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Full Length Research Paper

RT-PCR assays for the evaluation of the expression of *Listeria monocytogenes* virulence genes after cold and freezing shock

Hanene MILADI[1]*, Kamel CHAIEB[1], Mejdi SNOUSSI[1], Emna AMMAR[2] and Amina BAKHROUF[1]

[1]Laboratoire d'Analyses, Traitement et Valorisation des Polluants de l'Environnement et des Produits, Faculté de Pharmacie, rue Avicenne 5000 Monastir, Tunisie.
[2]Unité de Recherche Gestion des Environnements Urbains et Côtiers – LARSEN Ecole Nationale d'Ingénieurs de Sfax (Tunisie), B.P. « W » 3038 Sfax, Tunisie.

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Listeria monocytogenes **is a food-borne bacterial pathogen that is able to grow at refrigeration temperatures and resist freezing. This organism frequently colonizes and proliferates on preserved food products despite exposure to stress conditions induced by low storage temperatures. To investigate microbial virulence genes expression associated with cold shock, RT-PCR (Reverse Transcript PCR) assays was applied in three isolated strains *L. monocytogenes* and reference strain ATCC 19115. These included cDNAs for *L. monocytogenes* genes involved in previously described virulences genes (*hlyA*, *iap*, *flaA*, *fri*, *fbp*) who cold-adaptive response is *flaA*, cold shock response is *fri*, cell surface alterations were *fbp*, and *flaA*, and the general microbial stress response (σ^{β}) which contributed to such stress resistance. Transcription of virulence genes was significantly increased after cold and freezing stress of different virulence genes used in this study. Transcript level for the σ^{β} gene was significantly affected by the factor temperature. These findings therefore suggest important roles of virulence genes and σ^{β} factor to controls *L. monocytogenes* organisms against stress conditions associated with low storage temperatures.**

Key words: *Listeria monocytogenes*, cold stress, virulence, expression.

INTRODUCTION

Listeria monocytogenes is a food-borne pathogen that can cause an invasive human illness (Yvonne et al., 2007). It is the causative agent of listeriosis, a serious illness for which the young, elderly, and immunocompromised are especially at risk (Olsen et al., 2005). The control of this bacterium during production and storage of processed food products is one of critical measures in public protection against *L. monocytogenes* infection. This task however, remains tough due to environmental ubiquity, as well as, natural resistance of this bacterium to environmental stress (Arguedas-Villa et al., 2010; Gandhi and Chikindas, 2007).

L. monocytogenes is of particular concern for the food industry due to its wide distribution in the environment and its presence on raw and minimally processed foods. When contaminated food is stored for extended periods at low temperatures, *L. monocytogenes* is able to grow, leading to costly product recalls and posing a significant threat to public health (Duodu et al., 2010; Raimann et al., 2009). Consequently, a detailed understanding of the physiology of *L. monocytogenes* during cold shock and

*Corresponding author. E-mail: miladi_h@yahoo.fr. Tel: +216 97 776 410. Fax: +216 73 461 830.

growth is needed to allow for the design of effective strategies to prevent *L. monocytogenes* on refrigerated ready-to-eat foods (Yvonne et al., 2007).

L. monocytogenes is known for its ability to survive and proliferate in adverse environmental conditions, including pH acid, high osmolarity, refrigeration temperatures, and freezing (Henrike et al., 2004). While the stress by thermal shock of *L. monocytogenes* was described and reproduced experimentally to evaluate the impact of relatively low temperature (5°C) on the bacterial growth (Bergis, 2002). More recently, the survival of *L. monocytogenes* at least ten months on frozen salmon was reported (Miladi et al., 2008). Such a response of a bacterium to environmental changes involves activation of existing enzymes and enhanced rates of transcription of genes, resulting in enhanced levels of adaptive proteins (Henrike et al., 2004).

Nevertheless, it is assumed that virulence is not a stable property, and can be influenced by environmental conditions. Many environmental factors may increase or decrease the virulence expression of *L. monocytogenes* (Conte et al., 2000). For instance, temperature has been shown to be important for the expression of key virulence genes in *L. monocytogenes* (McGann et al., 2007; Duodu et al., 2010) although, transcriptome analysis has revealed that various *L. monocytogenes* genes are activated in response to cold growth. The precise role such genes in cold adaptation is not yet understood (Liu et al., 2002).

In *L. monocytogenes*, the role of sigma factor has been determined in response to adverse conditions; e.g., it plays a role in acid resistance of stationary-phase cells, in oxidative and osmotic stress resistance, in the response to carbon starvation, and in growth at low temperatures (Ferreira et al., 2001; Becker et al., 2000). A detailed study using transcript analysis by RT-PCR (Bubert et al., 1999) provided clear evidence that virulence genes are differentially transcribed in the different compartments of an infected host cell. Indeed, it has been reported that temperature downshift in *L. monocytogenes* induces a range of proteins which, may play a role in cryotolerance (Bayles et al., 1996). In other Gram positive bacteria, specific cold shock proteins (CSP) have been shown to be essential for survival at low temperatures (Wouters et al., 1999; Graumann et al., 1997). To adapt to low-temperature, *L. monocytogenes* develop many mechanisms including the changes in membrane lipid composition, the uptake of osmolytes and oligopeptides and the expression of cold shock proteins (CSPs) and cold acclimation proteins (CAPs) (Yvonne et al., 2007; Liu et al., 2002; Bayles and Wilkinson, 2000; Borezee et al., 2000). While CSPs and CAPs have been identified in *L. monocytogenes* (Yvonne et al., 2007), the functions of many of these proteins have not yet been clearly defined. One *L. monocytogenes* CSP that is highly synthesized during low-temperature exposure is ferritin (Fri) (Yvonne et al., 2007; Hébraud and Guzzo, 2000) required for efficient bacterial growth at early stages of the infection process (Fiorini et al., 2008; Polidoro et al., 2002). *L. monocytogenes* ferritin is a major cold shock protein which, is also strongly over-expressed after cold, heat shock or chemical stress (Hebraud and Guzzo, 2000).

L. monocytogenes can also swim by means of flagella-based motility in extracellular environments. *L. monocytogenes* strains are highly flagellated and motile at low temperatures, 30°C and below, and are typically not motile at 37°C or above (Yvonne et al., 2007; Bayles and Wilkinson, 2000). Previous studies have shown that flagella motility gene expression in *L. monocytogenes* is regulated by temperature and have shown that transcription of *L. monocytogenes flaA*, encoding flagellin, is down-regulated at physiological temperature (37°C) and contributes to virulence (Borezee et al., 2000).

Fibronectin has critical roles in eukaryotic cellular processes, such as adhesion, migration and differentiation. Dramsi et al. (2004) identified a new *L. monocytogenes* gene, *fbpA*, required for efficient colonization of host tissues. FbpA is seen a fibronectin-binding protein and present on the listerial surface that can mediate adherence to host cells but also modulate the protein levels of two virulence factors, listeriolysin and internaline B. These results point to the multiple contributions of FbpA to *L. monocytogenes* virulence.

The primary and the best characterized *L. monocytogenes* virulence determinant is listeriolysin (LLO) which is responsible for escape from a vacuole and thus entrance in the cytosol (Eneida et al., 2004; Dramsi and Cossart, 2002). LLO is a pore-forming, thiol-activated toxin that is pivotal for *L. monocytogenes* virulence. Initial studies on enzymatic activity shows that the amount of protein produced and gene expression have been conducted for the *hlyA* gene under different environmental conditions (Ripio et al., 1996; Leimeister-Wachter et al., 1992). Invasion-associated protein (*iap*) is implicated in the adherence to certain mammalian cells. The p60 protein is a suitable target in immunoassays as it is expressed in high amounts at various temperatures and growth phases (Bubert et al., 1997). In addition, p60 is already relatively high at low growth temperature, (Bubert et al., 1994).

Previous studies, using either semi or relative qRT-PCR approaches to study stress related gene expression changes in *L. monocytogenes* have been described (Kazmierczak et al., 2003; Sue et al., 2004; Wemekamp-Kamphuis et al., 2004). This study is therefore conducted to evaluate the expression level of five genes (*fri*, *flaA*, *fbp*, *hlyA* and *iap*) in reference strain and in three food isolated strains. The aim of this work was to study the response of *L. monocytogenes* after cold and freezing stress by analyzing expression levels of virulence genes and the gene encoding the stress- responsive

Table 1. Primer sequences used in this study

Primer	Amplicon size (pb)	Anneal temperature (°C)	Primer sequence 5' → 3'	References
Fla A	864	49	FlaA (F) : ATGAAAGTAAATACTAATATC FlaA (R) : TTAGCTGTTAATTAATTGAGT	This study
Fri	471	49	Fri (F) : ATGAAAACAATCAACTCAGT Fri (R): CTACTCTAATGGAGCTTTT	This study
Fbp	911	50	Fbp (F): ATGCAAACAAAATTGCACTG Fbp (R): GAATTCGCCGACAACTTACT	This study
HlyA	388	66	HlyA (F): GAATGTAAACTTCGGCGCAATCAG HlyA (R): GCCGTCGATGATTTGAACTTCATC	Garrec et al., 2003
Iap	453	46	iap (F): GAATGTAAACTTCGGCGCAATCAG iap (R) : GCCGTCGATGATTTGAACTTCATC	Medrala et al., 2003
σβ	780	49	sig β (F) : ATGCCAAAAGTATCTCAACCTGA sig β (R) : TTACTCCACTTCCTCATT	This study
16S rRNA	318	51	16S rRNA (F) : TTAGCTAGTTGGTAGGGT 16S rRNA (R) : AATCCGGACAACGCTTGC	O'Driscoll, 1997

alternative sigma factor.

MATERIALS AND METHODS

Bacterial strain isolation and biochemical characterization

Four strains of *L. monocytogenes* were used: one reference strain ATCC 19115 and three strains (S1, S2 and S3) were isolated from meat according to the standardised French method NF V 08-055 (AFNOR, 1999), for *L. monocytogenes* food detection (Miladi et al., 2008). The cells were preserved in 20% glycerol in medium at -80°C and cultivated on tryptic soy agar-yeast extract (BIO-RAD) at 37°C prior to use. Freshly grown colonies were used to inoculate 10 mL brain heart infusion broth (BHI) tubes and incubated at 37°C for 20 h.

Bacterial growth conditions and stress adaptation

L. monocytogenes strains were cultured by plating on tryptic soy agar-yeast extract (TSA-YE) (BIO-RAD) for 18 to 24 h at 37°C. Single colonies from each strain were used to inoculate 10 mL TSA-YE. The cultures were grown at 37°C with shaking (250 r.p.m.) to reach the exponential phase. To assess target virulence gene expression in the cold and freezing stress model, such exponential-phase cultures were pelleted by centrifugation (4000 g for 5 min). The supernatants were discarded and the pellets were once again resuspended in 10 mL of fresh TSA-YE broth. Each culture was further subdivided into 5 mL aliquots that were incubated at 37°C (control samples), at 4°C (cold stress) and at −20°C (freezing stress) for 4 h. After stress, 1.5 mL sample aliquots were centrifuged (5 min at 4,000×g) at 4°C (for cold and freezing stress) or room temperature (for control samples).

Thereafter, the samples were processed for total RNA isolation from control and stressed cells by SV total RNA isolation system (Promega, France) according to the manufacturer's instructions. The total RNA was quantified using an Ultraspec spectrophotometer (Ultraspec 2100 pro; Amersham Bio- sciences Europe GmbH, France) and its integrity was verified by 2% agarose gel electrophoresis and ethidium bromide staining.

RT-PCR for virulence genes expression

To study the expression level of *Listeria monocytogenes* virulence genes before and after cold and freezing shock, semi-quantitative RT-PCR method was used. First-strand cDNA was produced by reverse transcription (RT) using murine Moloney leukemia virus reverse transcriptase (Invitrogen) in conjunction with 100 ng total RNA and the reverse primer named of each tested genes of *L. monocytogenes* for 45 min at 42°C. The resultant cDNA was then used as template for polymerase chain reaction (PCR) analysis. The primers of *L. monocytogenes* control and virulence genes used in this study and their anneal temperatures were listed in Table 1.

The PCR mixture (25 µL) contained 1 µM forward and reverse primer, dNTP mix (100 µM each of dATP, dCTP, dGTP and dTTP), 1 U of Go *Taq* polymerase (Promega), 5 µl green Go *Taq* buffer (5X), 3 µl of resultant cDNA. PCR conditions of *hly*A and *iap* genes are performed as described elsewhere (Miladi et al., 2008). PCR for the *fri, flaA* and σ^B genes included each one an initial step (94°C for 5 min), followed by 30 denaturation cycles (94°C for 1 min), annealing (49°C for 1 min) and extension (72°C for 1 min), and concluded at the end of cycling by a final extension (72°C for 10 min). The PCR conditions of the *fbp* gene included an initial step (94°C for 5 min), followed by 30 cycles of denaturation (94°C for 1 min), annealing (50°C for 1 min) and extension (72°C for 1 min) and concluded at the end of cycling by a final extension (72°C for 10 min). Primers for the 16S rRNA (O'Driscoll, 1997) were used as controls.

PCR products (7 µL) were analyzed on 1% agarose gel stained with ethidium bromide (0.5mg/mL) at 100 V for 45 min and viewed under ultraviolet trans-illumination. The amplification products were photographed and their sizes determined with 100 bp molecular size marker (Promega). Quantitative analysis of DNA bands was performed using imaging software (Gene Tools, Sygene, UK).

Statistical analysis

Statistical analysis was performed using the S.P.S.S. 13.0 statistics

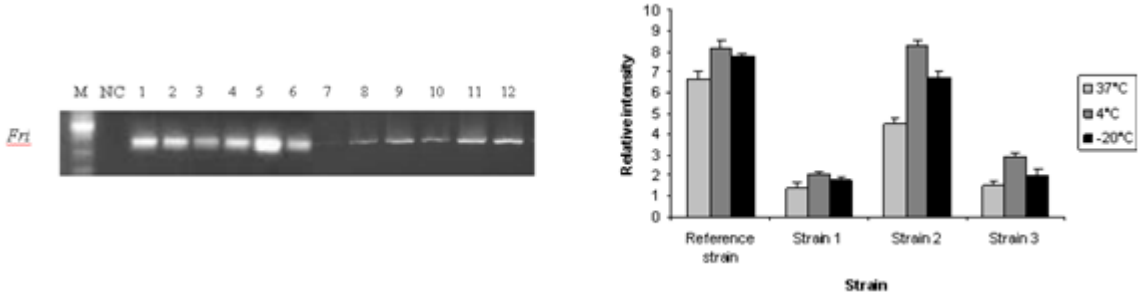

Figure 1. Agarose gel analysis (1%) of *fri* expression of *Listeria monocytogenes* before and after cold and freezing stress. (A) M, 100 bp DNA ladder (Promega); **NC**, negative control; **1**, Ref_{37}; **2**, Ref_{4}; **3**, Ref_{-20} ; **4**, $S2_{37}$; **5**, $S2_{4}$; **6**, $S2_{-20}$; **7**, $S1_{37}$; **8**, $S1_{4}$; **9**, $S1_{-20}$; **10**, $S3_{37}$; **11**, $S3_{4}$; **12**, $S3_{-20}$. The relative expression of *Fri* gene. (B)

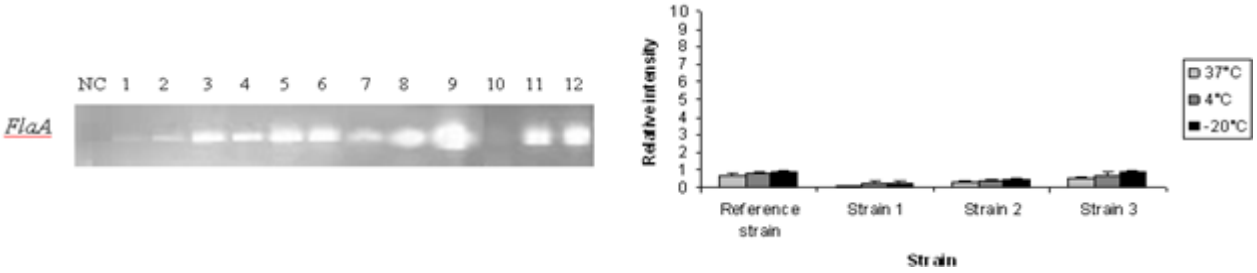

Figure 2. Aagarose gel analysis (1%) of *flaA* expression of *Listeria monocytogenes* before and after cold and freezing stress. (A) **NC**, negative control; **1**, $S1_{37}$; **2**, $S1_{4}$; **3**, $S1_{-20}$; **4**, $S2_{37}$; **5**, $S2_{4}$; **6**, $S2_{-20}$; **7**, $S3_{37}$; **8**, $S3_{4}$; **9**, $S3_{-20}$; **10**, Ref_{37}; **11**, Ref_{4}; **12**, Ref_{-20}. The relative expression of *fla*A gene (B).

package for Windows. The differences in the level of virulence genes expression were examined by the Friedman test, followed by the Wilcoxon signed ranks test. *P*-values of <0.05 were considered significant.

RESULTS

Detection of virulence genes in *Listeria monocytogenes* strains

All *L. monocytogenes* strains used in this study were positive for the five virulence genes and for alternative sigma factor *σβ* which contributes to the regulation of gene expression at low temperature.

Expression of virulence genes

After cold shock, we observed an increase in the expression level of different virulence genes used in this study. RT-PCR analysis indicated that under normal growth conditions (TSB-YE at 37°C), constitutive expression of all virulence genes was observed. Ferritin as a protective factor against stress conditions (Olsen et al., 2005). After cold and freezing stress, we observed an increase in the expression level of *fri* gene in *L. monocytogenes* and it was noticed that the expression level of this gene at 4°C increased more than those at -20 and 37°C in tested stains. However, after cold stress, the relative intensity is the highest and increased by 1.223 ± 0.366, 0.644 ± 0.176, 3.818 ± 0.299 and 1.392 ± 0.166 for reference strain, S1, S2 and S3, respectively (Figure 1) compared then at 37°C. On the other hand, we noted that the expression of *fri* gene at -20°C was more important than at 37°C. Statistical analysis revealed a significant difference between the level of expression at 37, 4 and -20°C (P <0.05).

L. monocytogenes strains are highly flagellated and motile at low temperatures, 30°C and below, and are typically not motile at 37°C or above (Way et al., 2004). After stress, it was observed *that* the expression level of *flaA* increased significantly (P < 0.05) in all tested strains. Furthermore, the presences of a significant amount of flaA transcript levels in different strains when grown at 37°C were observed (Figure 2).

Fbp appears as a novel multifunctional virulence factor of *L. monocytogenes*. After stress, a significant increase was observed in the expression of *fbp* gene (P <0.05) in three isolated strains. For reference strain, the level of

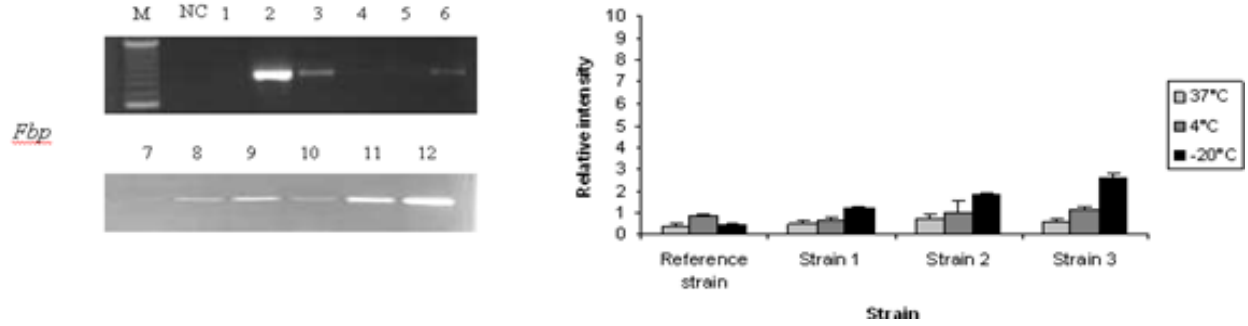

Figure 3. Agarose gel analysis (1%) of *fbp* expression of *Listeria monocytogenes* before and after cold and freezing stress. (A) **M**, 100 bp DNA ladder (Promega); **NC**, negative control; **1**, Ref_{37}; **2**, Ref_{4}; **3**, $Ref._{20}$; **4**, $S3_{37}$; **5**, $S3_{4}$; **6**, $S3._{20}$; **7**, $S1_{37}$; **8**, $S1_{4}$; **9**, $S1._{20}$; **10**, $S2_{37}$; **11**, $S2_{4}$; **12**, $S2._{20}$ The relative expression of *fbp* gene. (B).

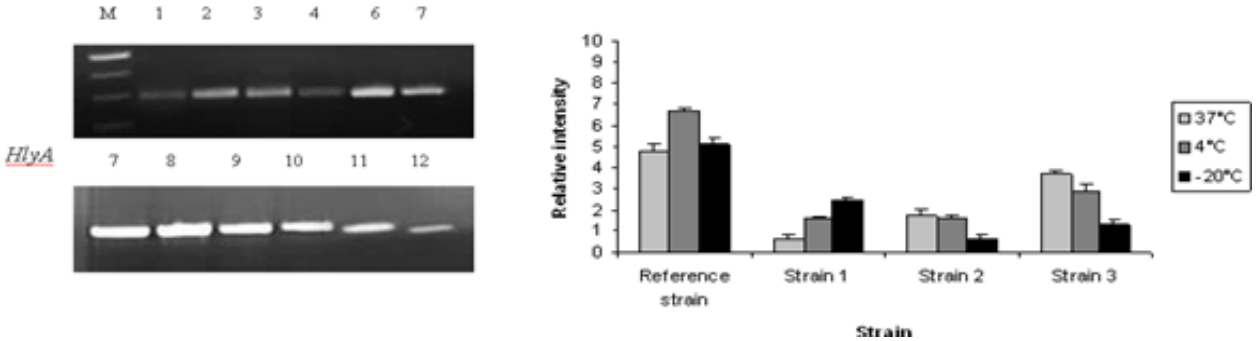

Figure 4. Agarose gel analysis (1%) of *hly*A expression of *Listeria monocytogenes* before and after cold and freezing stress. **(A)** M, 100 bp DNA ladder (Promega); 1, $S3_{37}$; 2, $S3_{4}$; 3, $S3._{20}$; 4, $S1_{37}$; 5, $S1_{4}$; 6, $S1._{20}$; 7, $S2_{37}$; 8, $S2_{4}$; 9, $S2._{20}$; 10, Ref_{37}; 11, Ref_{4}; 12, $Ref._{20}$. **(B)** The relative expression of *hly*A gene.

expression increased by 0.482 ± 0.119 after cold stress at 4°C which is more important than after freezing (Figure 3). For isolated strains, the expression levels of *fbp* gene are more important after freezing then after cold stress. The relative intensity increased by 2.364, 2.494 and 4.208 fold for S1, S2 and S3, respectively compared as 37°C (Figure 3). The *hlyA* expression for the reference strain was relatively high at 4°C, while the expression was partly repressed at freezing compared then at 37°C (Figure 4). The strains 1 were moderately induced at 4°C, as compared with 37°C and also more expressed at freezing.

In strain 2 and 3, the transcript levels for this gene were relatively repressed by 0.153 ± 0.066 and 0.828 ± 0.341, respectively at 4°C and by 1.116 ± 0.123 and 2.473 ± 0.493, respectively at -20°C. We noted also that the level of expression is the higher at 37°C (Figure 4). Statistical analysis revealed a significant difference between the level of expression of *hlyA* mRNA at 37, 4, and -20°C (P <0.05), respectively.

This study reveals that freezing (-20°C) enhanced transcription of *iap* gene in *L. monocytogenes* ATCC 19115 and in both strains 1 and 3. In contrast, in strain 2, the higher level of expression is at 4°C to be 2.158 ± 0.162 compared as at -20°C to be 1.645 ± 0.233 (Figure 5). Statistical analysis revealed a significant difference between its expression level at 37 and 4°C, and between the level of expression at 37 and -20°C (P <0.05) in different strains. σ^{β}, the stress responsive alternative sigma factor for RNA polymerase, has been confirmed in a separate study to contribute to the expression of a subset of *L. monocytogenes* virulence genes (Kazmierczak et al., 2003). Transcript levels for the σ^{β} gene were significantly affected by the factor temperature (P < 0.05) and the transcript levels of this gene were similar in the three isolated strains. The transcription increase as *L. monocytogenes* cells have incubated at 4°C and more increased after strains (S1, S2 and S3) were shocked at -20°C. A significant difference (P <0.05) in the level of expression of reference strain was noted, the higher transcription is at 4°C (Figure 6).

For housekeeping gene, the RT-PCR data presented here show that the transcription of 16s rRNA is not cold and freezing stress activated (Figure 7).

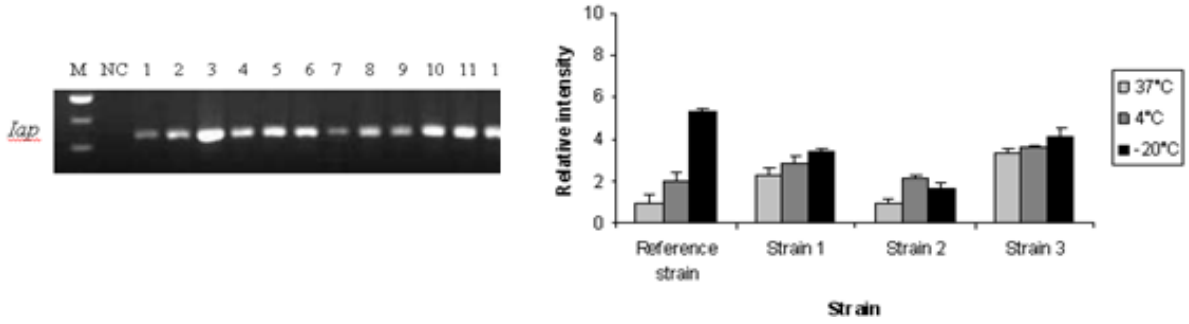

Figure 5. Agarose gel analysis (1%) of *iap* expression of *Listeria monocytogenes* before and after cold and freezing stress. **(A)** M, 100 bp DNA ladder (Promega); NC, negative control; 1, Ref_{37}; 2, Ref_{4}; 3, Ref_{-20}; 4, $S1_{37}$; 5, $S1_{4}$; 6, $S1_{-20}$; 7, $S2_{37}$; 8, $S2_{4}$; 9, $S2_{-20}$; 10, $S3_{37}$; 11, $S3_{4}$; 12, $S3_{-20}$. **(B)** The relative expression of *iap* gene.

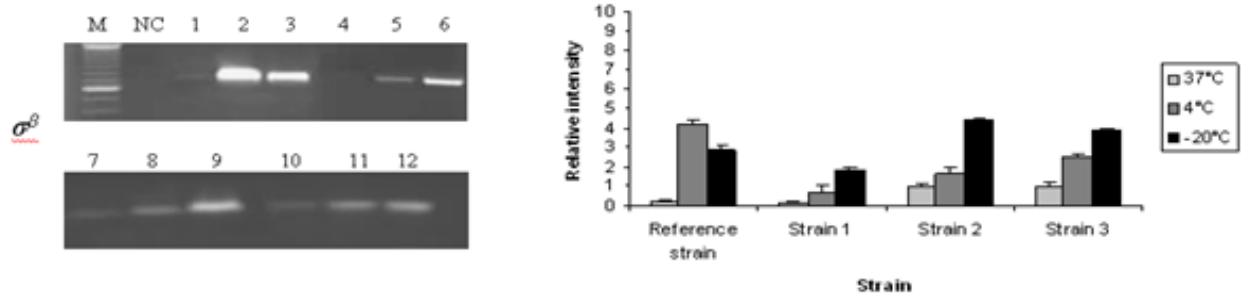

Figure 6. Agarose gel analysis (1%) of σ^{B} expression of *Listeria monocytogenes* before and after cold and freezing stress. **(A)** M, 100 bp DNA ladder (Promega); NC, negative control; 1, Ref_{37}; 2, Ref_{4}; 3, Ref_{-20}; 4, $S1_{37}$; 5, $S1_{4}$; 6, $S1_{-20}$; 7, $S2_{37}$; 8, $S2_{4}$; 9, $S2_{-20}$; 10, $S3_{37}$; 11, $S3_{4}$; 12, $S3_{-20}$. (**B)** The relative expression of σ^{B} gene.

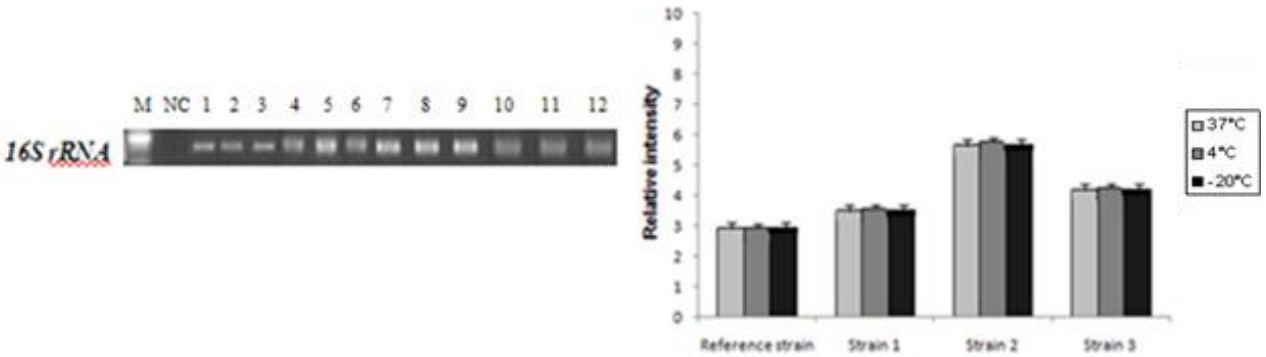

Figure 7. Agarose gel analysis (1%) of 16s rRNA expression of *Listeria monocytogenes* before and after cold and freezing stress. **(A)** M, 100 bp DNA ladder (Promega); NC, negative control; 1, Ref37; 2, Ref4; 3, Ref-20 ; 4, S137; 5, S14; 6, S1-20; 7, S237; 8, S24; 9, S2-20; 10, S337; 11, S34; 12, S3-20. **(B)** The relative expression of 16s rRNA gene.

DISCUSSION

All organisms respond to environmental stress by modifying the rate of transcription of certain genes. Our study shows an increase in the level of transcription of virulence genes of foodborne pathogen *L. monocytogenes* after cold and freezing stress. These results indicate that transcription enhanced in stationary phase at 4°C and after shocked at -20°C (Leimeister-Wächter et al., 1992). Recent study demonstrates that

the reference strain was less resistant to cold and freezing stress, compared to the strains isolated from meat (Miladi et al., 2008). This could be explained by the easy adaptation of these bacteria to starvation conditions in the initial food environment.

The transcription of *fri* gene, which encodes a major cold shock protein that appears to be involved in enhancing *L. monocytogenes* survival under conditions of cold stress. Consistently with a previous report (Hébraud and Guzzo, 2000), ferritin is highly transcribed during low-temperature exposure. Dussurget et al. (2005) provided also evidence that *L. monocytogenes* ferritin promotes adaptation to nutritional and thermal shifts, and contributes to virulence. Increased motility and flagellum production have long been known to be associated with the growth of *L*. *monocytogenes* at low temperatures (Leifson and Palen, 1955). *flaA* encodes the *L*. *monocytogenes* major flagellin protein FlaA (Dons et al., 1992). RT-PCR analyses indicate that trace amounts of flagellin are present in cells grown at 37°C. Liu et al. (2002) were able to detect *flaA* transcripts in bacteria grown at 10°C by Northern hybridization and much lower levels of transcription were present in bacteria grown at 37°C. The significance of increased flagellum production during growth at low temperatures is unclear. However, Gründling et al. (2004) indicate that temperature dependent motility and flagellation in *Listeria* is not only regulated on a transcriptional level but also, on a post transcriptional level. Despite the presence of a significant amount of *flaA* transcripts in 10 403S when grown at 37°C, no FlaA protein detectable on the bacterial surface by Western blot, but only a few (2%) 10 403S bacteria contained a single flagellum on the surface.

Also identified is mRNA for a novel *L*. *monocytogenes* fibronectin- binding protein expressed in response to reduced temperatures. *L*. *monocytogenes fbp* encodes a 24.6-kDa peptide (Liu et al., 2002) is more expressed after exposed to cold and freezing stress. These results are not in agreement with Liu et al. (2002) which show that any relationship between *fbp* mRNA expression and low-temperature growth of *L*. *monocytogenes* remains to be elucidated.

The expression pattern for the *hlyA* gene is seen as highly diverse among different strains of *L. monocytogenes*. The responses of four strains varied differently to the environmental conditions tested in this work. These results were in agreement with Rudi et al. (2003) who investigated the effect of growth temperature and medium on the *hlyA* expression and found that the expression pattern of *hlyA* was highly diverse among the different strains tested.

The σ^{β} gene product of *L. monocytogenes* is an alternative factor involved in transcriptional regulation in response to many environmental conditions, including low temperature, osmotic and acid stress (Ferreira et al., 2001). In this study, the cold and freezing stress induced significantly the expression levels of σ^{β} gene. As these results are in agreement with those of Becker et al. (2000) who also found increased transcript levels for σ^{β} - dependent genes at low temperature. As experimental evidence suggest that ***sigβ*** gene is crucial for survival and maintenance of *L. monocytogenes* under stressful conditions (Kim et al., 2005).

Results obtained in this study show that transcription of virulence genes was significantly affected by growth temperature. Since, the levels of the expression of five virulence genes increase after cold and freezing shock. In the other hand, the σ^{β} gene encoding the general tress sigma factor appear to be involved in the survival of *L. monocytogenes* at low temperature. This is of particular importance in the food industry where the control of this bacterium during production and storage of processed food products is one of critical measures in health protection against *L. monocytogenes* infection.

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