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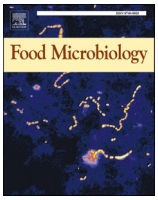

Influence of temperature on regulation of key virulence and stress response genes in Listeria monocytogenes biofilms

Sofia V. Poimenidou [a], Nelly Caccia [b], Spiros Paramithiotis [a,d], Michel Hébraud [b], George-John Nychas [c], Panagiotis N. Skandamis [a,*]

[a] Agricultural University of Athens, Department of Food Science and Human Nutrition, Laboratory of Food Quality Control and Hygiene. Iera Odos 75, 11855, Athens, Greece
[b] University Clermont Auvergne (UCA), Institut National de Recherche pour L'Agriculture, L'alimentation et L'environnement (INRAE), UMR Microbiologie, Environnement Digestif et Santé (MEDiS), Site de Theix, F-63122 Saint-Genès Champanelle, France
[c] Agricultural University of Athens, Department of Food Science and Human Nutrition, Laboratory of Food Microbiology and Biotechnology. Iera Odos 75, 11855, Athens, Greece
[d] Agricultural University of Athens, Department of Food Science and Human Nutrition, Laboratory of Food Process Engineering. Iera Odos 75, 11855, Athens, Greece

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ABSTRACT

Temperature is a major determinant of *Listeria (L.) monocytogenes* adherence and biofilm formation on abiotic surfaces. However, its role on gene regulation of *L. monocytogenes* mature biofilms has not been investigated. In the present study, we aimed to evaluate the impact of temperature up- and down-shift on *L. monocytogenes* biofilms gene transcription. *L. monocytogenes* strain EGD-e biofilms were first developed on stainless steel surfaces in Brain Heart Infusion broth at 20 °C for 48 h. Then, nutrient broth was renewed, and mature biofilms were exposed to 10 °C, 20 °C or 37 °C for 24 h. Biofilm cells were harvested and RNA levels of *plcA, prfA, hly, mpl, plcB, sigB, bapL, fbpA, fbpB, lmo2178, lmo0880, lmo0160, lmo1115, lmo 2089, lmo2576, lmo0159* and *lmo0627* were evaluated by quantitative RT-PCR. The results revealed an over-expression of all genes tested in biofilm cells compared to planktonic cells. When biofilms were further allowed to proliferate at 20 °C for 24 h, the transcription levels of key virulence, stress response and putative binding proteins genes *plcA, sigB, fbpA, fbpB, lmo1115, lmo0880* and *lmo2089* decreased. A temperature-dependent transcription for *sigB, plcA, hly,* and *lmo2089* genes was observed after biofilm proliferation at 10 °C or 37 °C. Our findings suggest that temperature differentially affects gene regulation of *L. monocytogenes* mature biofilms, thus modulating attributes such as virulence, stress response and pathogenesis.

1. Introduction

Listeria (L.) monocytogenes is a Gram-positive food-borne pathogen widely distributed in the environment and able to cause the severe food-borne disease, listeriosis. Despite its low incidence rate (0.46 cases per 100,000 population), listeriosis is a threatening infection for at-risk population groups; in 2019 the fatality rate of listeriosis in the EU was 17.6% making it one of the most serious food-borne diseases in EU (EFSA, 2021).

One of the most widely known mechanisms of this pathogen to spread in the food processing facilities and contaminate food products, is its ability to colonize abiotic surfaces forming biofilms and to persist cleaning and disinfection procedures (Halberg Larsen et al., 2014). Biofilms are multicellular communities that enclose aggregated cells into an extracellular polymeric substances (EPS) matrix (Flemming and Wingender, 2010; Guilbaud et al., 2015; Harmsen et al., 2010) and provide cells with unique defending properties against a variety of adverse conditions, including a wide range of antimicrobial agents (Chavant et al., 2004; Flemming et al., 2016; Stewart and William Costerton, 2001). Various environmental conditions may favor the development of a biofilm on abiotic surfaces, including temperature, growth medium, inter-strain variability and surface type (Giaouris and Nychas, 2006; Kadam et al., 2013; Nilsson et al., 2011; Poimenidou et al., 2009, 2016; Skandamis and Nychas, 2012). Once biofilms are

* Corresponding author. Lab. Food Quality Control & Hygiene, Dep. Food Science & Human Nutrition, Agricultural University of Athens, Iera Odos 75, 11855, Athens, Greece.
E-mail address: pskan@aua.gr (P.N. Skandamis).

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established in a food processing plant, they become a potential source of food contamination and spread of disease (Bridier et al., 2015; Halberg Larsen et al., 2014; Nakamura et al., 2013).

Temperature is a major determinant of *L. monocytogenes* biofilm development, and affects initial cell attachment, structure and thickness of a biofilm (Bonsaglia et al., 2014; Chavant et al., 2002; Poimenidou et al., 2016). A sudden temperature downshift was shown to induce a cold shock on *L. monocytogenes* cells, resulting in increased adhesion of the pathogen on abiotic surfaces (Lee et al., 2017). In addition, temperature differentially modulated virulence genes transcription in different *L. monocytogenes* strains (Duodu et al., 2010). To overcome the stress of low temperature conditions in the food industry, *L. monocytogenes* activates adaptive strategies that may include control of membrane fluidity, synthesis of cold shock proteins (Csp) and cold acclimatization proteins (Cap), uptake of carnitine and betaine as cryoprotectants, and the activation of the alternative sigma factor B (σ^B) (Becker et al., 2000; Santos et al., 2019; Wiktorczyk-Kapischke et al., 2021). The σ^B regulon controls the general stress response in *L. monocytogenes*, while the positive regulatory factor A (PrfA) controls virulence genes expression. PrfA-dependent genes include: *hly*, which encodes the pore-forming toxin listeriolysin O (LLO); *plcA* and *plcB* which encode phospholipases PlcA and PlcB; *mpl*, which encodes a metal-dependent protease required for PlcB maturation; and *actA*, which encodes the actin-recruiting protein required for bacterial movement and cell-to-cell spread (Camejo et al., 2011). The two regulons σ^B and PrfA interact and provide optimum fitness and survival outside and inside the host for *L. monocytogenes* (Gaballa et al., 2019; Nadon et al., 2002).

In a food processing environment, biofilm cells that have escaped cleaning and sanitation procedures undergo temperature changes that may significantly affect important functions such as stress response, adherence and virulence. Thus, the objective of the present study was to investigate the impact of temperature fluctuations on gene transcription of *L. monocytogenes* mature biofilms. The transcription level of sixteen genes representing different groups of *L. monocytogenes* physiological responses were evaluated by RT-qPCR before and after exposure of a mature biofilm to three different temperature conditions (*i.e.,* 10 °C, 20 °C and 37 °C). The different groups included: *(i)* genes involved in stress response, *ie*. *sigB*, which encodes the alternative factor σ^B; *bapL*, which is involved in cell adhesion; the PrfA-dependent genes (*plcA, prfA, hly, mpl, plcB)*, and *(ii)* genes coding for fibronectin binding proteins (*fbpA* and *fbpB*) and putative binding proteins (*lmo1115, lmo2178, lmo0880, lmo0160, lmo 2089, lmo2576, lmo0159* and *lmo0627)*.

2. Materials and methods

2.1. Bacterial strain and growth

L. monocytogenes strain EGDe was stored at −80 °C in Tryptic Soy Broth (TSA; LAB M, Lancashire, UK) supplemented with yeast extract (YE; LAB M, Lancashire, UK) and 20% glycerol (Applichem, Darmstadt, Germany). *L. monocytogenes* EGDe strain is a reference strain, which belongs to serotype 1/2a. Prior to the experiment, an aliquot was transferred to Brain Heart Infusion agar (BHI; LAB M) and incubated at 37 °C for 48 h to allow growth. A single colony was inoculated into 10 mL BHI broth (LAB M) and incubated under shaking (250 r.p.m.) overnight at 20 °C. Cells were harvested by centrifugation (Megafuge 1.0 R, Heraeus Instruments, Hanau, Germany) for 10 min at 20 °C at 2434 *g*, mixed with 200 μL RNAlater® solution (Ambion, Waltham, MA, USA) and stored at −80 °C until used for RNA extraction. For biofilm development on stainless steel surfaces, harvested cells were washed twice with quarter-strength Ringer solution (Ringer; LAB M) and properly diluted in BHI broth for further experiments.

2.2. Surfaces preparation

Round stainless steel surfaces (diameter 7.3 cm) were soaked for 10 min with 10% of a commercial surfactant, rinsed five times for 5 min each with hot tap water and five times for 5 min each with deionized water, and autoclaved for 15 min at 121 °C (Chavant et al., 2002).

2.3. Experimental growth conditions

BHI broth suspension (10 mL) containing approximately 10^6 *L. monocytogenes* cells/mL were poured on stainless steel surfaces placed in a Petri dish (diameter 8.7 cm) and incubated at 20 °C for 48 h. The mature biofilms thus formed (Chavant et al., 2002) were carefully rinsed twice with Ringer solution and 10 mL of fresh BHI broth were poured on each surface. Petri dish containing the developed biofilms with fresh nutrient medium was stored either at 10 °C, 20 °C or 37 °C for 24 h. At the end of the incubation time, ice-cold phenol-ethanol (5%: 95%; PanReac AppliChem) solution was added to cell suspensions for 5 min (Fink et al., 2012; Kyle et al., 2010). Biofilms were washed twice with 10 mL Ringer solution and finally 10 mL were poured to cover biofilm cells. Bacterial cells were harvested using a cell scrapper and the cell suspension was centrifuged (2434 *g*, sample temperature), mixed with 200 μL RNAlater® solution and stored immediately at −80 °C for RNA extraction.

2.4. RNA extraction and RT-qPCR analysis

RNA extraction was performed using the NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. The extracted RNA samples were treated with Ambion® TURBO DNA-free kit as described by the manufacturer. RNA concentration and quality were measured using a NanoPhotometer (Implen GmbH, Munich, Germany). SuperScript™ First-Strand Synthesis System for RT-PCR was used to synthesize first-strand cDNA from purified RNA using random hexamers. RT-qPCR was performed using the KAPA SYBR FAST qPCR kit Master Mix (2X) for ABI Prism (KapaBiosystems, Boston, MA, USA). Primer concentration was 1 μM. All primers are listed in Table 1. For the primers designed for this study we used the GeneLink™ Oligo Explorer 1.2 software. For each experimental condition transcription levels for the genes *plcA, prfA, hly, mpl, plcB, sigB, bapL, lmo1115, fbpA, fbpB, lmo2178, lmo0880, lmo0160, lmo 2089, lmo2576, lmo0159* and *lmo0627* were evaluated.

2.5. Statistical analysis

Three housekeeping genes were tested as internal controls: *rpoB, tpi,* and the intergenic spacer (IGS). Based on the transcription stability of the reference genes, assessed with NormFinder v0.953 (Andersen et al., 2004), IGS was selected to normalize the expression of the target genes. Control condition used for the normalization of each experimental condition is indicated in the corresponding figure. Data processing and C_t conversion to relative expression and $\log_2$-(fold change) values was performed according to Hadjilouka et al. (2016). Relative changes lower than 2 $\log_2$ (fold change) compared to corresponding control samples were not considered as significant transcription regulation. The statistical tool "Two Sample Assuming Unequal Variances *t*-Test" of Microsoft® Excel for Mac package was used to estimate significant differences ($P < 0.05$) in gene regulation levels.

3. Results and discussion

The aim of the present study was to investigate whether the temperature fluctuations that occur in a food processing facility affect gene transcription of a developed *L. monocytogenes* biofilm. We selected groups of genes that have an important contribution in pathogen stress response, virulence regulation, surface adherence and genes coding for

Table 1
Primer sequences used in the present study.

Gene	Primer sequence (5' - 3')	Amplicon size	*E*-value	Reference
IGS	F, GGCCTATAGCTCAGCTGGTTA R, GCTGAGCTAAGGCCCCGTAAA	135	2.00	Rantsiou et al. (2008)
rpoB	F, CGTCGTCTTCGTTCTGTTGG R, GTTCACGAACCACACGTTCC	82	2.01	This study
tpi	F, AACACGGCATGACACCAATC R, CACGGATTTGACCACGTACC	93	2.03	Veen et al. (2007)
plcA	F, TCCCAGAACTGACACGAGC R, GCATGCCGAATTTGCGTGAG	122	1.87	This study
prfA	F, CTATTTGCGGTCAACTTTTAATCCT R, CCTAACTCCTGCATTGTTAAATTATCC	100	2.09	Oliver et al. (2010)
hly	F, CTAACCTATCCAGGTGCTCTC R, TGTCTTGATTAGTCATACCTGGCA	127	1.98	This study
mpl	F, CGGTTATCCAGTATTCGGCG R, TTCCTCTGTGAGTGGAAGCG	230	1.84	This study
plcB	F, GGTGACTGATTACCGAGAAGG R, GTAGCCTGGAGGGTATGA	133	1.91	This study
sigB	F, GCCGCTTACCAAGAAAATGG R, AATATTTTCGGGCGATGGAC	91	1.91	This study
bapL	F, TGTATTACTGGGAAGCACCG R, TGTTACTCCGGGAAGTTTGG	233	1.98	This study
fbpA	F, GCTACCGAACACTTTTGCCG R,CTGCCGTTAAGTTTCCAGCG	207	2.09	This study
fbpB	F, CGCATACCGTTCTGCCAATG R, CGGCTCTGCTTCTTTGGTTG	157	2.01	This study
lmo0159	F, GGATTTCACGCTGCCAAGTC R, CCAGTTCGCCCTTAACATCG	170	1.92	This study
lmo0160	F, CTTATCCAGTGGGTTGTGCG R, TCAAACTCGCCGTGGAATGC	128	1.97	This study
lmo0627	F, GACTAAAGGTGGCGAAGGTA R, TAGAAACGGGCGTAGTTGTG	149	1.78	This study
lmo0880	F, GCTGCAACAGATTACGGGAG R, TCCATGAGAGGAAATCCGCC	209	1.99	This study
lmo1115	F, GCTGGAGAAGAGGCTAAAGA R, TTAGGAGAGTGCCATTGTCC	212	1.92	This study
lmo2089	F, GTACAAACTACGGGTGCTCG R, CAACGGTCGCTAAGTTTCCG	190	1.98	This study
lmo2178	F, CGAGTGATGTTAGTGCTGGG R, TGATAATGTCCCTGCATCGC	230	1.93	This study
lmo2576	F, CCAGAAGGATACACATTACC R, CGCCATTTTCATCCGTTGTG	235	1.87	This study

[1]Thermocycling conditions: initial denaturation at 95 °C for 20 s, 40 x (95 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s). Melting curve analysis: 95 °C for 15 s, then 60 °C for 1 min and raise to 95 °C at 0.3 °C/s.

putative binding proteins with to date unknown specific function. The transcription of the genes under study of *L. monocytogenes* biofilms developed at 20 °C for 48 h on stainless steel surfaces were first compared to the respective of planktonic cells. Then, mature biofilms at 20 °C were exposed to either 20 °C, 10 °C or 37 °C for further 24 h.

3.1. Gene transcription in biofilm cells vs. planktonic cells

L. monocytogenes cells grown in the biofilm state displayed a significant gene upregulation compared to planktonic state (Fig. 1). The $\log_2$-fold changes varied between 4.1 and 8.4 for genes coding for the general stress response factor SigB, biofilm adhesion protein L (BapL), fibronectin binding proteins FbpA and FbpB and genes of the *prfA* virulence gene cluster (*i.e. plcA, prfA, hly, mpl, plcB*) (Fig. 1A). Similarly, an over-transcription was observed for genes coding for putative binding proteins (Fig. 1B). A 11.6- and 9.3- $\log_2$-folds upregulation for the genes *lmo0880* and *lmo1115,* respectively, probably suggests a critical role for these genes in *L. monocytogenes* adherence to stainless steel.

These results are indicative of a transcriptional adjustment for *L. monocytogenes* when it transits from the free-floating living to the biofilm state. Attributes such as stress response are governed by σ^B (Chaturongakul et al., 2008) and its activation in biofilm cells may trigger *L. monocytogenes* functions such as resistance to adverse environmental conditions, antibiotic resistance and pathogenicity (Guldimann et al., 2016). Therefore, the risk of inadequate removal of *L. monocytogenes* biofilms in a food processing facility might result in complex phenomena including persistence, adaptation and cross-tolerance to antimicrobial factors (Lundén et al., 2003).

Another key factor for *L. monocytogenes* mode of life, previously characterized as a global regulator (de las Heras et al., 2011), is the virulence regulon *prfA*, also known as *Listeria* pathogenicity island 1 (LiPI-1). All genes comprising LiPI-1 were upregulated in the biofilm cells compared to planktonic cells. Known for the induction of intracellular parasitism (Vázquez-Boland et al., 2001), PrfA seems to be also an essential factor for *L. monocytogenes* biofilm formation, which is in line with previous studies (Lemon et al., 2010; Zhou et al., 2011). There is a complex interplay between the two master regulators, σ^B and PrfA, that induces biofilm formation, survival of the pathogen outside the host, in digestive tract and in the host cells (Gaballa et al., 2019). Therefore, it is essential to include in *L. monocytogenes* biofilm studies the PrfA regulon.

BapL is a LPXTG protein involved in adhesion of *L. monocytogenes* to abiotic surfaces (Jaglic et al., 2014). The gene encoding BapL *(lmo0495)* is an homologue to a biofilm associated protein (Bap) in *Staphylococcus aureus* (Cucarella et al., 2001). However, *bapL* is not present in all *L. monocytogenes* strains and its role in adhesion abilities of the pathogen is not clear. For instance, some BapL-negative strains have been shown to adhere to abiotic surfaces significantly better than BapL-positive strains (Jordan et al., 2008). The increased transcript levels of the gene *bapL* in biofilm cells compared to planktonic cells observed in the present study demonstrate the involvement of *bapL* in biofilm forming procedure for *L. monocytogenes* EGD-e strain.

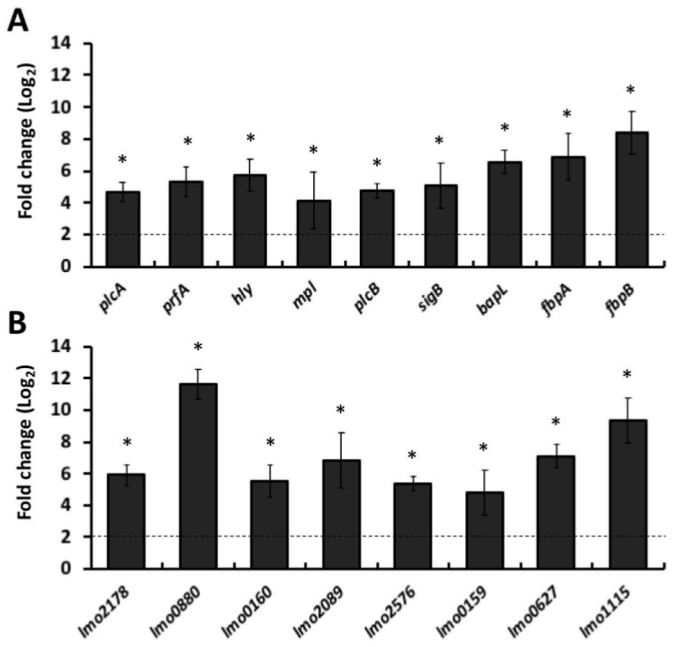

Fig. 1. Relative transcription of genes with known functions (A) and genes coding for putative binding proteins (B) in *L. monocytogenes* biofilms developed at 20 °C for 48 h. Transcript levels were normalized to IGS and compared to *L. monocytogenes* planktonic cells grown at the same conditions. Data represent mean ± stdev of two biological and two technical replicates. Asterisks (*) indicate significant upregulation of the target gene compared to 2-fold (log_2) change.

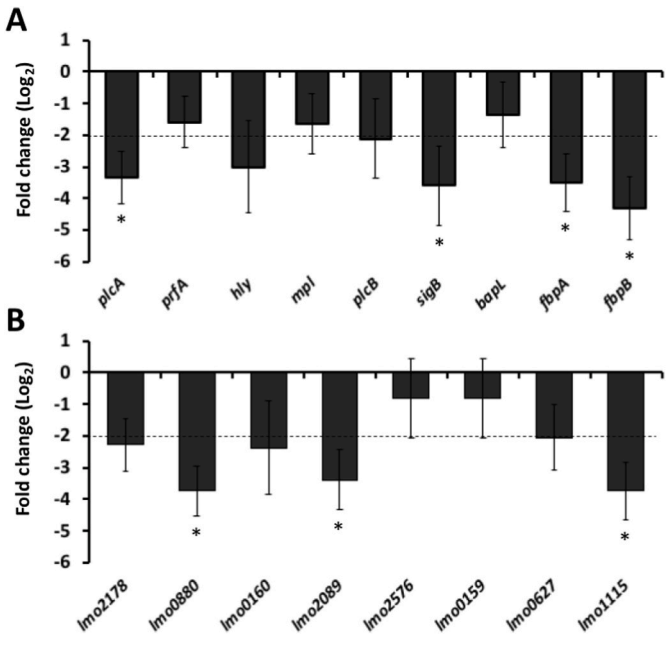

Fig. 2. Relative transcription of genes with known functions (A) and genes coding for putative binding proteins (B) in *L. monocytogenes* biofilms developed at 20 °C for 48 h and further exposed to 20 °C for 24 h. Transcript levels were normalized to IGS and compared to biofilms developed at 20 °C for 48 h. Data represent mean ± stdev of two biological and two technical replicates. Asterisks (*) indicate significant upregulation of the target gene compared to 2-fold (log_2) change.

We also investigated the transcription of genes encoding for fibronectin binding proteins (*ie., fbpA, fbpB*) and putative binding proteins. *FbpA* and *fbpB* presented a significant upregulation during *L. monocytogenes* biofilm formation, thus revealing a possible crucial role in biofilm development. Their induction within a biofilm might also stimulate the adherence of *L. monocytogenes* biofilm cells to fibronectin structures. Specifically for FbpA, it was shown that it affects the protein levels of two virulence factors, LLO and internalin B, and thus it is a multifunctional virulence factor of *L. monocytogenes* (Dramsi et al., 2004). Additionally, all genes coding for putative binding proteins also displayed an upregulation in biofilms compared to planktonic cells. Gene *lmo0880* codes for a cell wall LPXTG protein with unknown function and a collagen binding domain and presents a *sigB*-dependent regulation (Hain et al., 2008; Oliver et al., 2010) and a temperature-dependent transcription for *L. monocytogenes* cells grown in suspension (Veen et al., 2007). Its association with the biofilm mode of growth is also suggested in this study.

3.2. Impact of temperature changes on mature biofilms gene transcription

Biofilms developed at 20 °C for 48 h were subsequently incubated at 20 °C, 10 °C or 37 °C in renewed nutrient medium for 24 h. At 20 °C, *L. monocytogenes* displayed a general gene down-regulation (Fig. 2). Significant decrease in transcript levels was observed for genes *plcA, sigB, fbpA* and *fbpB* encoding virulence, stress response and fibronectin binding functions (Fig. 2A), and for genes *lmo0880*, *lmo2089* and *lmo1115* encoding putative binding proteins (Fig. 2B). At 10 °C, the pathogen exhibited a significant upregulation of *sigB* and *lmo2089* genes (Fig. 3), while at 37 °C a significant upregulation was observed for *plcA*, *hly* and *lmo 2089* (Fig. 4).

The results regarding the *sigB* transcription indicate a *sigB*-dependent surface attachment for *L. monocytogenes*; once the pathogen was established on the stainless steel surface, it displayed a notable *sigB* down-regulation. It should be noted here that a strain-specific involvement

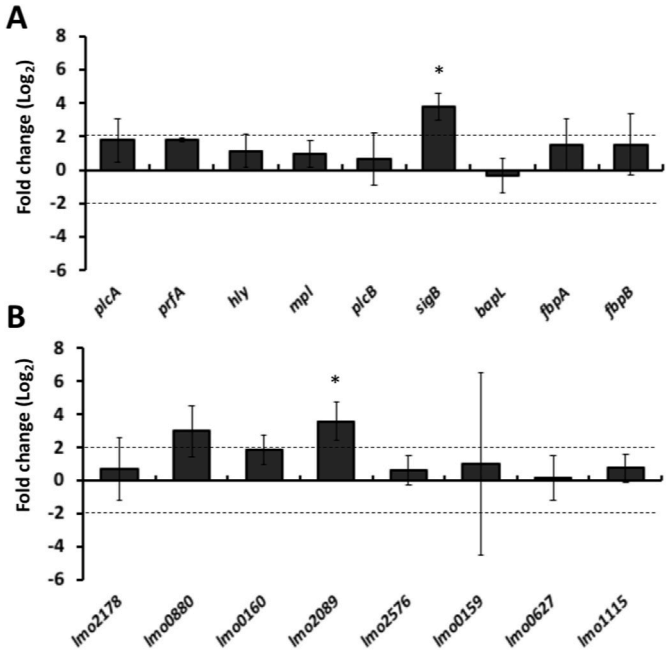

Fig. 3. Relative changes in transcription levels of genes with known functions (A) and genes coding for putative binding proteins (B) in *L. monocytogenes* cells biofilms developed at 20 °C for 48 h and further exposed to 10 °C for 24 h. Transcript levels were normalized to IGS and compared to biofilm exposure to 20 °C for 24 h. Data represent mean ± stdev of two biological and two technical replicates. Asterisks (*) indicate significant upregulation of the target gene compared to 2-fold (log_2) change.

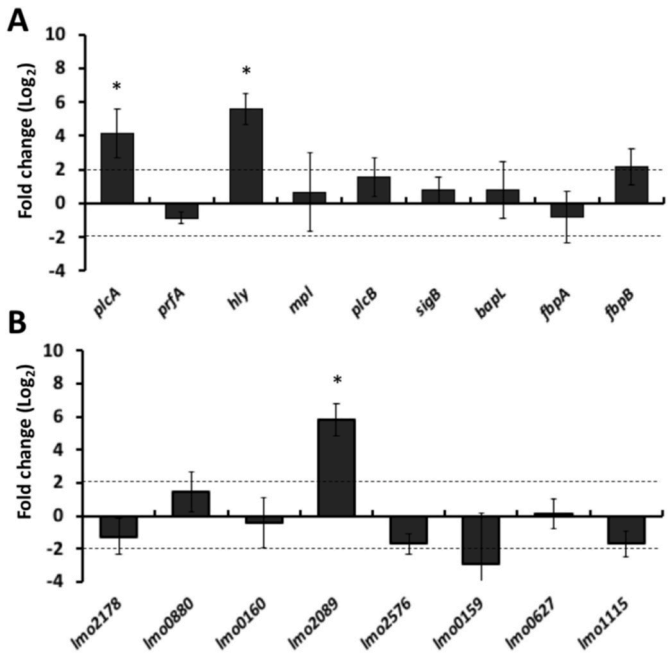

Fig. 4. Relative changes in transcription levels of genes with known functions (A) and genes coding for putative binding proteins (B) in *L. monocytogenes* biofilms developed at 20 °C for 48 h and further exposed to 37 °C for 24 h. Transcript levels were normalized to IGS and compared to biofilm exposure to 20 °C for 24 h. Data represent mean ± stdev of two biological and two technical replicates. Asterisks (*) indicate significant upregulation of the target gene compared to 2-fold (log_2) change.

of *sigB* in pathogen attachment is also possible, since in the study of Schwab et al. (2005) σ^B was not required for the initial surface attachment of *L. monocytog*enes 10403 S strain. In addition, the different *sigB* transcription at 10 °C and 37 °C revealed a temperature-dependent *sigB* transcription in *L. monocytogenes* biofilms. Those phenomena are relevant to food safety as the stress response and biofilm formation of *L. monocytogenes* may vary significantly along the food processing chain and may result in numerous practical implications for persistence and food contamination.

The downregulation of *plcA* at 20 °C and upregulation of *plcA* and *hly* at 37° were the only observed modifications for LiPI-1. These factors are relevant to *L. monocytogenes* virulence and are governed by the *prfA*, which is thermoregulated in *L. monocytogenes* planktonic cells (de las Heras et al., 2011). The findings here probably indicate that specifically *plcA* and *hly* have also a temperature-dependent role in biofilm proliferation. Price et al. (2018) also showed that *hly* affects *L. monocytogenes* cell aggregation and biofilm formation. Combined these results suggest an involvement of these genes in *L. monocytogenes* biofilms and lifecycle, which needs further investigation, also taking into consideration their pathogenicity relevance.

Interestingly, among genes encoding putative binding proteins, only *lmo2089* displayed a temperature-dependent transcription during biofilms proliferation with a significant up-regulation in mature biofilms exposed to 10 °C or 37 °C compared to 20 °C. What is so far known regarding these genes is that proteins encoded by *lmo0159, lmo0160, lmo0880, lmo2178* and *lmo2576* have a collagen binding domain (Bierne and Cossart, 2007). Collagen and fibronectin are structured and specialized proteins, respectively, contained in the extracellular matrix of host cells that are recognized by the bacterial adhesins, thus leading to bacterial adhesion on the surface of the host cells (Bierne and Cossart, 2007). Therefore, the induction of collagen or fibronectin binding proteins is expected to favor *L. monocytogenes* pathogenesis. Some of these genes were also shown to be absent from particular serotype or lineage strains. For instance, *lmo2576,* coding for a LPXTG protein with collagen binding domain, was shown to be absent in lineage I and IV and serotype 1/2b and 4b strains and present in serotype 1/2a strains (den Bakker et al., 2013; Zhang et al., 2003). *lmo2178* was found to be present in serotype 1/2a and absent in 1/2b and 4b strains (Zhang et al., 2003), while in the study of Doumith et al. (2004) it was found to be absent only in *L. ivanovii, L. seeligeri* and *L. grayi* strains. Therefore, the upregulation of these genes in biofilm cells might be related to serotype-specific prevalence in food premises and *L. monocytogenes* pathogenicity and needs to be further investigated.

4. Conclusions

It is well established that *L. monocytogenes* biofilm cells differ from planktonic cells and that certain environmental conditions may induce biofilm development. *L. monocytogenes* is an opportunistic pathogen, ubiquitous in nature, and conditions that allow biofilm development are very common in a food processing environment. The results presented here demonstrate that biofilm proliferation is a dynamic process as well. It was shown that temperature and nutrient fluctuations modulated molecular determinants of *L. monocytogenes* virulence and stress response within a developed biofilm. Genes encoding confirmed or putative fibronectin and collagen binding proteins, essential for pathogenesis, were also shown to be significantly affected in a temperature-dependent manner. Since aggregated cells tend to detach and disperse from a mature biofilm, the transcription profile of a *L. monocytogenes* biofilm may prove determinant for food contamination and subsequent pathogenesis. Further research in this area should investigate the impact of inadequate disinfection of abiotic surfaces on gene regulation of *L. monocytogenes* mature biofilms. An in depth understanding of the mechanisms used by the pathogen to survive antimicrobial treatments in food processing environments will contribute in developing more efficient disinfection procedures and improved food preservation protocols.

Acknowledgments

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