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PFGE characterisation and adhesion ability of *Listeria monocytogenes* isolates obtained from bovine carcasses and beef processing facilities

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ABSTRACT

Listeria monocytogenes is a pathogen capable of adhering to many surfaces and forming biofilms, which may explain its persistence in food processing environments. This study aimed to genetically characterise *L. monocytogenes* isolates obtained from bovine carcasses and beef processing facilities and to evaluate their adhesion abilities. DNA from 29 *L. monocytogenes* isolates was subjected to enzymatic restriction digestion (*AscI* and *Apal*), and two clusters were identified for serotypes 4b and 1/2a, with similarities of 48% and 68%, respectively. The adhesion ability of the isolates was tested considering: inoculum concentration, culture media, carbohydrate source, NaCl concentration, incubation temperature, and pH. Each isolate was tested at 10^8 CFU mL⁻¹ and classified according to its adhesion ability as weak (8 isolates), moderate (17) or strong (4). The isolates showed higher adhesion capability in non-diluted culture media, media at pH 7.0, incubation at 25 °C and 37 °C, and media with NaCl at 5% and 7%. No relevant differences were observed for adhesion ability with respect to the carbohydrate source. The results indicated a wide diversity of PFGE profiles of persistent *L. monocytogenes* isolates, without relation to their adhesion characteristics. Also, it was observed that stressing conditions did not enhance the adhesion profile of the isolates.

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1. Introduction

Listeria monocytogenes has been considered an emerging foodborne pathogen since 1980 due to sporadic cases and outbreaks of listeriosis that have been associated with the consumption of contaminated foods (Luber et al., 2011; McLauchlin, Mitchell, Smerdon, & Jewell, 2004). Considering the severity of symptoms and the high mortality rate (Allerberger & Wagner, 2010; Colodner et al., 2003; Jiang et al., 2008; Kerr, Dealler, & Lacey, 1988; Lamont et al., 2011; Warriner & Namvar, 2009), several studies were developed to identify the main sources of contamination and relevant foods associated with this pathogen and to characterise its virulence factors (Autio et al., 1999; Barbalho, Almeida, Almeida, & Hofer, 2005; Barros et al., 2007; Cordano & Rocourt, 2001; Jiang et al., 2008; Lecuit, 2005; Linnan et al., 1988; Nucera et al., 2010; Peccio, Autio, Korkeala, Rosmini, & Trevisani, 2003; Thevenot, Dernburg, & Vernozy-Rozand, 2006; Vitas, Aguado, & Garcia-Jalon, 2004; von Laer et al., 2009; Warriner & Namvar, 2009).

L. monocytogenes is usually associated with dairy, meat and ready-to-eat products (Autio et al., 1999; Cordano & Rocourt, 2001; Luber et al., 2011; Peccio et al., 2003; Rivoal et al., 2010; Vitas et al., 2004; von Laer et al., 2009) due to the utilisation of several types of equipment and utensils during processing and storage prior to refrigeration under conditions that favour contamination and growth (Gandhi & Chikindas, 2007). In addition, *L. monocytogenes* is relatively resistant to variations in pH and NaCl concentrations and several antimicrobial

Table 1

Description of sources, codification and serotypes of *Listeria monocytogenes* isolates obtained from beef processing facilities, bovine carcasses and end products (Barros et al., 2007).

Site	Specification	n	Codification	Serotypes
Non-food contact surfaces	Floor	3	En01, En02, En03	1/2a, 4b ^a
	Drain	1	En04	ns ^b
Food-contact surfaces	Plastic boxes	6	Ut01, Ut08 to Ut12	4b
	Tenderizer	2	Ut02, Ut03	4b
	Grinder	3	Ut04, Ut05, Ut07	1/2a
	Mixer	1	Ut06	4b
Bovine carcasses	–	9	Cc01 to Cc09	1/2a, 4b ^a
End products	Ground beef	1	Fp01	1/2a
	Sausages	3	Fp02, Fp03, Fp04	4b

^a En01: 4b; En02 and En03: 1/2a; Cc01 and Cc02: 1/2a; Cc03–Cc09: 4b.

^b ns: not serotyped.

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Table 2
Tested conditions for the adhesion ability analysis of *Listeria monocytogenes* isolates (according to methodology described by Djordjevic et al., 2002 and Stepanović et al., 2007).

Variable	Culture concentration	Culture media ^a	Supplements	pH	Incubation
Bacterial concentration	10 ¹ to 10 ⁹ CFU mL ⁻¹	TSB	–	7.0	37 °C for 24 h
Culture medium	10 ⁸ CFU mL ⁻¹	BHI	–	7.0	37 °C for 24 h
		BHI at 10%		7.0	
		TSB		7.0	
		TSB at 10%		7.0	
		Meat broth		7.0	
		Meat broth at 10%		7.0	
Carbohydrates	10 ⁸ CFU mL ⁻¹	Meat broth	Rhamnose 1.0%	7.0	37 °C for 24 h
			Glucose 1.0%	7.0	
			Maltose 1.0%	7.0	
			Fructose 1.0%	7.0	
NaCl	10 ⁸ CFU mL ⁻¹	Meat broth	NaCl 2.5%	7.0	37 °C for 24 h
			NaCl 5.0%	7.0	
			NaCl 7.5%	7.0	
			NaCl 10.0%	7.0	
pH	10 ⁸ CFU mL ⁻¹	Meat broth	HCl 0.1 N	5.0	37 °C for 24 h
			None	7.0	
			NaOH 0.1 N	9.0	
Temperature	10 ⁸ CFU mL ⁻¹	Meat broth	–	7.0	4 °C for 24 h
					10 °C for 24 h
					25 °C for 24 h
					37 °C for 24 h

^a TSB: trypticase soy broth; BHI: brain heart infusion broth; meat broth (per L): 10 g of peptone, 5 g of yeast extract, 5 g of NaCl (according to Freney et al., 1999).

substances, enhancing its ability to persist in food processing environments (Duffy & Sheridan, 1997; Gandhi & Chikindas, 2007; Lubber et al., 2011).

After adhesion to utensils and equipments, *L. monocytogenes* tends to form micro-colonies and then complex biofilms (Hood & Zottola, 1995; McLandsborough, Rodriguez, Perez-Conesa, & Weiss,

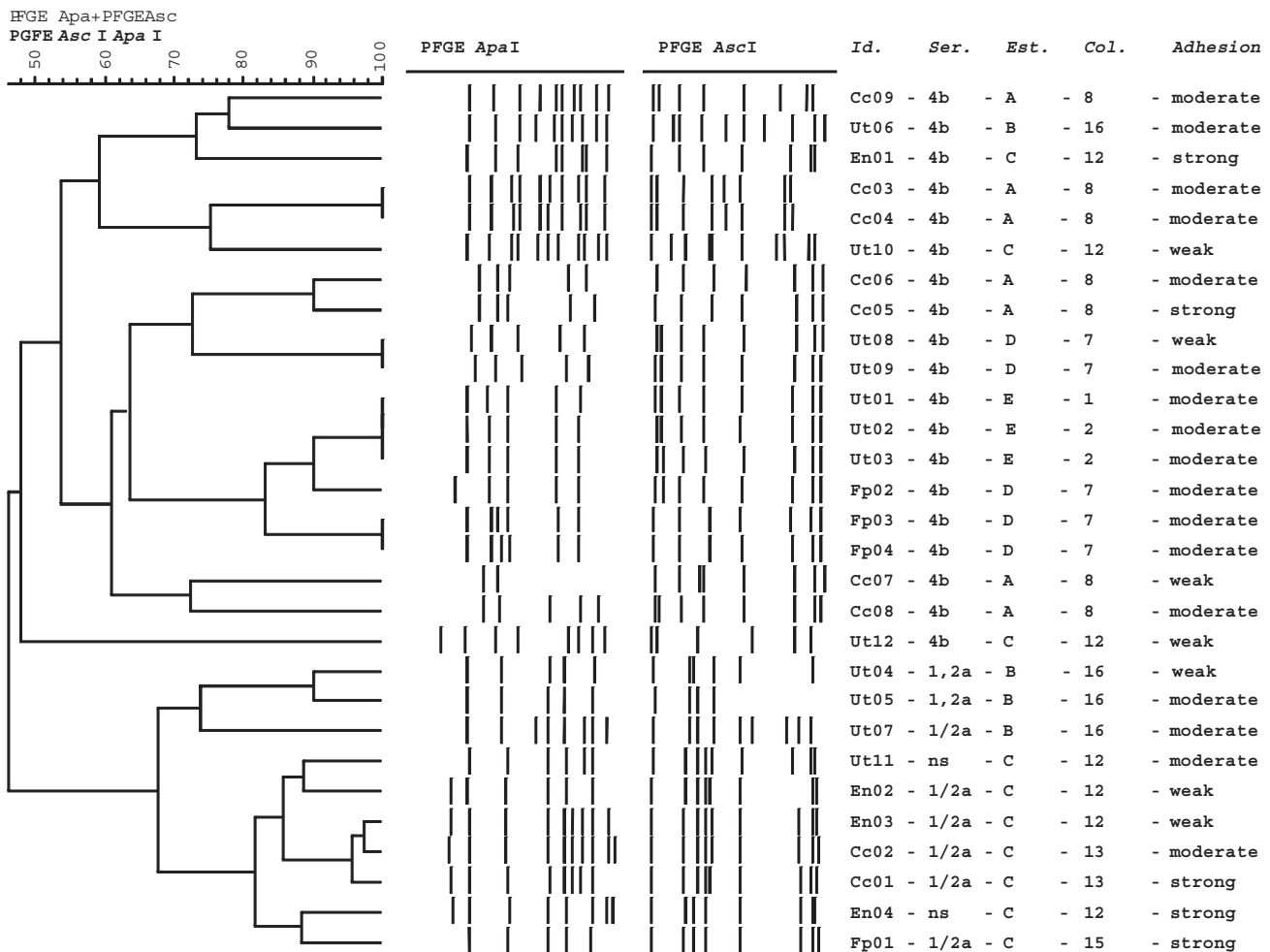


Fig. 1. Schematic representation of the obtained PFGE profiles after DNA macro-restriction (*ApaI* and *AscI*) of 29 *Listeria monocytogenes* isolates obtained from beef processing facilities, bovine carcasses and end products. Isolate identification (Id.), serotypes (Ser.), establishment (Est.) and collection period (Col.), and also the adhesion ability (Adhesion). Similarities between the identified PFGE profiles were estimated using the Dice coefficient (1% tolerance).

Table 3

Frequencies of *Listeria monocytogenes* isolates obtained from beef processing facilities, bovine carcasses and end products categorised according to their adhesion ability (according to methodology described by Djordjevic et al., 2002 and Stepanović et al., 2007).

Adhesion categories	Initial concentration of the tested isolate (CFU mL ⁻¹)								
	10 ⁹	10 ⁸	10 ⁷	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹
Absence	0	0	1	1	1	4	6	12	16
Weak	8	8	8	10	14	17	17	16	12
Moderate	17	17	18	18	14	8	6	1	1
Strong	4	4	2	0	0	0	0	0	0

2006; Takhistov & George, 2004). Fragments of mature biofilms can detach, releasing micro-colonies that are capable of adhering to new surfaces and begin the process again, causing new contamination spots (Møretro & Langsrud, 2004; Takhistov & George, 2004). Therefore, the biofilm cycle may support the persistence of specific strains in a specific environment (McLandsborough et al., 2006; Moltz & Martin, 2005; Takhistov & George, 2004). This persistence can be detected using molecular tools, in order to identify strains with identical genetic profiles on

different equipment and utensils (Graves & Swaminathan, 2001; Møretro & Langsrud, 2004; Senczek, Stephan, & Untermann, 2000; Slade, 1992). Among the different molecular methods used, pulsed field gel electrophoresis (PFGE) has been a very useful tool for determining relationships among bacterial isolates (Neves, Lourenco, Silva, Coutinho, & Brito, 2008; Senczek et al., 2000).

The aim of the present study was to characterise by enzymatic restriction *L. monocytogenes* isolates obtained from bovine carcasses and beef processing facilities and evaluate their adhesion potential under distinct conditions, in order to establish an association between these characteristics.

2. Materials and methods

2.1. L. monocytogenes isolates

In a previous study conducted by Barros et al. (2007), a culture collection of *Listeria* spp. strains was obtained from the environment, bovine carcasses, and meat products of beef processing facilities. The present study was conducted with 29 isolates that were identified as *L. monocytogenes*, obtained from five beef processing facilities (A, B, C,

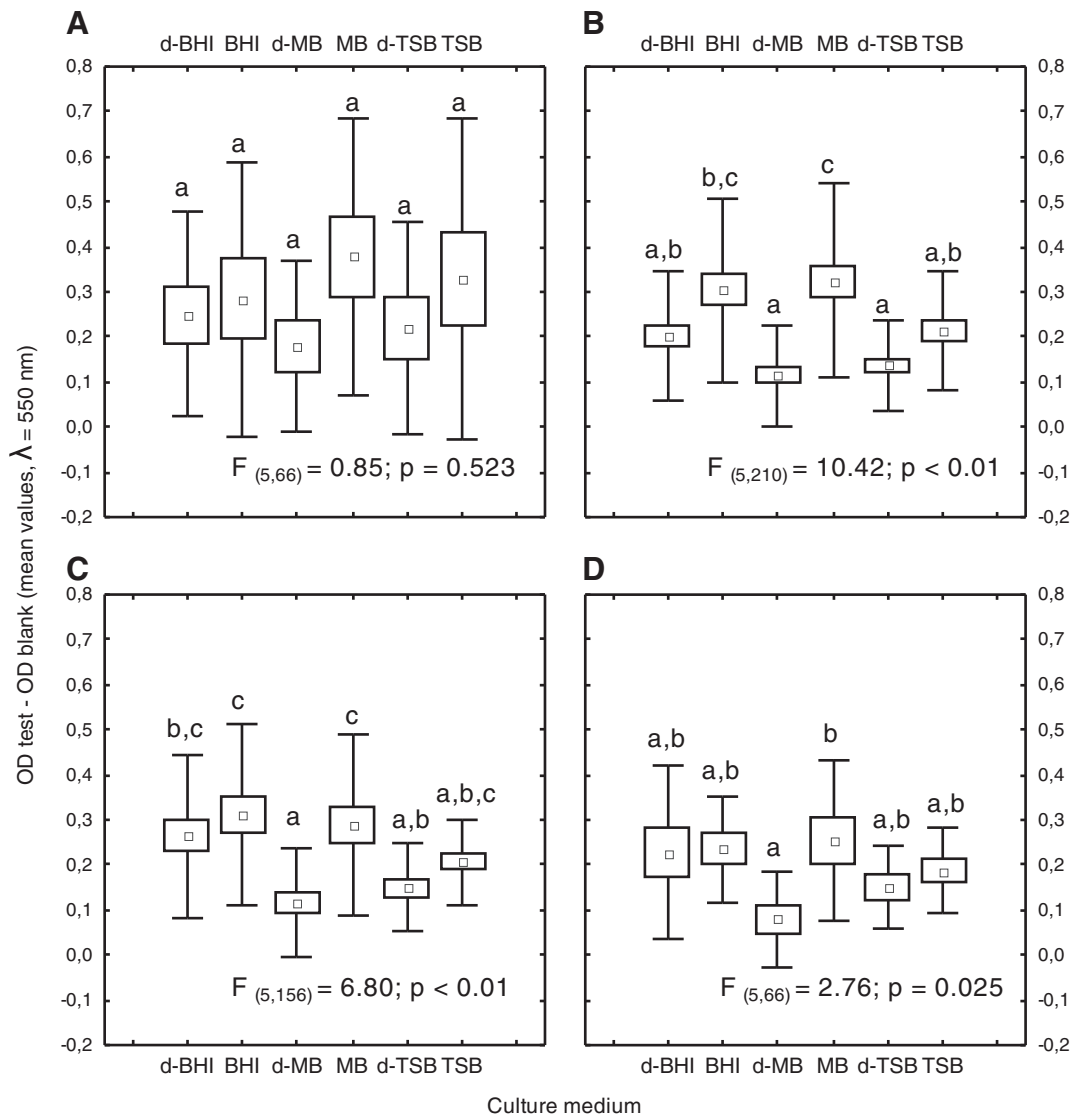


Fig. 2. Adhesion ability of the strains according to their origin and culture media. A: non-food contact surfaces; B: food contact surfaces; C: bovine carcasses; D: end products. Differences in mean values of absorbance between *L. monocytogenes* isolates and negative controls (OD_{test} minus OD_{blank}). BHI: brain heart infusion broth; TSB: trypticase soya broth; MB: meat broth; "d-": diluted. Mean values with distinct letters are significantly different by ANOVA (p<0.05).

D, and E) (Table 1). The isolates were kept lyophilised at -80°C until the point of use, when they were added to 10 mL of trypticase soy broth (TSB, Oxoid Ltd., Basingstoke, England), incubated at 30°C for 24 h, streaked on trypticase soy agar (TSA, Oxoid) and incubated at 30°C for 24 h. One isolated colony from each culture was transferred to TSB and incubated at 30°C until achieving turbidity similar to MacFarland scale 1, corresponding to approximately 10^8 CFU mL^{-1} .

2.2. PFGE typing of the isolates

All isolates were subjected to PFGE using the restriction enzymes *AscI* and *ApaI* (New England BioLabs, Massachusetts, USA) according to the protocol described by PulseNet (Graves & Swaminathan, 2001). The profiles obtained were analysed using BioNumerics software v. 3.0 (Applied Maths, Gand, Belgium), considering maximum optimization of 1%, and based on Dice similarity of bands, with maximum position tolerance of 1%. Unweighted Pair Group Method using Averages (UPGMA) was used for profile clustering and dendrogram

construction. *Salmonella enterica* serovar Braenderup (strain H9812, digested with *XbaI*) was used as a reference standard.

2.3. Adhesion ability

The adhesion ability of each isolate was evaluated according to the methodology described by Djordjevic, Wiedmann, and McLandsborough (2002), and recommendations of Stepanović et al. (2007). Briefly, $20\ \mu\text{L}$ of a culture of each *L. monocytogenes* isolate was transferred to each of five wells of a microtitre plate (U-shaped bottom) containing $180\ \mu\text{L}$ of a specific culture medium and incubated at specific conditions (Table 2). Culture media were discarded, and the wells were subjected to three consecutive washes with phosphate-buffered saline solution (PBS, pH 7.2) to remove non-attached cells. Adhered cells were fixed by the addition of methanol, and then the plates were air dried for 10 min. Crystal violet solution (1% w/v) was added to each well, and after 15 min the plates were washed with tap water. After drying, 95% ethanol was added to each well, and after 30 min, the absorbance was measured ($\lambda = 500\ \text{nm}$). All adhesion tests

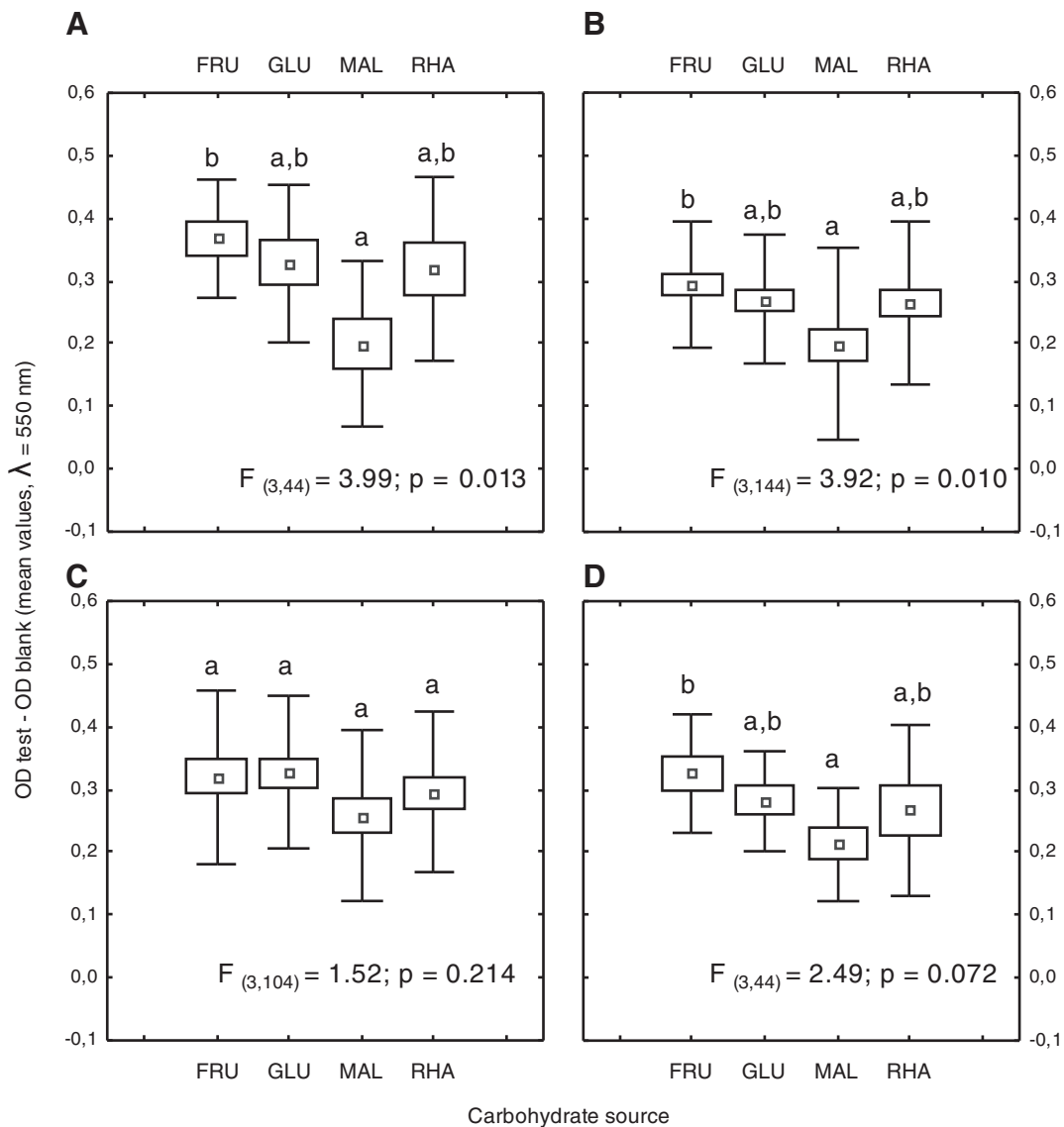


Fig. 3. Adhesion ability of the strains according to their origin and distinct carbohydrate sources. A: non-food contact surfaces; B: food contact surfaces; C: bovine carcasses; D: end products. Differences in mean values of absorbance between *L. monocytogenes* isolates and negative controls (OD_{test} minus OD_{blank}). FRU: fructose; GLU: glucose; MAL: maltose; RHA: rhamnose. Mean values with distinct letters are significantly different by ANOVA ($p < 0.05$).

were conducted in triplicate. In each test plate, five wells containing only the respective culture medium were used as negative controls. The first parameter evaluated was *L. monocytogenes* concentration, which varied from 10¹ to 10⁹ CFU mL⁻¹. Once this parameter was established, the interference of culture medium composition, carbohydrate sources, NaCl concentrations, pH, and temperatures was tested, with specific modifications to the adhesion test protocol, as detailed in Table 2.

2.4. Adhesion data analysis

The results for the first adhesion test (bacterial concentration of *L. monocytogenes* isolates) were evaluated according the adhesion classification by Stepanović et al. (2007). The results of the adhesion test conducted with the isolates at 10⁸ CFU mL⁻¹ were considered for this analysis, due to their superior adhesion performance. The optical densities (OD) of the five wells of each isolate were read after the end of adhesion protocol (as described before), and the mean OD was calculated

(OD test) and compared to the mean OD values of the negative controls (OD blank), adjusted according to the following equation:

$$\text{OD blank adj.} = \text{mean value of OD blank} + 3 \times (\text{standard deviation of OD blank}).$$

Based on the results, each isolate was categorised as follows:

- Absence of adhesion OD test ≤ OD blank adj.
- Weak adhesion OD blank adj. < OD test ≤ 2 OD blank adj.
- Moderate adhesion 2 OD blank adj. < OD test ≤ 4 OD blank adj.
- Strong adhesion OD test > 4 OD blank adj.

For further adhesion tests that considered different conditions (Table 2), the results were evaluated according to Djordjevic et al. (2002), as described above. The mean recorded OD value for each isolate (OD test) was subtracted from the mean OD values of the negative controls (OD blank) in each plate to assess significant

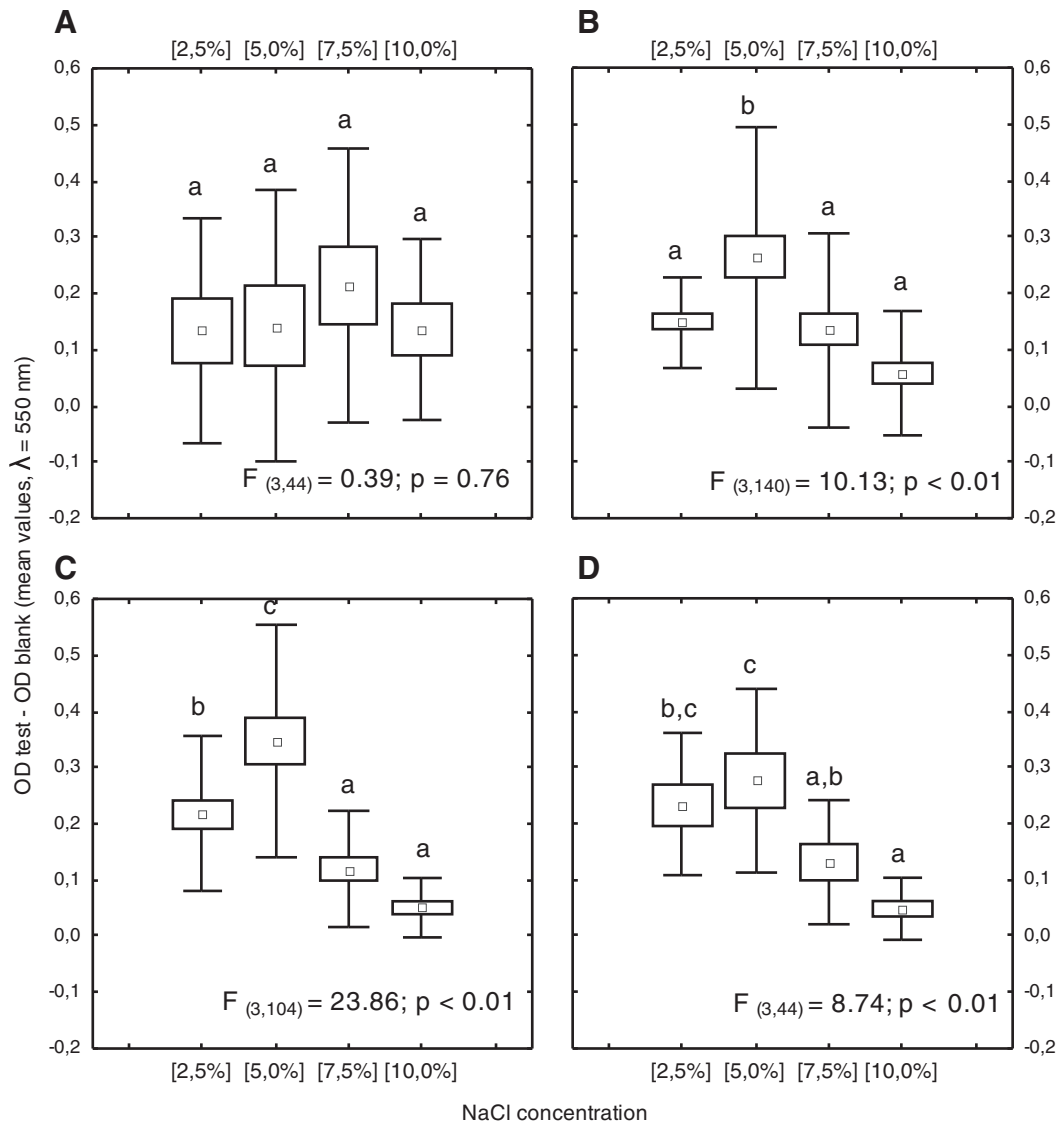


Fig. 4. Adhesion ability of the strains according to their origin and distinct NaCl concentrations. A: non-food contact surfaces; B: food contact surfaces; C: bovine carcasses; D: end products. Differences in mean values of absorbance between *L. monocytogenes* isolates and negative controls (OD_{test} minus OD_{blank}). Mean values with distinct letters are significantly different by ANOVA (p < 0.05).

differences. For this set of adhesion test, the isolates were grouped according to their origin (Table 1), and the adhesion results were compared by ANOVA ($p < 0.05$) using Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA). In addition, the presence of association between the adhesion abilities and the genetic profiles of the tested isolates was verified.

3. Results and discussion

Fig. 1 shows the genotyping profiles and serotypes of the *L. monocytogenes* isolates, the establishment of their origins, and their initial adhesion classifications. Considering the serotypes previously identified by Barros et al. (2007), a clear grouping can be observed for 4b and 1/2a serotypes. The 4b isolates presented at least 48% similarity, while 1/2a isolates presented 68%. Rivoal et al. (2010) analysed 196 *L. monocytogenes* isolates from eggs and found that serotype 1/2a was the most prevalent, presenting 61.7% similarity by PFGE. In a study with *L. monocytogenes* isolates obtained from fresh sausage, several serotypes were identified (1/2b, 1/2c, 4b) with no evident association among the 22 identified PFGE profiles (von Laer et al., 2009). A similar PFGE profile diversity, independent of the identified serotypes, was

observed in a retrospective study of *L. monocytogenes* isolates obtained from food and clinical samples (Nucera et al., 2010).

Table 3 shows the frequencies of *L. monocytogenes* isolates that were categorised according to distinct adhesion profiles with respect to their initial concentrations. The best adhesion performances were recorded when the isolates were inoculated at 10^8 and 10^9 CFU mL⁻¹, and a decreasing adhesion ability was recorded when the isolates were inoculated at lower concentrations (Table 3). Despite the observed variation, adhesion studies usually adopt an initial concentration of 10^8 CFU mL⁻¹ of the tested organism, independent of the microbial species (Djordjevic et al., 2002; Moltz & Martin, 2005; Stepanović et al., 2007). The initial concentration of the bacterial isolate can directly interfere with its adhesion ability and biofilm formation, demanding a standardisation of this variable for a proper evaluation of these characteristics under distinct conditions (Stepanović et al., 2007).

Although the *L. monocytogenes* PFGE profiles presented an evident association with the identified serotypes, they were not associated with the adhesion abilities of the isolates (Fig. 1). Despite not highlighting this association, PFGE allowed the identification of potential contamination routes and isolate persistence in the evaluated establishments.

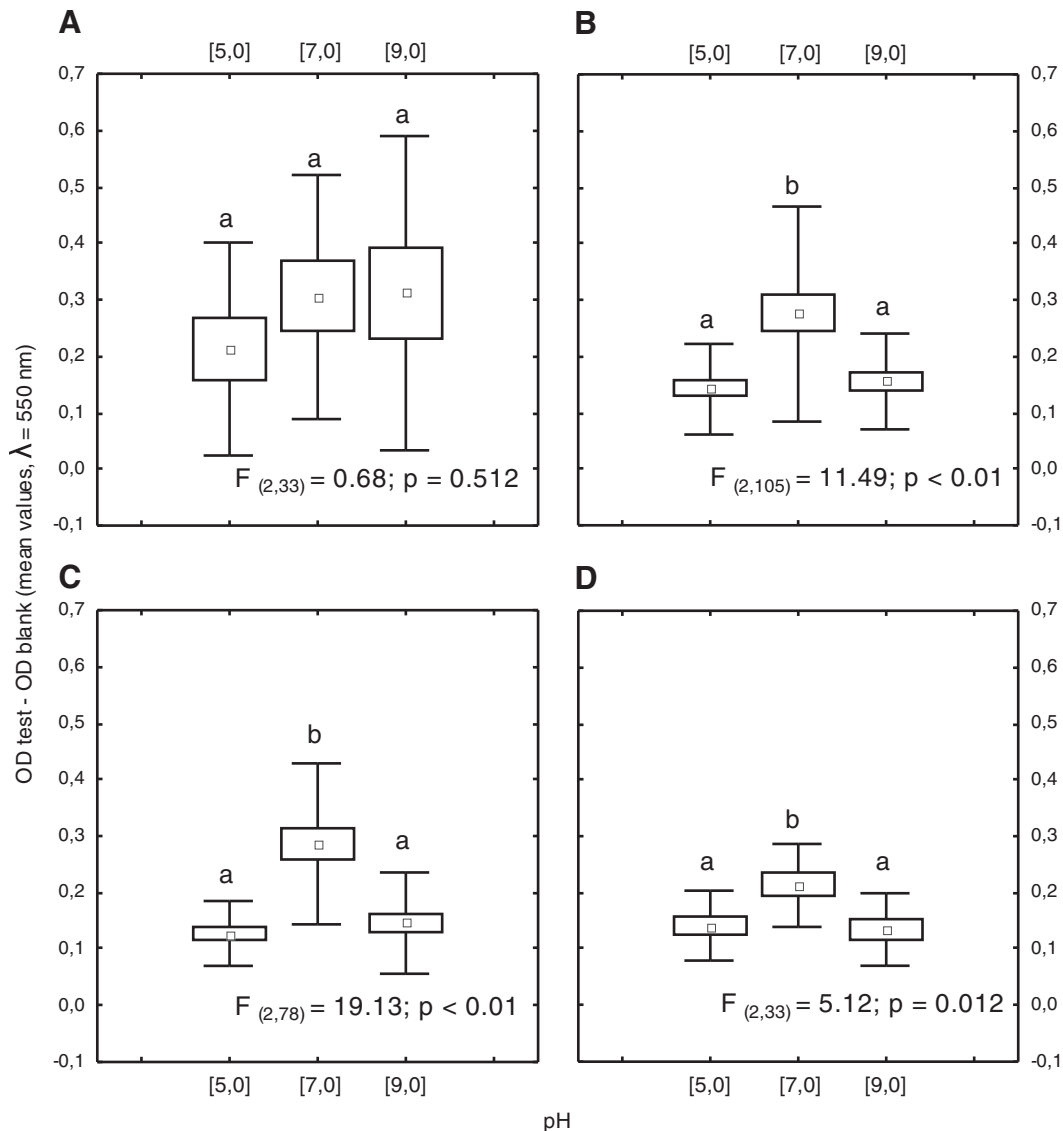


Fig. 5. Adhesion ability of the strains according to their origin and distinct pH values. A: non-food contact surfaces; B: food contact surfaces; C: bovine carcasses; D: end products. Differences in mean values of absorbance between *L. monocytogenes* isolates and negative controls (OD_{test} minus OD_{blank}). Mean values with distinct letters are significantly different by ANOVA ($p < 0.05$).

Isolate Fp01 from establishment C, for example, was obtained from final product (ground beef) and was categorised as possessing strong adhesion ability. This isolate also presented a similarity index $\geq 82\%$ with six other isolates (Cc01, Cc02, En02, En03, Ut11, and En04), all of which were obtained in the same establishment on previous sampling dates over a period of 5 months. At establishment E, Ut02 and Ut03 were detected in a tenderiser one month after Ut01 was detected in a plastic box for cuts storage. These three isolates presented 100% similarity by PFGE (Fig. 1) and were categorised as moderate in terms of adhesion. These results may suggest the persistence of *L. monocytogenes* strains in beef processing plant E and may suggest that their adhesion capacities play important roles in this persistence, even though those are not related to the genetic profile of the isolate (Jiang et al., 2008; Rivoal et al., 2010). The isolates obtained from establishment A presented higher genetic variability and distinct adhesion capacities (Fig. 1), indicating continuous contamination of the environment by new strains that may have arrived with bovine carcasses, as suggested by Thevenot et al. (2006) for swine carcasses.

The results for adhesion ability with respect to the tested variables (Table 2) are presented in Figs. 2 through 6. Poor adhesion performance

was observed when the isolates were cultivated in diluted culture media, with significant differences mainly seen for diluted meat broth ($p < 0.05$) (Fig. 2). It has been postulated that low levels of nutrients in the growth medium can be considered a stress factor for microorganisms, leading to adherence and biofilm formation (Stepanović, Ćirković, Ranin, & Švabić-Vlahović, 2004). However, the low level of nutrients did not enhance the adhesion of the *L. monocytogenes* isolates in the present study, as observed elsewhere (Moltz & Martin, 2005; Stepanović et al., 2004).

With regard to the carbohydrate source (Fig. 3), the isolates presented poor adhesion when they were cultivated in meat broth supplemented with maltose, but this difference was only significant when compared to fructose, apart from the end products group, where no differences were observed ($p < 0.05$). Isolate En02 was categorised as possessing weak adhesion ability (Fig. 1), but it presented high adhesion when cultivated in meat broth supplemented with fructose (data not shown). Our findings are consistent with Kim and Frank (1994), who reported that the carbohydrate source is not relevant for adhesion and biofilm formation.

In the current study, the isolates presented higher adhesion ability when they were cultivated in culture medium with 5% NaCl, but

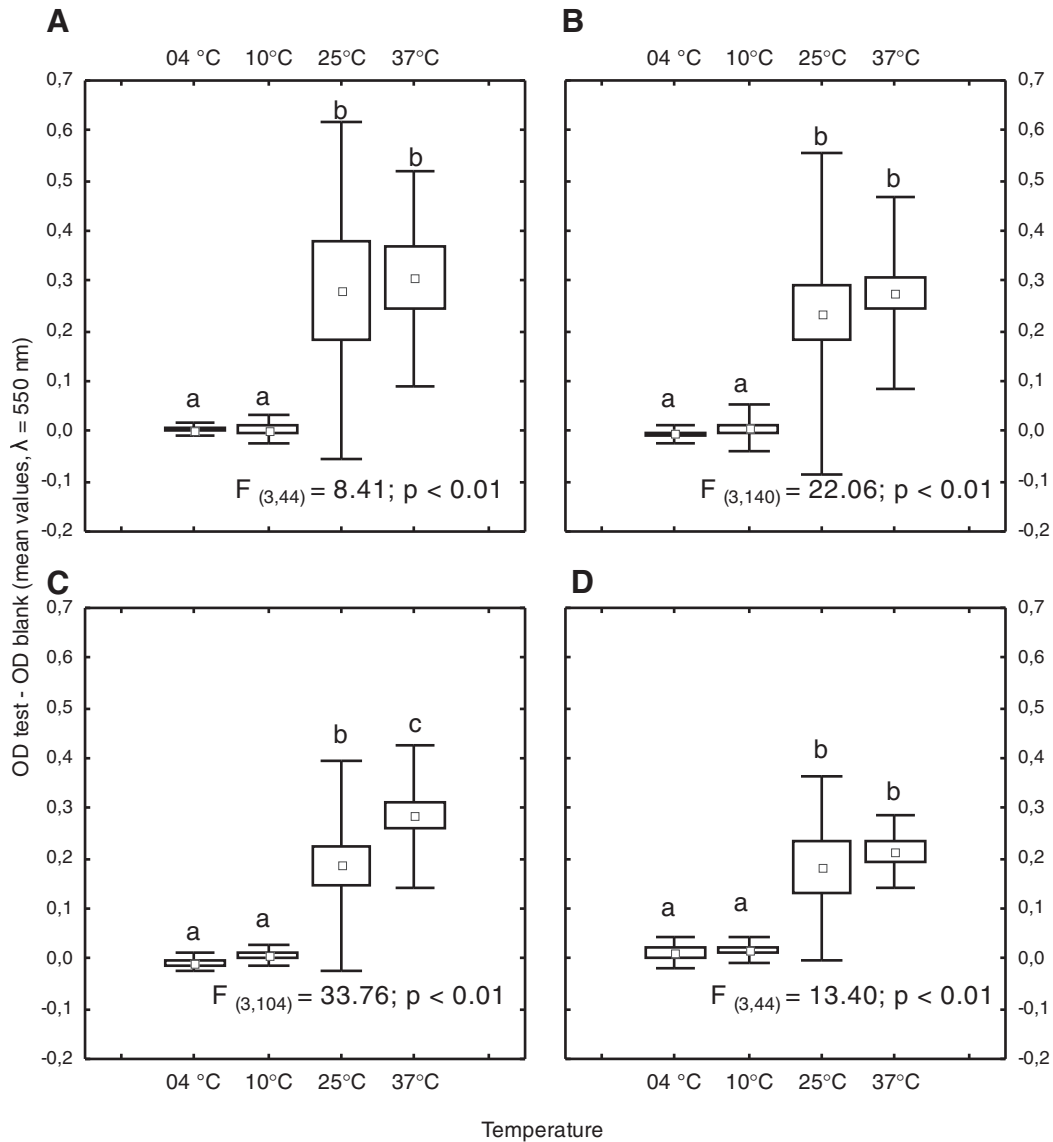


Fig. 6. Adhesion ability of the strains according to their origin and distinct incubation temperatures. A: non-food contact surfaces; B: food contact surfaces; C: bovine carcasses; D: end products. Differences in mean values of absorbance between *L. monocytogenes* isolates and negative controls (OD_{test} minus OD_{blank}). Mean values with distinct letters are significantly different by ANOVA ($p < 0.05$).

there were no significant differences between the isolates obtained from non-food contact surfaces ($p > 0.05$, Fig. 4). Interestingly, NaCl concentrations exceeding 5% have been reported to inhibit *L. monocytogenes* adhesion (Jensen, Larsen, Ingmer, Vogel, & Gram, 2007), even in those strains that are capable of multiplying in culture medium with up to 15% NaCl (Caly, Takilt, Leuret, & Tresse, 2009).

With regard to pH variation, the isolates presented higher adhesion at pH 7.0, except the isolates obtained from non-food contact surfaces ($p < 0.05$, Fig. 5). However, En01 was categorised as possessing strong adhesion ability (Fig. 1) and presented higher adhesion at pH 9.0 (data not shown); considering its origin (beef processing facility floor), these results suggest that the continuous use of alkaline sanitizers in this establishment may have selected this strain with resistance to this environmental condition, enhancing its adhesion characteristics as observed by Belessi, Gounadaki, Psomas, and Skandamis (2011). The results obtained differ from those obtained by Stopforth, Samelis, Sofos, Kendall, and Smith (2002), who found no influence of pH on the adhesion ability and biofilm formation of *L. monocytogenes*.

Finally, the isolates presented higher adhesion ability when cultivated at 25 °C and 37 °C than at lower temperatures ($p < 0.05$) (Fig. 6). These results corroborate the idea that temperature is a determining factor for *L. monocytogenes* adhesion, in agreement with that observed in a similar study (Moltz & Martin, 2005). However, it must be noted that in this study, the adhesion tests were conducted for the same period of incubation, independent of the tested temperature.

The results obtained allowed the establishment of relationships between the genetic profiles of persistent *L. monocytogenes* isolates and their serological identities. In counterpart, the adhesion characteristics of the isolates did not show a relationship with their genetic profiles. Finally, it was observed that typical stressing conditions of beef processing environments (low levels of nutrients, pH variations and low temperature) did not induce the adhesion of *L. monocytogenes* isolates.

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