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Short communication

Expression levels of the agr locus and prfA gene during biofilm formation by *Listeria monocytogenes* on stainless steel and polystyrene during 8 to 48 h of incubation 10 to 37 °C

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ABSTRACT

The objective of this study was to compare the gene expression levels of the *agr* locus and *prfA* gene during adhesion and biofilm formation by four L. *monocytogenes* isolates (2 biofilm-forming and 2 non–forming) on stainless steel and polystyrene surfaces at different temperatures (10 °C, 20 °C and 37 °C), and times (8 h, 12 h, 24 h and 48 h). The *agrA* and *prfA* genes were expressed at higher levels than the *agrBCD* genes. The levels of *agr* locus expression were higher in the biofilm-forming strains, and the greatest difference between biofilm-forming and non–forming isolates was observed for the *agrB*, *agrC* and *agrD* genes. However, no difference in the expression of the prfA gene was seen among the isolates, independent of the biofilm-forming ability. Maximum expression of the *agr* locus and *prfA* gene was observed at 37 °C, whereas expression was lowest at 10 °C. The *agr* locus, and particularly the *agrB*, *agrC* and *agrD* genes, is important in the initial adhesion phase of biofilm production by L. *monocytogenes*, with this expression independent of *prfA*. In addition, the *agr* locus and *prfA* gene expression levels were strongly influenced by time and temperature.

1. Introduction

Listeria monocytogenes is a ubiquitous, Gram-positive, non-spore forming and facultative intracellular pathogen that causes listeriosis, a food-borne disease, which is responsible for a high mortality (20–30%) in population risk groups (Farber and Peterkin, 1991; Gande and Muriana, 2003; Gilbreth et al., 2005).

This pathogen is a particular threat to the food industry as it can survive for long periods under environmental conditions such as high salt and low pH that are adverse to other relevant food microorganisms. In addition, it can multiply under refrigeration and persist on equipment and surfaces of processing plants by forming biofilms (Di Bonaventura et al., 2008; Lukinmaa et al., 2004; Pieta et al., 2014; Uhitil et al., 2004). However, the ability to produce biofilm varies among L. *monocytogenes* isolates, which may be due to the suppression or overactivation of genes whose products confer adaptive advantages in terms of adherence to abiotic surfaces.

L. monocytogenes has a wide repertoire of transport proteins and regulatory genes related to its ability to colonize a wide variety of ecosystems (Folsom and Frank, 2006; Glaser et al., 2001). Among these genes is *prfA*, a key transcriptional activator, regulating most of the expression of known virulence genes in L. *monocytogenes*, and the highly conserved locus *agr* (accessory regulatory gene) with orthologs in several Gram-positive bacteria (Autret et al., 2003; Garmyn et al., 2012; Luo et al., 2013; Paspaliari et al., 2014). The locus *agr* is composed of four genes, co-transcribed in an operon (*agr BDCA*), which encode four proteins. *agrB* is responsible for the expression of a membrane protein, whose function is to export the peptide expressed by *agrD*, which is transformed into a mature auto-inducing peptide (AIP). AgrC (histidine kinase) and AgrA (auto-regulatory) form a transduction signal of a two-component system. When the extracellular AIP concentration reaches a certain threshold, the system is activated by quorum sensing, through the detection of AgrC-AgrA, exerting regulatory effects on target substrates and inducing its own production (positive auto-regulation)

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(Autret et al., 2003; Riedel et al., 2009; Rieu et al., 2007;). The activated system regulates the genes encoding the adhesins required for biofilm formation (Paspaliari et al., 2014; Travier et al., 2013; Worthington et al., 2012).

In this sense, studies showed that mutations of *agr* locus genes decrease the ability of L. *monocytogenes* to adhere to abiotic surfaces and to form biofilm, and may also affect the infective capacity (Chang et al., 2012; Guariglia-Oropeza et al., 2014; Riedel et al., 2009).

In this study, new questions will be answered, which were not fully addressed by other studies. New methods will be used under different conditions. The objective of this study was to compare the levels of gene expression of the *agr* locus (*agrB*, *agrD*, *agrC,* and *agrA*) and of the *prfA* gene, in first hours of adhesion of biofilm formation, by L. *monocytogenes* isolates of different serotypes, selected for their ability to produce biofilm (biofilm forming and non-forming) and their origins (food or processing environment), on different surfaces (stainless steel and polystyrene) at different times (8 h, 12 h, 24 h, 48 h) and temperatures (10 °C, 20 °C, 37 °C).

2. Materials and methods

2.1. Bacterial strains

Four isolates of L. *monocytogenes*, from the bacterial library of the Food Microbiology Laboratory of the Department of Agroindustrial Science and Technology of the Federal University of Pelotas (UFPel), were selected for their ability to produce biofilm (biofilm forming and non-forming) and their origins (food or processing environment) (Table 1). The capacity of biofilm formation was previously evaluated according to Stepanovic et al. (2007) and Djordjevic et al. (2002). Aside from these criteria, the serotype of the isolates (1/2a, 1/2b) was also taken into account, because they are known to be related to high percent of cases of listeriosis (Farber and Peterkin, 1991).

2.2. Purification and characterization of strains

Isolates stored at −70 °C were recovered in Tryptone Soy Broth (TSB, Oxoid CM0129) with 0.6% Yeast Extract (YE, Oxoid LP0021), and incubated at 37 °C for 24 h. Subsequently, a Tryptone Soy Agar (TSA, Oxoid CM0131) containing 0.6% YE plate was streaked from the TSB culture, and incubated at 37 °C for 24 h. From this culture, a scraping of each isolate from the TSA plate were transferred and suspended in test tubes containing 3 mL of 0.85% saline solution (Vetec), standardizing the inoculum to a 0.5 scale of MacFarland (Laborclin), corresponding to approximately 1.5×10^8 CFU/mL.

2.3. Assessment of gene expression under biofilm-forming and planktonic conditions

Two surface types were tested: stainless steel (SS - AISI 304 stainless steel coupons, diameter 8 cm - local manufacturer- high quality roughness stainless steel polished AISI 304) and polystyrene (PS - Petri plates - 8 cm in diameter - Kasvi), according to the protocol proposed by Rieu et al. (2007), with adaptations. Stainless steel is typically present in food processing environments. Polystyrene was chosen because is the material found in Petri plates, is not typically present in food processing environments, but is good plastic polymer model to biofilm formation assays. Petri dishes (with and without SS coupons) were filled with 9 mL of TSB-YE 1% glucose and 1 mL of the standardized inoculum (10^8 CFU/mL) and incubated at 10 °C, 20 °C and 37 °C, for 8 h, 12 h, 24 h, and 48 h. After completion of each period, the culture medium was removed and the coupons or the Petri dishes were washed twice with 10 mL of phosphate-buffered saline (PBS, pH 7.3 ± 0.2; Laborclin) to remove unbound cells, and then dried at 37 °C. Thereafter, the adhered cells were removed using two sterile swabs, which were inserted into test tubes containing 0.85% saline solution (10 mL), and vortexed for 1 min. A 1 mL aliquot was transferred to an Eppendorf microtube and centrifuged at 6048rcf (g) for 5 min at 4 °C for concentration the cells (the concentration varied from 10^3 to 10^6 CFU/mL, determined by plate counting). This variation is due to verified viable cell concentrations. Under unfavorable conditions for growth, the minimum concentrations were found and under favorable conditions the highest concentrations were observed.

The planktonically grown cells of L. *monocytogenes* were subjected to the similar treatments as used in the evaluation of biofilm formation (biofilm formation conditions - BFC, at 10 °C - commonly used cooling temperature, 20 °C - room temperature and 37 °C - this temperature not typically relates to biofilms on stainless steel or polystyrene in food processing facilities, but is best condition temperature for majority bacteria and is the host temperature, for 8 h, 12 h, 24 h, and 48 h). Each L. *monocytogenes* strain was separately cultured in a microfuge tube containing 900 µL of TSB-YE and 100 µL of standardized inoculum (10^8 CFU/mL). After the different incubation periods, the removed planktonic cells were centrifuged at 6048rcf (g) for 5 min at 4 °C, in aim of precipitate the cells and separate them from the medium, to reach the desired cell concentration for all treatments (the concentration varied from 10^3 to 10^6 CFU/mL, as previously explained).

2.4. RNA isolation and cDNA synthesis

The mRNA was extracted from L. *monocytogenes* biofilm-forming and planktonic cells using the RiboPure™-Bacteria Kit (Part Number AM 1925-, Ambion, Life Technologies Corporation, Carlsbad, CA - USA), according to the manufacturer's protocol. The RNA extracted from each sample was quantified and tested for purity by calculating the A_{260}/A_{280} ratio in a spectrophotometer (NanoVue Version 4282 V2.0.4). Extracted RNA with a purity of 1.8–2.0 was used for cDNA synthesis. The cDNA synthesis was performed with a High Capacity cDNA Reverse Transcription Kit (Part Number 4368814, AppliedBiosystems, Life Technologies Corporation, Carlsbad, CA - USA), according to the manufacturer's instructions.

2.5. Primers, validation and expression profile of the agr locus and prfA gene by real-time PCR (RT-qPCR)

The oligonucleotides are listed in Table 2.

Each primer pair was validated by means of dissociation curves and the average efficiency was 103 ± 15%. The expression was validated and evaluated with the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems).

Table 1
Isolates of L. *monocytogenes* selected for evaluation of the expression of genes of the *agr* locus and *prfA* gene during biofilm formation.

Isolate	Origin		Serotype	Biofilm-forming ability[a]
LS006	Processing environment	Storage box for meat products	1/2c	Non-forming
LS020		Drain of the cooling chamber of the chicken slaughterhouse	1/2b	Biofilm-forming
LA003	Food	Sheep carcass	1/2a	Non-forming
LA039		Frozen chicken purchased in retail	1/2a	Biofilm-forming

[a] Classification according to Stepanovic et al. (2007) and Djordjevic et al. (2002).

Table 2
Oligonucleotide sequences used to quantify the expression of genes of the *agr* locus and *prfA* gene in L. *monocytogenes*.

Gene	Sequence (5′ → 3′)	Fragment size (bp)	Theoretical MT (°C)	R^2	Efficiency	Reference
agrA	(F) GCAAGCAGAAGAACGGATTTCCAA	726	62.90	0.99	2.17	Rieu et al. (2007)
	(R) CGCTGTCTCAAAAAACAAGATAT		57.40			
agrB	(F) CGGCAGACACAGAAAGTTTG	612	60.40	0.99	2.07	Rieu et al. (2007)
	(R) TGCGAATGGTATTAGCAACG		58.40			
agrC	(F) GGGGTCAATCGCAGGTTTTG	1293	62.40	0.96	2.24	Rieu et al. (2007)
	(R) CTTTAAGTTCGTTGGTTGCCGTA		61.00			
agrD	(F) AAATCAGTTGGTAAATTCCTTTCTAG	159	58.30	0.99	1.98	Rieu et al. (2007)
	(R) AATGGACTTTTTGGTTCGTATACA		57.70			
prfA	(F) GGAGCATGTGGTTTAATTCG	193	58.00	0.83	1.85	França et al. (2012)
	(R) CCAACTAAATGCTGGCAACT		58.20			
16S rRNA	(F) GGTAGCCTGTTCGCTAATGA	199	58.10	0.87	1.84	França et al. (2012)
	(R) TAACCAATGGGATCCACAAG		57.90			

R^2 (correlation coefficient) between the threshold cycle (TC) and the slope* (endogenous gene); Efficiency = $10^{(-1/\text{slope}_*)}$. *The slope of a standard curve is commonly used to estimate the PCR amplification efficiency of a real-time PCR reaction and its value is given by the equation of the line y = mx + b (m = slope).

Triplicate samples were placed in 96-well plates and covered with optical adhesive. The gene expression level was determined by relative quantification (RQ) and calculated based on the threshold cycle (ΔΔCt) described by Pfaffl (2001), using software 7500 2.0.4 (Software 7500, 2010). The planktonic cells were a standard control for comparison of the results of the relative quantification (RQ) and the 16S rRNA gene was used for normalization.

2.6. Statistical analysis

The relative quantification (RQ) was calculated and the control results were subjected to ANOVA, followed by Tukey's mean test, with STATISTICA 7.0, to detect significant ($p < 0.05$) differences comparing the expression levels of biofilm forming and non-forming isolates (Statsoft, 2004).

3. Results and discussion

In this study, the expression levels of the genes of the *agr* locus and of the *prfA* gene in L. *monocytogenes* isolates with and without biofilm-forming ability were compared during adhesion and biofilm formation at different temperatures and incubation times. Planktonically grown cultures (PC) were used as controls under the same incubation conditions to determine differences in expression of the same genes.

Four isolates of L. *monocytogenes* (two isolated from processing surfaces and two from food) were used, of which two were biofilm producers and the other two not. The biofilm-forming strains belonged to serotypes 1/2a and 1/2b and the non-biofilm forming strains to serotypes 1/2a and 1/2c.

The expression of genes of the *agr* locus and *prfA* gene in biofilm-forming strains and non-forming isolates of L. *monocytogenes* is shown in Fig. 1. In general, the *agrA* and *prfA* genes were expressed at higher levels ($p < 0.05$) than the *agrB, agrC* and *agrD* genes, with no significant ($p > 0.05$) differences between the expression of these two genes. The expression of the *prfA* gene was not significantly ($p > 0.05$) different among the isolates, regardless of their biofilm-forming ability.

The non-biofilm-forming strains LS006 and LA003 had lower expression levels of the *agr* locus genes ($p < 0.05$) than the biofilm-forming strains LS020 and LA039. However, the *agrA* gene of isolate LA003 had expression levels similar to those of the biofilm-forming strains ($p > 0.05$).

There was no difference ($p > 0.05$) in the expression of *agrBCD* genes among non- biofilm forming isolates, which had lower expression levels than the biofilm-forming strains. Similarly, the biofilm-forming strains LS020 and LA039 did not differ ($p > 0.05$) from each other in *agrBCD* gene expression.

A difference could be detected in the gene expression of the *agr* locus, especially of the *agrBCD* genes, between biofilm-forming and non-forming isolates, with higher expression of these genes in the biofilm-forming strains. These results suggest an effective participation of the *agr* locus, mainly of the *agrBCD* genes, in the initial phase of biofilm formation by L. *monocytogenes* isolates on abiotic surfaces. Other authors, using mutant L. *monocytogenes* strains (*agrA*- and *agrD*-), also observed a reduction in their biofilm-forming ability compared to the parent strains (Chang et al., 2012; Garmyn et al., 2012; Riedel et al., 2009; Rieu et al., 2007), corroborating the results of this study. Temperature influenced the expression of all analyzed genes (Fig. 2). At 10 °C, the genes of the *agr* locus were less expressed ($p < 0.05$) than at 37 °C, and the individual expression of the genes of this locus also differed significantly ($p < 0.05$). The expression level of the *agrA* gene was higher ($p < 0.05$) than that of the other genes of the *agr* locus at almost all temperatures tested, except for 37 °C, where it was similar to that of the *agrB* gene ($p < 0.05$). The expression levels of the *prfA* gene also differed as a function of the evaluated temperatures, and were higher at 37 °C than at 10 °C ($p < 0.05$), regardless of whether the isolate was biofilm-forming or non-forming.

In addition to the influence of temperature on the overall gene expression of the *agr* locus and *prfA* gene, a difference was detected in the expression of these genes among the L. *monocytogenes* isolates, as a function of this parameter (Fig. 2). The isolate LS006, which was not a biofilm producer, had the lowest mean expression for the analyzed genes, especially at low temperatures. The biofilm-forming strains LS020 and LA039 had the highest expression levels, mainly at 37 °C for *agrA*. At 20 °C, isolate LA039 had higher expression levels of *agrA* than the non-biofilm forming isolates. However, the same was not observed for isolate LS020, although both are biofilm producers.

The *agrA* gene had the highest expression level of all evaluated genes, mainly at 37 °C. The highest expression observed for the *agrA* gene corroborates the study of Garmyn et al. (2012), who suggested that the peptide produced by *agrA*, aside from effectively participating in the initial adhesion of L. *monocytogenes* to surfaces, may also be involved in other functions, e.g., regulating the transport of nitrogen, amino acids, purines, and pyrimidinase, which could explain its higher expression levels.

In contrast, expression of the other genes of the locus (*agrBCD*) was similar ($p > 0.05$). Furthermore, these genes were similarly expressed ($P > 0.05$) in both the biofilm-forming (LS020 and LA039), and non-biofilm-forming strains (LS006 and LA003). However, there were differences ($p < 0.05$) comparing the biofilm-forming and non-forming isolates.

Unlike our findings, Rieu et al. (2009) reported similar expression levels between *agr* locus genes in planktonic L. *monocytogenes* isolates and during biofilm formation, with a higher level of *agrD* and a lower level of *agrA* expression. These differences between Rieu et al. (2009) and our study can be explained by the environmental induction that the L. *monocytogenes* genes undergo, including the agr locus, since the

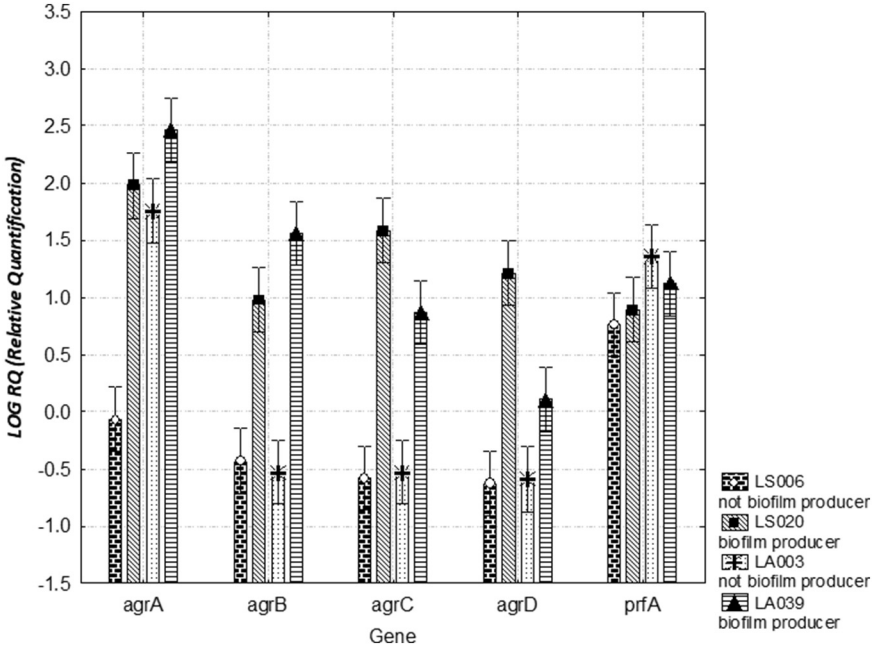

Fig. 1. Relative quantification of the overall gene expression of the *agr* locus and *prfA* gene in L. *monocytogenes* isolates during biofilm formation. The results are the average of all conditions.

experimental conditions in the Rieu et al. (2009) work were different from those used in our study.

Although the sample size may seem too small to conclude anything about the origin of the isolates, there was no relationship between the levels of locus expression and the origin (processing or food environment) of the isolates, nor with the serotype. Other studies (used a number of samples similar to or less than the one used in this study) indicated an interaction between biofilm-forming ability and different L. *monocytogenes* serotypes (Borucki et al., 2003; Djordjevic et al., 2002; Kadam et al., 2013; Lee et al., 2013; Nilsson et al., 2011). Thus, our results highlight the importance of the *agr* locus for biofilm formation, considering that its expression levels were directly related to the intrinsic capacity of the isolate to produce biofilm, rather than with a specific serotype of L. *monocytogenes*.

Another interesting response was observed in the behavior of the *prfA* gene, which had similar expression levels in the biofilm-forming and non-forming isolates. The *prfA* gene is a key transcriptional activator, which regulates the expression of most of the known virulence genes of L. *monocytogenes* (Luo et al., 2013). Some authors suggested a possible synergistic action between the *agr* system and regulatory *prfA*, by suppression of the *agrA* or *agrD* gene, which could affect the expression of some genes related to the virulence of L. *monocytogenes* one being the proper regulator *prfA* (Garmyn et al., 2012; Autret et al., 2003; Riedel et al., 2009).

Other studies pointed out that there may be an indirect participation of the regulator *prfA* in biofilm formation (Lemon et al., 2010; Lemon et al., 2007; Travier et al., 2013; Zhou et al., 2011). Zhou et al. (2011) describe that *PrfA* might exert effects on L. *monocytogenes* homeostasis by indirectly regulating expression of genes involving transporters, metabolic enzymes, regulators, and proteins of unknown function.

According to Luo et al. (2013), the suppression of the *prfA* gene could alter the expression of regulatory genes involved in biofilm formation, reducing the biofilm-forming ability of L. *monocytogenes*. However, expression of *prfA* for the biofilm-forming and non-forming isolates was similar, suggesting in that case an interaction between the *prfA* gene and the *agr* locus during biofilm formation. This would be related to the expression of *agrA,* rather than to the whole *agr* system, since *agrA* and *prfA* had similar expression levels. This can be justified

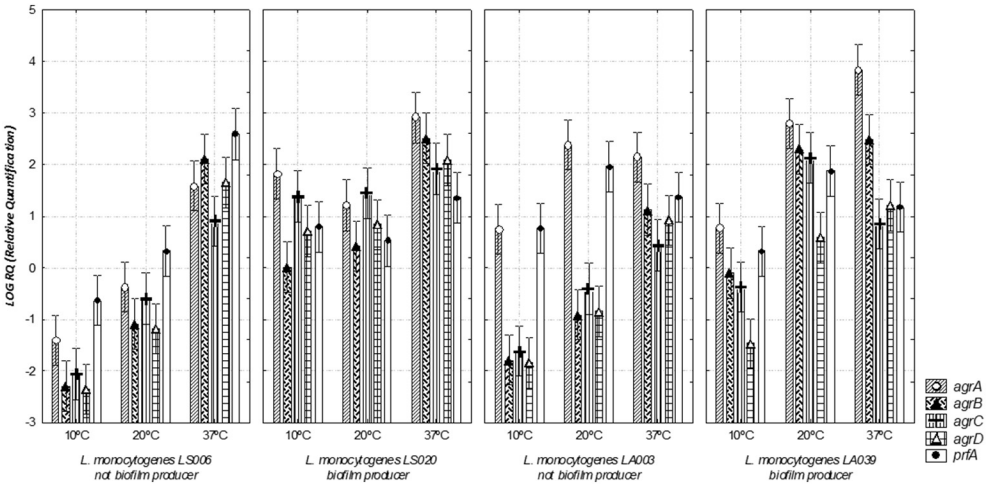

Fig. 2. Relative quantification of the gene expression of the *agr* locus and *prfA* gene in *L. monocytogenes* isolates during biofilm formation as a function of temperature.

by the study by Lemon et al. (2010), where mutant strains of the *prfA* gene showed low capacity for biofilm formation, however, this difference was more pronounced after the first hours of contact, which was not contemplated in this study.

The temperature and time variables affected the expression of genes of the *agr* locus and of the *prfA* gene, both for biofilm-forming and non-forming isolates of L. *monocytogenes*. Temperature is an important environmental stimulus in the regulation of the gene expression of L. *monocytogenes* (Garmyn et al., 2012). In this sense, the highest expression levels of the *agr* locus were found at 37 °C, as also reported elsewhere (Pieta et al., 2014; Garmyn et al., 2012; Harmsen et al., 2010). The expression levels of the *agrA* gene were highest at 37 °C in the biofilm-producing isolates and lowest for *agrD* at 10 °C in the non-biofilm-producing isolates, suggesting the critical role of temperature in the regulation of some genes of the *agr* locus.

Some studies described the importance of flagellar motility for biofilm formation by L. *monocytogenes* (Harmsen et al., 2010; Lemon et al., 2007; Tremoulet et al., 2002), which has expression regulated above 25 °C and repressed 37 °C (Lemon et al., 2007; Tremoulet et al., 2002). However, some authors stated that L. *monocytogenes* isolates can express genes responsible for motility and chemotaxis at temperatures higher than or equal to 37 °C, and may even be regulated by *prfA* (Bigot et al., 2005; Djordjevic et al.,2002; Gamble and Muriana, 2007; Garmyn et al., 2012; Luo et al., 2013). Thus, in the biofilm formation by isolates whose *agr* locus and *prfA* gene were expressed at significantly higher levels at 37 °C, the flagellum may have participated, or this initial adhesion may have occurred passively, independent of the flagellum (Tresse et al., 2009).

The influence of incubation time on the overall gene expression of the genes of the *agr* locus and *prfA* gene in biofilm-forming and non-forming isolates of *L. monocytogenes* is also shown in Fig. 3. The incubation time also influenced the expression levels of genes of the *agr* locus and of the *prfA* gene (Fig. 3), which did not differ significantly ($p > 0.05$) when compared to each other at the same incubation times. The expression levels of all genes were higher at 12 and 24 h, and lowest after 48 h of incubation. In addition, comparing individually, there was no difference in gene expression between 12 and 24 h ($p < 0.05$).

The expression of these genes was lowest for isolate LS006, a non-biofilm producer, after 48 h of incubation ($p < 0.05$). However, isolate LA003, another a non-biofilm former, also had a low overall gene expression after 48 h of incubation, but did not differ significantly from the biofilm-forming LS020 after the same time.

The levels of overall expression of the evaluated genes ($p < 0.05$) were highest for the biofilm-forming strains (LS020 and LA039 - $p > 0.05$) after 24 h. However, no differences in overall gene expression were observed comparing the biofilm-forming and non-forming isolates ($p > 0.05$) after 8 and 12 h of incubation. As with temperature, the incubation time influenced the expression of the *prfA* gene, regardless of whether the isolate was a biofilm producer or not.

In relation to the incubation period, the gene expression levels compared, were highest after 24 h, both for the *agr* locus and *prfA* gene, corroborating other studies, indicating the participation of the *agr* locus in the initial phase of biofilm formation by L. *monocytogenes* (Luo et al., 2013; Rieu et al., 2009), as well as higher levels of *prfA* expression in L. *monocytogenes,* producing biofilm in the first 24 h (Zhou et al., 2011). Thus, since biofilms formed by L. *monocytogenes* are considered mature after 48 h of incubation with the surface (Lee et al., 2013), the decline in *agr* locus expression after 48 h observed in this study indicates the importance of this system in the first hours of adhesion, mainly between 12 and 24 h. In this sense, it is most likely that the *agr* locus can also indirectly regulate the expression of pro-proteins, i.e., the precursor proteins required for cell adhesion to abiotic surfaces (Rieu et al., 2009).

The highest number of L. *monocytogenes* virulence factors was regulated at 37 °C (Garmyn et al., 2012) and the expression of the *prfA* regulator was higher at this temperature. Low expression levels of this gene were observed at lower temperatures, in both biofilm-forming as well as non-forming isolates. Moreover, the expression of *prfA* at 37 °C did not differ from that at 20 °C, as confirmed by Garmyn et al. (2012) when evaluating this gene in *agrD* mutant strains, and by Pieta et al. (2014), who reported *prfA* expression at even lower temperatures (7 °C).

There was no relationship between the tested surfaces (SS and PS) and the levels of gene expression ($p > 0.05$), regardless of the biofilm-forming ability of the isolates (Fig. 4).

Bacteria adhesion to these surfaces, mainly in the early stages of biofilm formation, can be influenced by the physico-chemical properties of the surface, the characteristics of the microorganism, and the nutrient contents of the medium (Takhistov and George, 2004). Surface hydrophobicity may affect the rate of bacterial adhesion, since hydrophobic interactions tend to increase with the increasing non-polar nature of the surfaces involved (Simões et al., 2010; Zeraik and Nitschke, 2010). In this way, hydrophobic surfaces could favor colonization by eliminating the water layer present at the interface (Simões et al., 2010; Zeraik and Nitschke, 2010). A positive correlation between bacterial adhesion and biofilm formation according to the hydrophobicity of the material was demonstrated by Takahashi et al. (2010), suggesting that changes in the surface material could have an impact on the expressed genes, among which are genes associated with cell motility and with surface structures, as well as regulatory genes (Chang et al., 2012). However, in this study, no influence of the different materials (polystyrene and stainless steel) on the gene expression of the *agr* locus and *prfA* gene was observed during initial adhesion of L. *monocytogenes*, independent of the properties of the material (Fig. 4).

Besides these observations, previously reported, integrating two

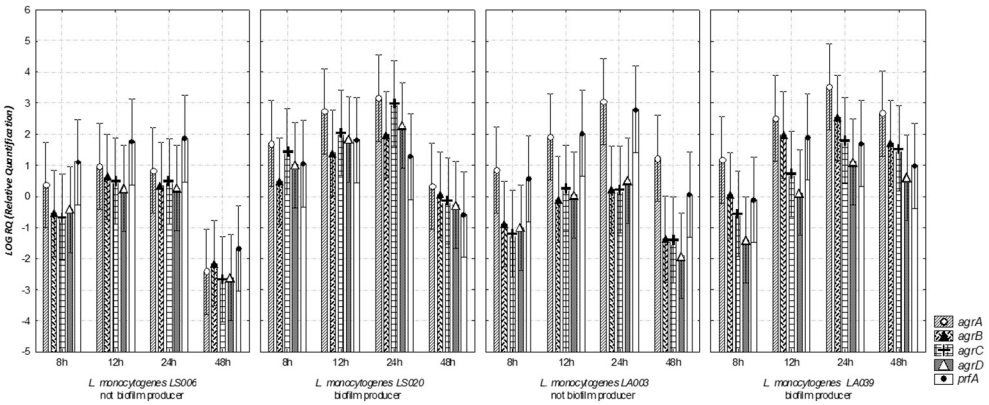

Fig. 3. Relative quantification of genes of the *agr* locus and *prfA* gene in *L. monocytogenes* isolates as a function of incubation time during biofilm formation.

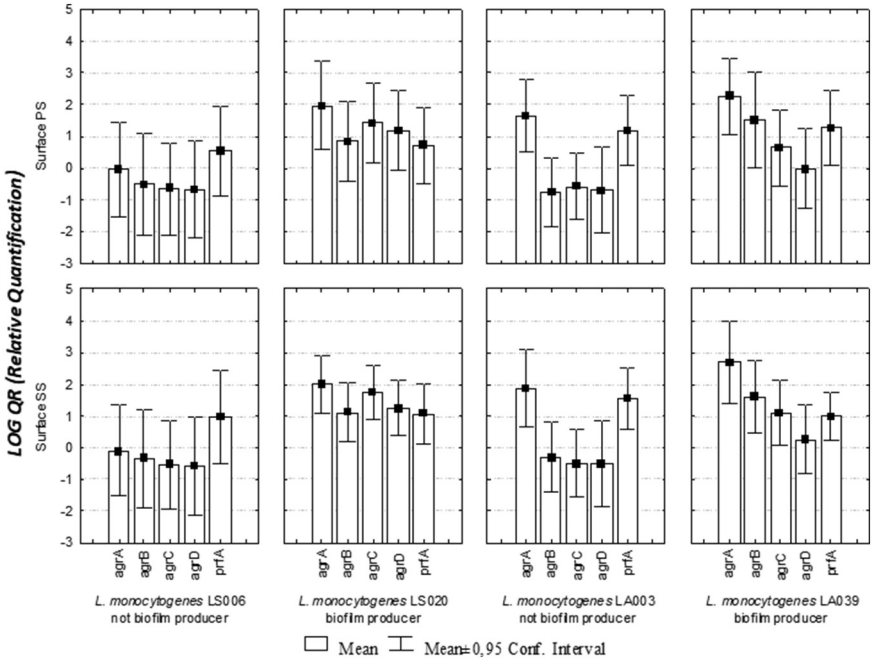

Fig. 4. Relative quantification of genes of the *agr locus* and *prfA* gene in *L. monocytogenes* isolates as a function of surfaces biofilm formation.

variables (gene and isolates, temperature and gene, temperature and isolates, incubation time and gene, incubation time and isolates), more highlights integrating three variables could be verified in our study (gene, temperature and incubation time; gene, incubation time and isolates; gene, temperature and isolates).

Superior expression ($p < 0.05$) of *agrABCD* and *prfA* genes was verified at 37 °C after 12 and 24 h and of the gene *agrA* at 20 °C after 12 and 24 h. Considering the isolates, the expression of *agrA* gene after 12 and 24 h to 37 °C was superior ($p < 0.05$) for the isolates LS020 and LA039 (biofilm-forming) or a 20 °C for the isolates LA003 and LA039 (origin from food). However, *prfA* expression was significantly greater ($p < 0.05$) after 12 and 24 h for isolate LA003 and for isolate LS006 at 37 °C (both non-biofilm-formers).

Lower expression ($p < 0.05$) of the *agrABCD* genes occurred in the isolate LS006 (non-biofilm-forming from processing environment) at 10 °C, principally after 48 h of incubation. Also under these conditions of time and temperature, the same was observed for the isolate LA003 (non-biofilm-forming from food), however only for the *agrBCD* genes. Based on these findings, the *agr* locus has an important function in both adhesion and biofilm formation by L. *monocytogenes* isolates, particularly of the *agrBCD* genes. The expression of the *agr* locus was not related to the expression of the *prfA* gene, nor was there any interference of the serotype or the evaluated origins of the isolates. Moreover, the expression levels of the genes of this locus and *prfA* gene were strongly influenced by temperature and surface incubation time, but unaffected by the tested incubation surfaces (stainless steel and polystyrene).

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