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ORIGINAL ARTICLE

Transcriptional analysis of genes related to biofilm formation, stress-response, and virulence in *Listeria monocytogenes* strains grown at different temperatures

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Abstract The genus *Listeria* includes eight different species, but only *Listeria monocytogenes* is associated to human illness. This microorganism is capable of growing in temperatures ranging from 4 °C to 37 °C and forming biofilms on processing sites, which is of great concern in the food industry. In this current work, the transcription of genes related to biofilm formation, stress-response, and virulence in two strains of *L. monocytogenes*, serotypes 1/2a and 4b, growing at 7 °C and 37 °C, was analyzed by quantitative real time PCR (qPCR). For both serotypes, a temperature of 7 °C significantly increased ($P<0.05$) the transcription level of *sigB*, *prfA*, *luxS*, *sufS*, *sufU*, *ltrC* and *flaA* genes when compared to growth at 37 °C, whereas transcription of the *hly* gene did not vary significantly at the temperatures tested. Incubation at 7 °C increased the transcriptional level of the *actA* gene only in *L. monocytogenes* serotype 1/2a. On the other hand, *L. monocytogenes* serotype 4b showed significantly increased transcription of the *degU* gene at 7 °C. Interestingly, expression of the *agrA* gene, which is involved in adhesion and biofilm formation on abiotic surfaces, was not detected in serotype 4b, and its transcription level was lower at 7 °C in serotype 1/2a. These results demonstrate that the two studied *L. monocytogenes* serotypes, which are responsible for many human listeriosis cases, have different molecular mechanisms at temperatures of 7 °C and 37 °C.

Keywords *Listeria monocytogenes* · Temperature · *agrA* · *hly* · *actA* · qPCR

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Introduction

Among the eight species of the genus *Listeria*, *Listeria monocytogenes* is the only one associated with human illness. Additionally, *L. monocytogenes* is a psychrotrophic microorganism, distributed widely in the environment, with low infectious doses and is the causative agent of listeriosis—a severe disease, affecting mainly the elderly, children, newborn, pregnant women and immunocompromised individuals. Thirteen different serotypes have been described for this bacterium, with serotypes 1/2a, 1/2b and 4b being responsible for most human cases of listeriosis (Pan et al. 2010). Of these serotypes, 4b has been involved in most major outbreaks (37–64 % of cases) and is associated with abortion, whereas most *L. monocytogenes* strains isolated from food sources or food processing plants belong to serotype 1/2a (Kathariou 2002; Hofer et al. 2006; Nes et al. 2010).

Listeria monocytogenes can adhere to and form biofilms on several materials, such as stainless steel, glass and polymers (Habimana et al. 2009); however, little is known about the molecular mechanisms responsible for these processes (Suo et al. 2012). Once formed, biofilms are very difficult to be completely eliminated, thus compromising the sanitary quality in various food industries and resulting in many problems such as damage to equipment, product contamination and energy losses (Trachoo 2003). Several environmental factors that are commonly related to food and food processing plants, such as temperature, concentration of salt and sugar, pH, and the presence of nutrients, are important in the initial adhesion and subsequent biofilm formation by *L. monocytogenes* (Moretro and Langsrud 2004).

Listeria monocytogenes has evolved over a long period of time to acquire a diverse set of genes and corresponding proteins, each with its own unique properties and functions in the survival and pathogenicity of this microorganism (Liu 2006). One example is the transcriptional regulator PrfA—an important transcription factor that controls the passage of bacteria from the extracellular to the intracellular form, and which upregulates the transcription of several virulence genes. Previous studies have indicated this factor as an important regulator of biofilm formation in this organism (Lemon et al. 2010). In addition to the virulence genes, there are some stress-response genes that, in the case of *L. monocytogenes*, are controlled by the transcription factor SigmaB (SigB) (Van der Veen and Abee 2010), and previous studies have demonstrated its involvement in adaptation of this microorganism to low temperatures (Becker et al. 2000).

Temperature is often used as a signal for controlling the transcription of virulence genes required for infection or genes necessary for persistence in the environment. However, very little is known about the molecular mechanisms that allow bacteria to adapt and respond to temperature fluctuations (Kamp and Higgins 2011). For example, flagellar motility, which is important for survival of the pathogen outside the host and colonization of the host during the early stages of infection, is temperature-dependent in *L. monocytogenes* (O'Neil and Marquis 2006), which is a non-motile microorganism at mammalian physiological temperature (Gründling et al. 2004). Kamp and Higgins (2011) also reported that many pathogenic bacteria, when exposed to the mammalian physiological temperature of 37 °C, undergo changes in transcription and translation of its genetic material, with large effects on physiology, survival and virulence.

Survival of *L. monocytogenes* in acidic environments, such as the human stomach, is vital to its transmission, and several studies have demonstrated the importance of investigating the connectivity between acid stress, low temperature gene induction, and the virulence properties of this microorganism. According to Ivy et al. (2012), growth of *L. monocytogenes* at 7 °C increases susceptibility to an artificial gastric fluid, when compared to growth at 30 °C or 37 °C, suggesting that temperatures commonly encountered during food storage and distribution affect the ability of *L. monocytogenes* to survive gastric transit and, ultimately, cause disease. Another study demonstrated a substantial reduction in the transcription of the *iap* virulence gene, involved in macrophage invasion, during growth of *L. monocytogenes* at 5 °C in the presence of 7 % NaCl (Burall et al. 2012). On the other hand, Czuprynski (2005) showed that *L. monocytogenes* grown at 4 °C or 37 °C exhibited similar infection phenotypes upon intragastrical inoculation, and it has also been reported that *prfA* and other virulence factors are transiently induced by acidification at lower temperatures (10 °C, 18 °C and 25 °C), as well as at the mammalian body temperature of 37 °C (Neuhaus et al. 2013).

Based on this data, the aim of this study was to perform a transcriptional analysis by quantitative real time polymerase chain reaction (qPCR) of *agrA*, *degU*, *luxS*, *prfA*, *hly*, *actA*, *flaA*, *sigB*, *ltrC*, *sufS* and *sufU* genes that are related to biofilm formation, stress-response, and virulence in two strains of *L. monocytogenes*, i.e., serotypes 1/2a and 4b, growning at 7 °C and 37 °C.

Materials and methods

Bacterial strains

L. monocytogenes strains used here were isolated from cheese at the National Agricultural Laboratory of Rio Grande do Sul (LANAGRO/RS) from the Ministry of Agriculture, Livestock and Supply (MAPA/Brazil), and serotyped at the Instituto Oswaldo Cruz (Rio de Janeiro, Brazil) as serotypes 1/2a and 4b (Mello et al. 2008). Bacteria were activated by cultivation on brain heart infusion (BHI) (Himedia, Mumbai, India) broth supplemented with 0.6 % yeast extract (Himedia), for 18–24 h at 37 °C (Imperial III Incubator, Lab-line), under agitation (30 rpm) in a benchtop shaking incubator (SI-600, Lab Companion). Cultures were then inoculated onto plates containing tryptone soya agar (TSA) (Himedia) medium supplemented with 0.6 % yeast extract and incubated for 48 h at 37 °C. Culture purity was confirmed by Gram staining and verification of colony morphology on solid selective medium for *Listeria* (*Listeria* enrichment broth, LEB) (Acumedia, Lansing, MI) with 2 % agar (Sigma, St. Louis, MO).

Total RNA isolation

Total RNA of *L. monocytogenes* strains was isolated by the cetyltrimethylammonium bromide method (CTAB method), modified for bacterial cells (Salter and Conlon 2007). Bacterial strains were grown in tryptone soya broth (TSB)

(Himedia) medium at two different temperatures: 7 °C (test condition—cold temperature) and 37 °C (control condition—optimum growth temperature), and when the inoculum reached values of optical density (OD), measured in a UV/Visible Spectrophotometer (Ultrospec 3100 Pro, Amersham Biosciences, Little Chalfont, UK), of between 0.3 and 0.4 at 600 nm (exponential growth phase of the bacterium, corresponding to bacterial counts equal to 10^7 CFU/mL), 1.5 mL was collected and centrifuged at 13,000 rpm for 3 min (Centrifuge 5415, Eppendorf, Germany), repeating this procedure three times. After washing the cells with 300 μL 1X TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, reagents from Sigma-Aldrich, St. Louis, MO) and centrifugation at 13,000 rpm for 3 min, the pellet formed was suspended in 100 μL 1X TE buffer, and 3 μL lysozyme (10 mg/mL, Sigma-Aldrich) were added, with incubation at 37 °C for 20 min in a water bath (Dubnoff NT232, Nova Técnica, Bairro Santa Rosa Ipês, Brazil). This material was centrifuged again at 13,000 rpm for 3 min, and 300 μL EB-CTAB (100 mM Tris-HCl pH 9.0, 2.0 % CTAB, 25 mM EDTA, 2.0 M NaCl, 2.0 % polyvinylpyrrolidone (PVP)-40, 0.5 g/L spermidine, all reagents from Sigma-Aldrich) were added, with incubation at 60 °C for 20 min. After this step, 400 μL of a chloroform-isoamyl alcohol (24:1) (Sigma-Aldrich) solution was mixed with the material, which was then centrifuged at 12,000 rpm for 5 min. From the aqueous phase, 300 μL was removed and transferred to a new microtube, and this solution, with 300 μL isopropanol (Sigma-Aldrich), was incubated at −20 °C for at least 30 min, in a conventional freezer. After centrifugation at 12,000 rpm and 4 °C, for 15 min (Centrifuge 5415R, Eppendorf), the supernatant was discarded and 300 μL 70 % ethanol (Sigma-Aldrich) was added with further centrifugation at 4 °C and 12,000 rpm for 3 min. Ethanol was discarded, and the RNA was air-dried for at least 30 min. At the end, the RNA was suspended in 50 μL 1X TE buffer and spectrophotometer readings were performed at 260 and 280 nm in quartz cuvettes to verify the quality of the material obtained. RNA isolation was performed in triplicate for each bacterial strain, at each of the two different temperatures.

cDNA synthesis

An initial treatment with the enzyme DNase was carried out to eliminate the presence of DNA molecules extracted along with the RNA. For this, the amount of total RNA from each sample was calculated and standardized using the absorbance value at 260 nm and RNAs. In 10 μL of a solution containing DNase I enzyme (Fermentas, Fisher Scientific, Pittsburgh PA), 10X reaction buffer [100 mM Tris-HCl (pH 7.5 at 25 °C), 25 mM $MgCl_2$, 1 mM $CaCl_2$ (Fermentas)] and water, was added 1 μg RNA. After incubation at 37 °C for 30 min, 1 μL EDTA (Fermentas) was also added, with further heating at 65 °C for 10 min. Initiating the synthesis of complementary DNA, the second step consisted of adding 1 μL oligodT (20) 10 mM (Invitrogen, Carlsbad, CA), with incubation at 70 °C for 10 min, and the last step was characterized by the addition of a 25 μL reaction mix (5 μL 5X reaction buffer, 1.25 μL of each dATP/dCTP/dGTP/dTTP 10 mM, 25 U RNasin®, 200 U M-MLV reverse transcriptase enzyme, water to final volume, all reagents from Promega, Madison, WI) and heating at 40 °C for 60 min. Conventional PCR experiments (MyCycler™ Thermal Cycler, Bio-Rad, Hercules, CA) were performed with all samples, including samples treated only with the enzyme DNase, to verify whether cDNA synthesis was realized correctly.

Quantitative real time PCR

Relative gene expression was quantified by quantitative real time PCR (qPCR). Primers for genes analyzed in this study (*agrA*, *degU*, *luxS*, *prfA*, *hly*, *actA*, *flaA*, *sigB*, *ltrC*, *sufU* and *sufS*; see Supplemental Table 2 for functions of the coded proteins) were designed using the GenScript tool (available in http://www.genscript.com/) using *L. monocytogenes* genome sequence (Table 1), and *rpoB*, *16SrRNA* (Van der Veen and Abee 2010) and *gap* genes were tested as candidate constitutive genes for data normalization (Supplemental Table 1). For qPCR experiments, a mix containing 0.1 mM of each primer (sense and anti-sense, Invitrogen), 25 mM dNTPs (Promega), 1X reaction buffer (Invitrogen), 3 mM $MgCl_2$ (Invitrogen), 1X SYBR Green (Bio-Rad), 0.25 U of Platinum *Taq* DNA polymerase enzyme (Invitrogen) and water to complete the final volume of 10 μL, was prepared. A dilution curve with cDNA of the samples (dilutions 1:25, 1:50, 1:75, 1:100) was performed, to verify which concentration showed better efficiency in the qPCR experiments, and 10 μL of each diluted cDNA was added to 10 μL reaction mix, giving a total final volume of 20 μL. For amplification of complementary DNA, ABI-7500 (Applied Biosystems) and 96-wells polystyrene microplates (PCR® Microplate PCR-96-MB-C, Axygen Scientific, Union City, CA) were utilized, and all experiments were performed in biological triplicates and experimental quadruplicates.

Relative gene expression analysis

Housekeeping genes *rpoB*, *gap* and *16SrRNA* were tested as candidates for qPCR data normalization using the algorithm NormFinder (Andersen et al. 2004) (available at http://www.mdl.dk/publicationsnormfinder.htm)—which identifies the best normalizing gene among a set of candidates according to their expression stabilities. Relative expression of the genes studied was analyzed and calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), and statistical analyses were

Table 1 Sequences of primers used in quantitative real time PCR (qPCR) transcriptional analysis, with sizes of amplified fragments and annealing temperatures

Gene	Nucleotide sequence	Amplicon size (bp)	Annealing temperature (°C)
agrA	5′ CGGGTACTTGCCTGTATGAA 3′	149	58.65
	5′ TGAATAGTTGGCGCTGTCTC 3′		59.03
degU	5′ GGCGCGTATATTCATCCAC 3′	150	58.96
	5′ TACCTCGCACTCTCTATGCG 3′		59.20
luxS	5′ CATTTGATGGCAGAACTTGC 3′	127	59.28
	5′ TGATTTCGAGTGCATCATCA 3′		58.73
prfA	5′ GGAAGCTTGGCTCTATTTGC 3′	145	59.07
	5′ ACAGCTGAGCTATGTGCGAT 3′		58.65
hly	5′ AGCTCATTTCACATCGTCCA 3′	124	59.24
	5′ TGGTAAGTTCCGGTCATCAA 3′		58.97
actA	5′ AGAAATCATCCGGGAAACAG 3′	147	58.98
	5′ CCTCTCCCGTTCAACTCTTC 3′		58.87
flaA	5′ GTAAGCATCCAAGCGTCTGA 3′	148	59.03
	5′ AAGAATCAGCATCAGCAACG 3′		59.03
sigB	5′ TGGTGTCACGGAAGAAGAAG 3′	135	58.85
	5′ TCCGTACCACCAACAACATC 3′		59.27
ltrC	5′ TACGGCGTCGATGAGATACT 3′	144	58.35
	5′ GAATGTGTGAACGGCGATAC 3′		59.01
sufS	5′ GAATTTGGCGGAGAAATGAT 3′	137	58.98
	5′ TCTGCCAAGTAATCAATCGC 3′		58.87
sufU	5′ TTCAGAAATGGTGCAAGGTC 3′	135	58.70
	5′ ATCGCTCTCTCCATTGCTTT 3′		59.03

performed by one-way analysis of variance (ANOVA), to verify whether there was statistically significant difference, at a significance level of 5 %, between ΔCt (difference between Ct value of the studied gene and Ct value of the constitutive gene) values of growth at 7 °C and 37 °C, for the two strains and all genes studied. For statistically significant difference ($P<0.05$) during growth at 7 °C, compared to growth at 37 °C, the gene was considered to be more transcribed ($2^{-\Delta\Delta Ct}>1$) or less transcribed ($2^{-\Delta\Delta Ct}<1$).

Results and discussion

The results of relative gene expression ($2^{-\Delta\Delta Ct}$) of two serotypes of *L. monocytogenes,* 1/2a and 4b, grown at 7 °C and 37 °C, are shown in Fig. 1.

Transcriptional analysis of virulence and stress-response genes

For both serotypes, growth at a temperature of 7 °C significantly increased ($P<0.05$) the transcription level of the *sigB* and *prfA* genes ($2^{-\Delta\Delta Ct}_{prfA/55}=100.02\pm29.70$; $2^{-\Delta\Delta Ct}_{prfA/47}=6.58\pm0.69$; $2^{-\Delta\Delta Ct}_{sigB/55}=3.54\pm0.30$; $2^{-\Delta\Delta Ct}_{sigB/47}=10.24\pm1.53$). Cold temperatures represent additional stresses for *L. monocytogenes* in food processing environments and induce the activity of SigmaB factor (Becker et al. 2000), as demonstrated in the present study. According to Ollinger et al. (2009), *prfA* and other virulence genes controlled by this transcription factor, such as *hly*, *mpl*, *plcA*, *plcB* and *actA* (Supplemental Fig. 1), are transcribed more highly at 37 °C, the optimum growth temperature for *L. monocytogenes*. However, *prfA* can also be transcribed at low temperatures, and the transcription factor PrfA can switch between an active and inactive form, depending on the growth temperature (Renzoni et al. 1997). Complementary, *sigmaB* and *prfA* are transcribed in response to environmental conditions, and their activity can be influenced by different pH, temperature and growth phase conditions of the bacteria (Larsen et al. 2010a). At the same time, it is important to note that the adaptation to environments outside the host tends to increase the virulence of *L. monocytogenes* and other pathogens, due to various stress conditions that the organism may have to face in such situations (Neuhaus et al. 2013; Burall et al. 2012).

The hemolysin gene *hly*, considered essential for virulence of *L. monocytogenes*, was transcribed equally well at 7 °C and 37 °C in both serotypes tested ($2^{-\Delta\Delta Ct}_{hly/55}=1.27\pm0.26$; $2^{-\Delta\Delta Ct}_{hly/47}=0.22\pm0.13$). These results indicate that *hly* is important to the maintenance of *L. monocytogenes* in both cold and optimum growth temperatures. After *L. monocytogenes* internalization into the host cells by proteins called internalins (encoded by *inlA* and *inlB* genes), two other proteins, listeriolysin O (LLO, encoded by the *hly* gene) and phosphatidyl-inositol-phospholipase C (PI-PLC, encoded by the *plcA* gene), disrupt the phagosomal membrane, allowing the bacteria to escape to the cytosol. Ben Slama et al. (2013) analyzed the transcription level of virulence genes *hlyA*, *iap*, *fri* and *flaA* by semi-quantitative reverse transcriptase PCR in artisanal cheese slices inoculated with *L. monocytogenes* strains, incubated for 6 months at −20 °C. Those authors demonstrated that all genes were transcribed after the incubation time, with transcription levels that were affected by freezing while the *hlyA* transcription rate was slightly lower than other genes measured. Larsen et al. (2010b) investigated how different growth conditions could influence the ability of *L. monocytogenes* to invade the epithelial cell lines Caco-2 and INT-407, and their results indicated that *L. monocytogenes* could invade Caco-2 cells even after 4 weeks of storage at chilled temperatures.

The transcription levels of the *actA* gene did not vary significantly between the temperatures of 7 °C and 37 °C for serotype 4b ($2^{-\Delta\Delta Ct}_{actA/47}=1.36\pm0.85$); however, for serotype

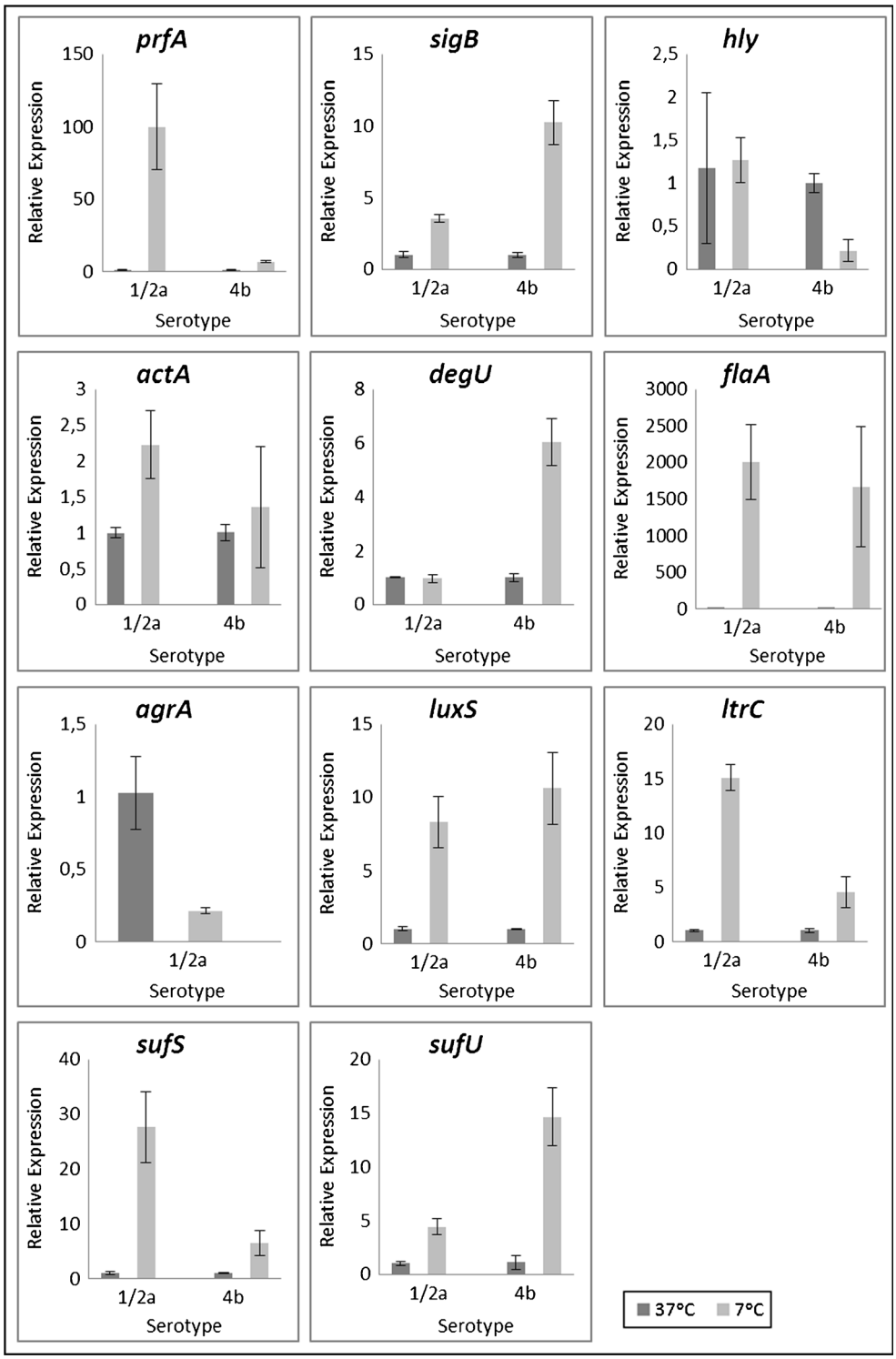

Fig. 1 Relative expression of *agrA*, *degU*, *luxS*, *prfA*, *hly*, *actA*, *flaA*, *sigB*, *ltrC*, *sufS* and *sufU* of *Listeria monocytogenes* serotypes 1/2a and 4b grown at 7C and 37C. Data were normalized to the expression of the constitutive gene *gap*. *Errors bars* denote standard deviations

1/2a, this gene was transcribed significantly more at 7 °C ($2^{-\Delta\Delta Ct}_{actA/55}$=2.23±0.47), agreeing with the result obtained for *prfA*, which controls the transcription of several virulence genes, including *actA*. Intracellular mobility and spread from one cell to another requires the presence of a surface protein called ActA (encoded by *actA* gene), which is co-transcribed with phosphatidylcholine-phospholipase C protein (PC-PLC, encoded by the *plcB* gene), and is responsible for the formation of the actin tails that propel the bacterium towards the cytoplasmic membrane (Liu 2006).

There are several two-component systems (TCS) found in *L. monocytogenes*, including VirR, CheY and DegU, an

important regulator that has been shown to be necessary for thermo-tolerance, virulence, biofilm formation, and motility (Gueriri et al. 2008). For the serotype 1/2a, the *degU* gene was transcribed equally at both temperatures ($2^{-\Delta\Delta Ct}_{degU/55}$=0.97 ±0.14), whereas for serotype 4b, this gene was transcribed more at 7 °C ($2^{-\Delta\Delta Ct}_{degU/47}$=6.04±0.87). This result, mainly for serotype 4b, is in accordance with what was observed for *sigB* and *prfA*, which are genes involved in the regulation of stress response and virulence, as they were all transcribed more at 7 °C, supporting the involvement of DegU in fundamental mechanisms of pathogenicity, resistance, and persistence of *L. monocytogenes* in the environment. DegU was also identified as a positive activator of flagellum biosynthesis, which is not expressed at 37 °C (Williams et al. 2005). At low temperatures, this protein activates transcription of *gmaR* gene, which encodes GmaR protein—a transcriptional activator of the *flaA* gene responsible for flagellum synthesis and motility (Murray et al. 2009).

In the present study, the *flaA* gene presented a higher transcription level at 7 °C for both serotypes ($2^{-\Delta\Delta Ct}_{flaA/55}$= 1,999.72±512.13; $2^{-\Delta\Delta Ct}_{flaA/47}$=1,668.11±823.33). According to Kamp and Higgins (2011), upon entering the host, *L. monocytogenes* represses transcription of flagella genes in response to mammalian physiological temperature—negative regulation of flagella motility being necessary for virulence.

Cold temperatures are used widely in food industries. As a psychrotrophic microorganism, the presence and multiplication of *L. monocytogenes* becomes a major concern to such establishments, since growth in freezing or cooling temperatures constitutes the main infection threat for foodborne *Listeria* (Shin et al. 2010). The gene *ltrC*—a stress-response gene involved in *L. monocytogenes* growth and adaptation at cold temperatures (Chan et al. 2007)—was transcribed significantly more at 7 °C for both serotypes studied ($2^{-\Delta\Delta Ct}_{ltrC/55}$=15.07±1.20; $2^{-\Delta\Delta Ct}_{ltrC/47}$=4.54± 1.41), as also observed for the *sigB* gene. According to Chan et al. (2007), some genes regulated by SigmaB factor were shown to be involved in *L. monocytogenes* adaptation at low temperatures, including the *ltrC* gene, whose transcription was demonstrated to be SigmaB-dependent at 4 °C. In the same work, the *ltrC* transcription level was higher in *L. monocytogenes* strain serotype 1/2a than in a mutant strain *ΔsigB*.

In respect to *sufS* (cysteine desulfurase) and *sufU* genes, which are involved in the biosynthesis of iron-sulfur clusters ([Fe-S]), by supplying sulfur, and scaffold of [Fe-S] clusters, respectively, it can be noted that they were both transcribed more at 7 °C in the two strains studied ($2^{-\Delta\Delta Ct}_{sufS/55}$=27.64± 6.49; $2^{-\Delta\Delta Ct}_{sufS/47}$=6.44±2.31; $2^{-\Delta\Delta Ct}_{sufU/55}$=4.42±0.73; $2^{-\Delta\Delta Ct}_{sufU/47}$=14.67±2.68). These genes have been associated with a number of biological processes, including pathogenicity and microbial virulence (Riboldi et al. 2013).

Transcriptional analysis of genes related to biofilm formation

The development and maturation of a biofilm requires complex molecular mechanisms, involving communication between the cells. In the case of *L. monocytogenes*, the *agr* system also plays an important role during the early stages of biofilm formation. Interestingly, the presence of the *agrA* gene was not detected in serotype 4b, whereas for serotype 1/2a, the gene was transcribed significantly less at 7 °C ($2^{-\Delta\Delta Ct}_{agrA/55}$=0.21±0.02). Some studies show that deletion of the *agrA* gene affects *L. monocytogenes* adhesion to abiotic surfaces, and also indicate the involvement of the *agr* system at the beginning of biofilm formation, but not at later stages of development (Rieu et al. 2008). Thus, the absence of *agrA* transcription can reduce *L. monocytogenes* initial adhesion to surfaces, but not enough to directly affect subsequent biofilm formation. Previous works (Borucki et al. 2003; Harvey et al. 2007) detected significant differences between biofilm formation by *L. monocytogenes* strains of different serotypes, demonstrating greater activity by strains of the lineage II (serotypes 1/2a and 1/2c) in comparison to lineage I strains (serotypes 1/2b and 4b). This is in accordance to Folsom et al. (2006), who demonstrated that strains of serotypes 1/2a and 4b differ in their biofilm development. It has also been reported previously that deletion of the *agrA* gene decreases the transcription of genes controlled by SigmaB and PrfA factors, reducing the ability of *L. monocytogenes* to generate an infectious processes, and increasing the growth rates of planktonic cells at 37 °C leading, to biofilm formation at this temperature (Garmyn et al. 2012). This same study showed increased transcription of 39 regulators, including *agrA*, with increasing temperatures.

Another gene analyzed was *luxS*, since few works have investigated it in *L. monocytogenes*, and there is some inconsistency in the literature regarding the influence of this gene in biofilm development (Daines et al. 2005; McNab et al. 2003). It has already been established that expression of *luxS* can influence biofilm formation negatively (Sela et al. 2006). For both serotypes studied here, *luxS* gene showed a higher transcription levels at 7 °C in comparison to the results obtained at 37 °C ($2^{-\Delta\Delta Ct}_{luxS/55}$=8.34±1.75; $2^{-\Delta\Delta Ct}_{luxS/47}$=10.62±2.47). These findings were, exactly opposite of those observed for the *agrA* gene, which positively influences biofilm formation.

In conclusion, most of the genes studied here showed increased expression at 7 °C when compared to the transcriptional levels observed at 37 °C. These findings were identified in both, *L. monocytogenes* serotypes studied. Collectively, the results reported here demonstrate the microorganism's ability to activate several genetics mechanisms under stress conditions, in order to retain viability and pathogenicity upon exposure to challenging situations. Furthermore, these results reinforce the importance of controlling the presence of *L. monocytogenes* in establishments where a cold chain is

used widely, such as the dairy and ready-to-eat food industries. It is also interesting to note that the two studied serotypes, which are responsible for many cases of human listeriosis, presented different molecular mechanisms at temperatures of 7 °C and 37 °C, and this indicates the importance of continuing such experiments with other relevant genes.

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