment of *L. monocytogenes* in processing control points should be continuously monitored to guarantee the safety of products.

Acknowledgements This work was financially supported by the São Paulo Research Foundation (FAPESP 2014/20590-0).

References

- Abdollahzadeh E, Ojagh SM, Hosseini H, Irajian G, Ghaemi EA (2016) Prevalence and molecular characterization of *Listeria* spp. and *Listeria monocytogenes* isolated from fish, shrimp, and cooked ready-to-eat (RTE) aquatic products in Iran. *LWT Food Sci Technol* 73:205–211. doi:10.1016/j.lwt.2016.06.020
- Abee T, Wouters JA (1999) Microbial stress response in minimal processing. *Int J Food Microbiol* 50(1–2):65–91. doi:10.1016/S0168-1605(99)00078-1
- APHA (2013) Compendium of methods for the microbiological examination of foods. American Public Health Association (APHA), Washington DC
- Baert L, Vandekinderen I, Devlieghere F, Coillie E, Debevere J, Uyttendaele M (2009) Efficacy of sodium hypochlorite and peroxyacetic acid to reduce murine norovirus 1, B40-8, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 on shredded iceberg lettuce and in residual wash water. *J Food Prot* 72(5):1047–1054
- Bartolomeu DAFS, Dallabona BR, Macedo REF, Kirschnik PG (2011) Contaminação microbiológica durante as etapas de processamento de filé de tilápia (*Oreochromis niloticus*). *Arch Vet Sci* 16(1):21–30. doi:10.5380/avs.v16i1.22788
- Beltrame CA, Martelo EB, Mesquita RA, Barbosa J, Steffens C, Toniazzo G, Valduga E, Cansian RL (2015) Adhesion of *Listeria monocytogenes* to cutting board surfaces and removal by different sanitizers. *J Verbrauch Lebensm* 10(1):41–47. doi:10.1007/s00003-014-0923-7
- Blackman IC, Frank JF, Frank F (1996) Growth of *Listeria monocytogenes* as a biofilm on various food-processing surfaces. *J Food Prot* 8(8):827–831. doi:10.4315/0362-028X-59.8.827
- Boari CA, Pereira GI, Valeriano C, Silva BC, Morais VM, Figueiredo HCP, Piccoli RH (2008) Bacterial ecology of tilapia fresh fillets and some factors that can influence their microbial quality. *Ciênc Tecnol Aliment (Campinas)* 28(4):863–867. doi:10.1590/S0101-20612008000400015
- Brazil (2001) Regulamento técnico sobre os padrões microbiológicos para alimentos. Resolução No 12, de 2 de janeiro de 2001. Diário Oficial da República Federativa do Brasil, Brasília
- Carbonera N, Cappelletti BM, Espírito-Santo MLP (2011) ISO 22000/HACCP associated with the presence of microorganisms in the processing of tilapia (*Oreochromis niloticus*) fillets. *Qual Assur J* 14:50–60. doi:10.1002/qaj.489
- Carpentier B, Cerf O (2011) Review—Persistence of *Listeria monocytogenes* in food industry equipment and premises. *Int J Food Microbiol* 145(1):1–8. doi:10.1016/j.ijfoodmicro.2011.01.005
- Cartwright EJ, Jackson KA, Johnson SD, Graves LM, Silk BJ, Mahon BE (2013) Listeriosis outbreaks and associated food vehicles, United States, 1998–2008. *Emerg Infect Dis* 19(1):1–9. doi:10.3201/eid1901.120393
- Chae MS, Schraft H, Truelstrup Hansen L, Mackereth R (2006) Effects of physicochemical surface characteristics of *Listeria monocytogenes* strains on attachment to glass. *Food Microbiol* 23(3):250–259. doi:10.1016/j.fm.2005.04.004
- Chapman JS (2003) Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *Int Biodeterior Biodegrad* 51(4):271–276. doi:10.1016/S0964-8305(03)00044-1
- Chen B-Y, Wang C-Y, Wang C-L, Fan Y-C, Weng I-T, Chou C-H (2016) Prevalence and persistence of *Listeria monocytogenes* in ready-to-eat tilapia sashimi processing plants. *J Food Prot* 79(11):1898–1903. doi:10.4315/0362-028X.JFP-16-149
- Cruz CD, Silvestre FA, Kinoshita EM, Landgraf M, Franco BDGM, Destro MT (2008) Epidemiological survey of *Listeria monocytogenes* in a Gravilax salmon processing line. *Braz J Microbiol* 39:375–383
- De-Beer D, Srinivasan R, Stewart PS (1994) Direct measurement of chlorine penetration into biofilms during disinfection. *Appl Environ Microbiol* 60(12):4339–4344
- Doumith D, Buchrieser C, Glaser P, Jacquet C, Martin P (2004) Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. *J Clin Microbiol* 42(8):3819–3822. doi:10.1128/JCM.42.8.3819-3822.2004
- EFSA (2016) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. *EFSA J* 14(12):4634. doi:10.2903/j.efsa.2016.4634
- FAO (2016) FAO yearbook. Fishery and aquaculture statistics 2014. Food and Agriculture Organization of the United Nations, Rome
- Ferreira V, Wiedmann M, Teixeira P, Stasiewicz MJ (2014) *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. *J Food Prot* 77(1):150–170. doi:10.4315/0362-028X.JFP-13-150
- Gaulin C, Lê M, Shum M, Fong D (2011) Disinfectants and sanitizers for use on food contact surfaces. National Collaborative Centre for Environment Health. http://www.nccch.ca/sites/default/files/Food-Contact-Surface-Sanitizers_Aug_2011.pdf. Accessed 18 Feb 2017
- Hansen CH, Vogel BF, Gram L (2006) Prevalence and survival of *Listeria monocytogenes* in Danish aquatic and fish-processing environments. *J Food Prot* 69(9):2113–2122. doi:10.4315/0362-028X-69.9.2113
- Havelaar AH, Brul S, Jong A, Jonge R, Zwietering MH, Kuile BHT (2010) Future challenges to microbial food safety. *Int J Food Microbiol* 139:79–94. doi:10.1016/j.ijfoodmicro.2009.10.015
- IBGE (2015) Produção da Pecuária Municipal. Ministério do Planejamento, Desenvolvimento e Gestão, Rio de Janeiro

- Jamali H, Paydar M, Ismail S, Looi CY, Wong WF, Radmehr B, Abedini A (2015) Prevalence, antimicrobial susceptibility and virulotyping of *Listeria* species and *Listeria monocytogenes* isolated from open-air fish markets. BMC Microbiol 15:144. doi:[10.1186/s12866-015-0476-7](https://doi.org/10.1186/s12866-015-0476-7)
- Jami M, Ghanbari M, Zunabovic M, Domig KJ, Kneifel W (2014) *Listeria monocytogenes* in aquatic food products—a review. Compr Rev Food Sci Food Saf 13:798–813. doi:[10.1111/1541-4337.12092](https://doi.org/10.1111/1541-4337.12092)
- Junior PG, Assunção AWA, Baldin JC, Amaral LA (2014) Microbiological quality of whole and filleted shelf-tilapia. Aquaculture 433:196–200. doi:[10.1016/j.aquaculture.2014.06.015](https://doi.org/10.1016/j.aquaculture.2014.06.015)
- Kitis M (2004) Disinfection of wastewater with peracetic acid: a review. Environ Int 30(1):47–55. doi:[10.1016/S0160-4120\(03\)00147-8](https://doi.org/10.1016/S0160-4120(03)00147-8)
- Kramarenko T, Roasto M, Meremäe K, Kuningas M, Pölsama P, Elias T (2013) *Listeria monocytogenes* prevalence and serotype diversity in various foods. Food Control 30:24–29. doi:[10.1016/j.foodcont.2012.06.047](https://doi.org/10.1016/j.foodcont.2012.06.047)
- Langsrud S, Sidhu MS, Heir E, Holck AL (2003) Bacterial disinfectant resistance: a challenge for the food industry. Int Biodeterior Biodegrad 51:283–290. doi:[10.1016/S0964-8305\(03\)00039-8](https://doi.org/10.1016/S0964-8305(03)00039-8)
- Lawrence LM, Harvey J, Gilmour A (1993) Development of a random amplification of polymorphic DNA typing method for *Listeria monocytogenes*. Appl Environ Microbiol 59:3117–3119
- Mann CM, Markham JL (1998) A new method for determining the minimum inhibitory concentration of essential oils. J Appl Microbiol 84(4):538–544
- Russell AD (2003) Similarities and differences in the responses of microorganisms to biocides. J Antimicrob Chemother 52(5):750–763. doi:[10.1093/jac/dkg422](https://doi.org/10.1093/jac/dkg422)
- Saá-Ibusquiza P, Herrera JJR, Vázquez-Sánchez D, Cabo ML (2012) Adherence kinetics, resistance to benzalkonium chloride and microscopic analysis of mixed biofilms formed by *Listeria monocytogenes* and *Pseudomonas putida*. Food Control 25(1):202–210. doi:[10.1016/j.foodcont.2011.10.002](https://doi.org/10.1016/j.foodcont.2011.10.002)
- Shimojima Y, Ida M, Nakama A, Nishino Y, Fukui R, Kuroda S, Hirai A, Kai A, Sadamasu K (2016) Prevalence and contamination levels of *Listeria monocytogenes* in ready-to-eat foods in Tokyo, Japan. J Vet Med Sci 78(7):1183–1187. doi:[10.1292/jvms.15-0708](https://doi.org/10.1292/jvms.15-0708)
- Sneath PHA, Sokal RR (1973) Numerical taxonomy: the principles and practice of numerical classification. Freeman, San Francisco
- Stanga M (2010) Sanitation: cleaning and disinfection in the food industry. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. doi:[10.1002/9783527629459](https://doi.org/10.1002/9783527629459)
- Struelens MJ, Bauernfeind A, Van-Belkum A, Blanc D, Cookson BD, Dijkshoorn L, El-Solh N, Etienne J, Garaizar J, Gerner-Smidt P, Legakis N, de-Lencastre H, Nicolas MH, Pitt TL, Römmling U, Witte W, Legakis N (1996) Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. Clin Microbiol Infect 2:2–11. doi:[10.1111/j.1469-0691.1996.tb00193.x](https://doi.org/10.1111/j.1469-0691.1996.tb00193.x)
- Välimaa A-L, Tilsala-Timisjärvi A, Virtanen E (2015) Rapid detection and identification methods for *Listeria monocytogenes* in the food chain—a review. Food Control 55:103–114. doi:[10.1016/j.foodcont.2015.02.037](https://doi.org/10.1016/j.foodcont.2015.02.037)
- Vallim DC, Hofer CB, Lisbôa RDC, Barbosa AV, Rusak LA, Reis CMF, Hofer E (2015) Twenty years of *Listeria* in Brazil: occurrence of *Listeria* species and *Listeria monocytogenes* serovars in food samples in Brazil between 1990 and 2012. Biomed Res Int 2015:540204. doi:[10.1155/2015/540204](https://doi.org/10.1155/2015/540204)
- Verran J, Packer A, Kelly P, Whitehead KA (2010) The retention of bacteria on hygienic surfaces presenting scratches of microbial dimensions. Lett Appl Microbiol 50(3):258–263
- Vogel BF, Jørgensen LV, Ojeniyi B, Huss HH, Gram L (2001) Diversity of *Listeria monocytogenes* isolates from cold-smoked salmon produced in different smokehouses as assessed by Random Amplified Polymorphic DNA analyses. Int J Food Microbiol 65:83–92
- Walter EHM, Nascimento MS, Kuaye AY (2009) Efficacy of sodium hypochlorite and peracetic acid in sanitizing green coconuts. Lett Appl Microbiol 49(3):366–371. doi:[10.1111/j.1472-765X.2009.02670.x](https://doi.org/10.1111/j.1472-765X.2009.02670.x)
- Wernars K, Boerlin P, Audurier A, Russell EG, Curtis GDW, Herman L, Mee-Marquet N (1996) The WHO multicenter study on *Listeria monocytogenes* subtyping: random amplification of polymorphic DNA (RAPD). Int J Food Microbiol 32:325–341. doi:[10.1016/S0168-1605\(96\)01146-4](https://doi.org/10.1016/S0168-1605(96)01146-4)
- Wiedmann-al-Ahmad M, Tichy HV, Schön G (1994) Characterization of *Acinetobacter* type strains and isolates obtained from wastewater treatment plants by PCR fingerprinting. Appl Environ Microbiol 60:4066–4071
- Wu S, Wu Q, Zhang J, Chen M, Yan Z, Hu H (2015) *Listeria monocytogenes* prevalence and characteristics in retail raw foods in China. PLoS ONE 10(8):e0136682. doi:[10.1371/journal.pone.0136682](https://doi.org/10.1371/journal.pone.0136682)
- Wulff G, Gram L, Ahrens P, Vogel BF (2006) One group of genetically similar *Listeria monocytogenes* strains frequently dominates and persists in several fish slaughter- and smokehouses. Appl Environ Microbiol 72(6):4313–4322. doi:[10.1128/AEM.02288-05](https://doi.org/10.1128/AEM.02288-05)