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ORIGINAL RESEARCH

A comparison of gene expression of *Listeria monocytogenes in vitro* and in the soft cheese Crescenza

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*This study focuses on the expression analysis of virulence and stress response genes (*plcA, hly, iap *and* sigB*) in 11 different strains of* Listeria monocytogenes. *The first step of this study considered an* in vitro *analysis in synthetic laboratory medium. According to statistical analysis, significant differences emerged for genes* sigB *and* plcA. *Subsequently, a soft cheese at two temperatures (4 and 12 °C) and for different times (24 and 48 h) was evaluated. Significant expression differences emerged for the condition at 12 °C after 48 h. No association could be observed between gene expression profile and origin of the different strains.*

Keywords *Listeria monocytogenes*, virulence gene expression, soft cheese, RT-qPCR.

INTRODUCTION

Listeria monocytogenes is a Gram positive bacterium recognised as an important human foodborne pathogen capable of colonising multiple host tissues and causing a range of clinical conditions. Once inside the human stomach, *L. monocytogenes* overcomes the acid environment, moves across the intestine, enters the blood circulation and migrates to other parts of the body where it grows and stimulates a range of host reactions and clinical diseases. High mortality rates occur predominantly among pregnant women, the immunocompromised and the elderly (Kathariou 2002).

It is an ubiquitous bacterium, commonly isolated from foods of animal origin, but also from many different environments, especially from soil, water, vegetation, sewage, animal feeds, farm and food-processing environments (Sauders and Wiedmann 2008). Due to its physiological characteristics, it has the capacity to survive at low temperatures and in acidic and sodium chloride stress (Liu *et al.* 2002; Gardan *et al.* 2003; Olesen *et al.* 2009), thus representing a particular problem especially for the safety of refrigerated and ready-to-eat (RTE) foods consumed without reheating and cooking (Kathariou 2002). As reported by Duodu *et al.* (2010), not all strains of *L. monocytogenes* have the same capacity to cause disease, and it is assumed that virulence is not a stable property and can be influenced also by environmental conditions (Cossart and Toledo-Arana 2008). In foods, in the environment, as well as in animals during infection, *L. monocytogenes* is exposed to numerous stress signals that may strongly influence its pathogenicity. Stresses caused by refrigeration, heat, acid and salt are of special relevance to the physiological status and virulence of this pathogen in food (Kathariou 2002). Temperature and acid conditions have been shown to have important impact on the expression of virulence genes (Conte *et al.* 2000; McGann *et al.* 2007; Olesen *et al.* 2009). In particular, the stress response alternative sigma factor σ^B transcribes genes contributing to bacterial survival under conditions of environmental stress such as low temperature, low pH, elevated osmolarity and entry into stationary phase (Becker *et al.* 1998, 2000; Cetin *et al.* 2004). Furthermore, differences in virulence may also be attributable to the serotypes because the vast majority of human listeriosis cases are caused

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by three serotypes (1/2a, 1/2b and 4b) (Schuchat *et al.* 1991).

Few studies have investigated gene transcription for *L. monocytogenes* in real food matrices. In a study of 2008, gene transcription was investigated after growth in ultra-high-temperature-processed skim milk (Liu and Ream 2008). Similarly, in 2010, two different papers investigated gene transcription in food. Olesen *et al.* (2010) analysed standard liver patè and liver pates with reduced NaCl content and/or added organic acids (Ca-acetate and Ca-lactate), to determine whether a reduction of the NaCl content of the standard liver patè and the addition of organic acids could change the relative transcription of virulence genes. Duodu *et al.* (2010) examined the influence of temperature storage conditions of two *L. monocytogenes* strains grown on salmon pointing out how the abuse temperature could be implicated in the increasing of the infective potential of low virulence strains in salmon. Despite the results obtained through the above studies, very little is known about the virulence expression of different strains in the different kinds of food.

To provide a contribution to this issue, the aim of this study was to examine the gene expression levels of strains of *L. monocytogenes* isolated from different sources. First, an *in vitro* condition, BHI broth, was tested, followed by the study of gene expression directly in soft cheese under different conditions and, in particular, at refrigeration temperature and at 12 °C, considered as abuse temperature. A Crescenza cheese was chosen for this experiment being a soft, creamy cheese widely consumed in the North of Italy. It is made from cow's milk and produced by thermophilic acidifying starters. This study focused on the expression of genes that are known to be associated with stress response and virulence. In particular, the sigma factor, encoded by *sigB* gene, regulates stress response genes, while the capacity of *L. monocytogenes* to escape from the single-layer membrane vacuole is associated with two virulence molecules: listeriolysin O and phosphatidylinositol-phospholipase C, respectively, encoded by *hly* and *plcA* genes (Liu *et al.* 2007). The *iap* gene, which encodes the p60 protein, a major extracellular protein associated with the invasion of phagocytic cells, was also taken into consideration.

MATERIAL AND METHODS

In vitro conditions: BHI broth

Bacterial strains

Serotype and source of isolation for the different strains used in the *in vitro* experiments are reported in Table 1. Strains isolated from food were identified with the use of qPCR as reported in Rantsiou *et al.* (2008).

Stock cultures were stored at −80 °C in BHI (Oxoid, Milan, Italy) supplemented with glycerol (30% vol/vol).

BHI cultures

The strains were resuscitated from −80 °C in BHI at 37 °C overnight and then streaked on BHI agar. One single colony was inoculated in 10 mL of BHI broth overnight. The overnight cultures were subjected to RNA extraction as described below and enumerated in Oxford plates (Oxoid, Milan, Italy) as suggested by the manufacturer. The experiment was performed in triplicate.

RNA extraction

One mL of the overnight culture was mixed with an equal volume of RNALater (Ambion, Monza, Italy), to block cell transcription, and centrifuged. The pellet was re-suspended in 50 μL of lysozyme (50 mg/mL, Sigma, Milan, Italy) and lyzed for 30 min at 37 °C. After this step, nucleic acid extraction was performed using Master-Pure™ Complete DNA and RNA Purification kit (Epicenter, Madison, WI, USA). The DNase-free RNase (Ambion) was adopted to eliminate co-extracted DNA. The presence of residual DNA in the RNA preparation was evaluated by qPCR. In case of positive signals, a second treatment was performed. The quantity of purified RNA was determined using absorbance reading on a NanoDrop® ND-1000 spectrophotometer (Celbio, Milan, Italy). The RNA quality was analysed by agarose gel (1.2% w/vol) electrophoresis.

cDNA synthesis

Prior to reverse transcription, the DNase was inactivated at 75 °C for 10 min, in 0.5 M EDTA to stabilize RNA during heating. One μg of RNA was reverse transcribed using the M-MLV enzyme (Promega, Milan, Italy), as suggested by the manufacturer. In the reaction, 1 μL of Random Hexamers (Promega) was used. One μL of cDNA was loaded in the qPCR.

qPCR amplification conditions

Amplifications were performed in a final volume of 25 μL in the Chromo4 Real-Time PCR Detection System (Bio-Rad, Milan, Italy). Specific PCR primers and Taqman probe were designed for the four genes, *sigB*, *iap*, *plcA* and *hly* (Table 2), considering DNA sequences of *L. monocytogenes* downloaded from GeneBank database and aligned by the ClustalW program, available at http://www.ebi.ac.uk/clustalw/. Primers and probes were synthesised by Sigma.

The conditions for amplification were for all genes: primers at 400 nM and probe at 250 nM, the concentration of $MgCl_2$ was 8 mM and the polymerase used was purchased from Euroclone (Fluomix for probe, Celbio, Milan, Italy). As reference gene, the intergenic spacer (IGS) region was selected, and primers IGS1 (5′-GGCCTATAGCTCAGCTGGTTAGAG-3′) and IGS2 (5′-GCTGAGCTAAGGCCCCGTAAAAGGG-3′) were used in combination with the probe IGS (5′-FAM-ATAAGAAATACAAATAATCATACCCTTTTAC-TAMRA-3′) (Rantsiou *et al.* 2008). The amplification

Table 1 Strains used in the gene expression analysis

Strain	*Serotype*	*Isolated from*	*Source*
EGDe	1/2a	Reference Strain	DIVAPRA, University of Turin, Italy
NCTC 10527	4b	International Collection Strain	DIVAPRA, University of Turin, Italy
Scott A	4b	Human Epidemic isolate (U.S. Food and Drug Administration Cincinnati, OH).	DIVAPRA, University of Turin, Italy
3	3c	Meat for sausage production	Animal Pathology Department, University of Turin, Italy
5	4b	Minced meat	Animal Pathology Department, University of Turin, Italy
19	1/2b	Fresh sausage	Animal Pathology Department, University of Turin, Italy
36	1/2a	Pork meat	Animal Pathology Department, University of Turin, Italy
18	3a	Soft cheese	Animal Pathology Department, University of Turin, Italy
70	1/2a	Soft cheese	Animal Pathology Department, University of Turin, Italy
162	4b	Semihard cheese	Animal Pathology Department, University of Turin, Italy
V7	n.s.	Human	Food Science Department, University of Naples, Italy

n.s., not serotyped.

cycle adopted for the IGS was as follows: initial denaturation at 95 °C for 10 min, 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s. These conditions were used also for the other genes, but for *sigB*, *iap* and *plcA* an annealing temperature of 50 °C was used, while for *hly* the annealing temperature was 54 °C. The cycle was repeated 50 times. All qPCRs were performed for each gene in triplicate.

Listeria monocytogenes gene expression in cheese

The trend of expression of the genes included in this study was also evaluated in food at different conditions, and in particular at two temperatures: 4 °C (refrigeration storage temperature) and 12 °C (abuse temperature), for two different times (24 and 48 h). Soft Italian cheese (Crescenza) was artificially contaminated with *L. monocytogenes* strains EGDe, 3, 36, 18, 70, 162 and V7 also used for the *in vitro* study. A negative control, without inoculated strains, was prepared and included in the analysis.

For each strain, two colonies were inoculated in two tubes with 15 mL of BHI and incubated overnight at 37 °C. Ten grams of Crescenza cheese was then inoculated with the strains in a sterile beaker to obtain values of 10^7–10^8 colony forming units (cfu)/g of *L. monocytogenes* in the sample. Crescenza presented a pH of 5.5 with a salt content of about 1% (w/w). A total of four beakers were inoculated: two for the 4 °C and two for the incubation at 12 °C. The overnight culture of *L. monocytogenes* used to contaminate food samples was enumerated on Oxford plate to determine the exact cfu spiked in the sample, and the RNA was also extracted as described before.

Food sampling

After 24 and 48 h, 20 mL of RNALater solution (Ambion) was added in 5 g of inoculated cheese from each beaker and homogenised in a stomacher machine. This addition was performed to stop the transcription of DNA, as reported for the *in vitro* experiments. At the same time, the samples were analysed by classical microbiological methods by plating on Oxford agar (Oxoid).

Table 2 Primers and probes for the different genes analysed in this work

Primer name	*Sequence (5'-3')*
iap F	ACAATACTAATACACCATCTAA
iap R	GAGCTTCAGCAATAATAGC
Probe *iap*	HEX-ATGCTAATCAAGGTTCTTCCAACAATAACAG-TAMRA
hly F	GCATCATCAAGCGTACGTTCC
hly R	AATGAGCCAAGCTGGTTAAGCT
Probe *hly*	HEX- GAAAAATATGCTCAAGCTTATCCAAATG-TAMRA
sigB F	TTGGAATATTGGTTTACTTGG
sigB R	CAATTGTTGGCACAGCAAA
Probe *sigB*	HEX-TGTTCATTACAAAAACCTAGTAGAGTCCAT-TAMRA
plcA F	CTAGAAGCAGGAATACGGTACA
plcA R	ATTGAGTAATCGTTTTCAAT
Probe *plcA*	HEX-AATTTATTTAAATGCATCACTTTCAGGT-TAMRA

RNA Extraction

Twenty mL of each stomacher bag was transferred in a 50 mL tube. After a rest of 5 min, to allow a separation of the cheese matrix, 1 mL of solution was submitted to nucleic acid extraction in triplicate. After centrifugation at 29 200 x g for 5 min, RNALater was discarded, and the pellets were mixed with 50 µL of lysozyme (50 mg/mL) and 25 µL of proteinase K (50 mg/mL, Sigma) and heated at 50 °C for 30 min. After this treatment, nucleic acids

extraction was performed by using the Master-Pure™ Complete DNA and RNA Purification Kit (Epicenter) following the manufacturer's instructions. The DNase-free RNase (Ambion) was adopted to eliminate DNA as described in the *in vitro* conditions, and the presence of residual DNA in the RNA preparation was evaluated by qPCR.

RT-qPCR analysis of L. monocytogenes virulence genes

As done for the BHI cultures, prior to reverse transcription, the DNase was inactivated at 75 °C for 10 min with 0.5 M EDTA. cDNA was synthesised using 13 μL RNA in a final volume of 30 μL as described above. One μL of cDNA was loaded in the qPCR.

Data analysis

The comparative expression (CE) was determined by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001), using the IGS region as reference.

Data were subjected to one-way ANOVA, and Duncan test was used to determine differences at $P < 0.05$, using the statistical software, STATISTICA 7.0 for Windows (Statsoft, Tulsa, USA).

RESULTS AND DISCUSSION

Taking into consideration the *in vitro* results, after an overnight incubation at 37 °C in BHI broth, all the strains presented counts of about 10^9 cfu/mL. Comparative gene expression for each strain was determined in relation to the EGDe strain, according to the $2^{-\Delta\Delta C_t}$ method. The comparative expression values obtained were treated statistically and the results are presented in Table 3. Significant differences emerged for genes *sigB* and *plcA*. Regarding *sigB*, it showed higher levels of expression in the strains 3 and 36, isolated from meat products. On the other hand, different results emerged for *plcA* gene that showed higher level of expression in strains 70 and 5 (isolated, respectively, from dairy and meat products), while lower values were observed for strain V7, of human origin. No significant differences were observed for genes *iap* and *hly*.

Regarding the gene expression *in situ*, in order to optimise the RNA extraction method and to allow the measurement of the transcription levels of the selected genes in the soft cheese, the combined use of RNALater solution and Epicenter kit was applied. Counts for all strains remain at the level of the inoculum (about 10^7–10^8 cfu/g) both after 24 and 48 h of storage at 4 and 12 °C (data not shown). The CE was calculated for each strain in the different conditions using as control the values obtained from the same strain in BHI. From the results of ANOVA and Duncan test of the CE values (Table 4), it can be seen that significant differences emerged for all the genes for the condition 12 °C for 48 h, while at the same temperature after 24 h, only the gene *plcA* showed significant differences between the strains. At 4 °C, no significant differences emerged. Focusing on the condition at 12 °C for 48 h, in the case of *sigB* gene, strain 70 presented the highest values of expression, while the gene *plcA* resulted more expressed in strain 36, result that was also observed at 24 h. Strain EGDe presented higher values of expression for both *iap* and *hly* genes.

As reported above, it is assumed that virulence is not a stable property, and it can be influenced by environmental conditions, and temperature has been shown to be important for the expression of virulence genes in *L. monocytogenes* (McGann *et al.* 2007). The goal of this study was to test how the virulence genes are expressed *in vitro* and in a food matrix, analysing a soft cheese at different conditions: 4 °C (refrigeration temperature) and 12 °C (abuse temperature). These temperatures were chosen based on the property of *L. monocytogenes* to grow at refrigeration temperature and for the risk that it represents for RTE foods. Furthermore, we focused on long-term stress (24 and 48 h), to simulate the conditions in which *L. monocytogenes* is present in real food products. As reported by Olesen *et al.* (2009), the majority of the studies concerning *L. monocytogenes* virulence as a result of exposure to food-related stress only deal with short-term shock or adaptation (maximum 1 h), but this does not reflect the virulence status of *L. monocytogenes*: as a matter of fact in most cases, *L. monocytogenes* is present in the food product for several hours or days before the consumption.

The preliminary investigation performed *in vitro* showed statistically significant differences ($P < 0.05$) in the expression for genes *plcA* and *sigB,* while for the *iap* and *hly* genes, the observed differences were not significant. Furthermore, it was shown that the EGDe strain is expressing the studied genes at higher level with respect to the other strains considered. Strains 19, V7, 3 and 36 are the exception to this general observation because they showed higher expression for the *sigB* gene.

From the *in situ* experiments, more differences were observed between the strains. It appears that the incubation at 12 °C triggers a diversification in the expression of the genes studied for the strains considered. In this condition, it is possible to observe the different behaviour of the different strains and the different level of transcription of the different genes for the same strain. Overall, the effect of low temperature (both 4 and 12 °C) resulted in downregulation of the expression of the studied genes. This was also confirmed in Duodu *et al.* (2010) where an abuse temperature could be involved in the increasing of virulence gene expression. However, for certain strains, significant upregulation, with respect to the BHI at 37 °C condition, was observed. In particular, we can notice how strains EGDe and 70 present high value of expression for gene *sigB*, likewise, strains 36 and V7 for gene *plcA*.

Regarding the temperature, it is known that PrfA protein, which coordinates transcription of the *prfA* virulence gene

Table 3 *In vitro* expression of different strains isolated from different sources: results of variance analysis and Duncan test performed on parameters obtained by comparative expression results

	5	*Scott A*	*162*	*70*	*NCTC 10527*	*18*	*EGDe*	*19*	*V7*	*3*	*36*	*sig.*
sigB	0,14[a]	0,25[a]	0,36[a]	0,44[a]	0,79[a]	0,95[a]	1,00[a]	1,03[a]	1,51[a]	2,90[b]	3,22[b]	*
plcA	0,63[a]	0,20[bc]	0,07[bc]	0,75[a]	0,15[bc]	0,12[bc]	1,00[a]	0,17[bc]	0,00[bc]	0,33[bc]	0,19[bc]	*
iap	0,02	0,03	0,48	0,47	0,27	0,63	1	0,24	1,77	0,59	0,43	ns
hly	0,92	0,17	0,23	0,94	0,45	0,77	1	0,97	0,35	0,33	0,14	ns

Values with different superscript letters for each gene are significantly different, $P < 0,05$. Values without letters are not significant. *$P \leq 0,05$; ns not significant.

Table 4 *In situ* expression of different strains isolated from different sources: results of variance analysis and Duncan test performed on parameters obtained by comparative expression results

4 °C 24 h					*12 °C 24 h*				
Strains	*sigB*	*plcA*	*iap*	*hly*	*Strains*	*sigB*	*plcA*	*iap*	*hly*
18	0,08	0,18	0,80	0,06	18	0,09	0,019[a]	0,02	0,00
70	2,09	0,08	0,04	0,00	70	10,21	0,067[a]	0,02	0,00
EGDe	5,53	0,00	0,03	0,02	EGDe	1,41	0,009[a]	0,04	0,03
V7	0,19	na	na	0,11	V7	0,20	n[a]	0,04	0,04
162	0,02	na	0,00	0,01	162	0,06	0,04[a]	0,27	0,01
36	0,07	0,47	0,01	0,00	36	0,09	0,82[b]	0,03	0,00
3	0,06	0,00	0,01	0,01	3	5,11	0,07[a]	0,005	0,006
sig.	ns	ns	ns	ns	sig.	ns	*	ns	ns
4 °C 48 h					*12 °C 48 h*				
18	0,05	0,01	0,13	0,00	18	0,015[a]	0,031[a]	0,13[a]	0,001[a]
70	0,11	0,00	0,01	0,00	70	12,64[c]	0,29[a]	0,11[a]	0,006[a]
EGDe	1,13	0,01	0,08	0,01	EGDe	2,92[b]	0,094[a]	0,63[b]	0,116[c]
V7	1,74	0,00	0,03	0,80	V7	0,53[a]	0,258[a]	0,48[b]	0,017[a]
162	0,05	0,17	0,13	0,07	162	0,57[a]	0,18[a]	0,004[a]	0,035[ab]
36	0,04	0,17	0,01	0,00	36	0,08[a]	1,73[b]	0,04[a]	0,06[b]
3	0,09	0,44	0,01	0,07	3	0,04[a]	0,06[a]	0,01[a]	0,02[a]
sig.	ns	ns	ns	ns	sig.	*	*	*	*

Values with different superscript letters for each gene are significantly different, $P < 0,05$. Values without letters are not significant. *$P \leq 0,05$; ns not significant.

na, no expression detected.

cluster, including the *plcA* and *hly* genes analysed in this study, is temperature dependent, showing no expression below 30 °C (Johansson *et al.* 2002). Different studies showed, under laboratory conditions, that the production of listeriolysin O and other virulence factors are repressed at temperatures below 25 °C (Leimeister-Wachter *et al.* 1992) suggesting that virulence genes may be repressed when bacteria are growing in cold-stored foods. However, *plcA* and *prfA*P2 promoters do not appear subjected to this type of thermoregulation and therefore are likely to contribute to the expression of some PrfA-dependent genes at temperature below 30 °C (Miner *et al.* 2007). This may explain our results, in which gene *plcA* showed a high level of expression also at refrigeration temperature in strain 36, isolated from meat. Nevertheless, the repression of gene expression at 4 °C does not affect proper expression of the genes following infection (in response to the body temperature), and strains may differ in their ability to resume growth at 37 °C following cold storage; it was shown that clinical isolates resume growth more readily than strains derived from meat (Avery and Buncic 1997).

Concerning the transcription of *sigB*, it has been demonstrated that it is important for the adaptation to various environmental stress conditions and its activity increases by exposing *L. monocytogenes* to cold shock (Chan *et al.* 2007). In our experiment, *L. monocytogenes* was exposed to a cold shock when transferred from BHI to cheese and incubated at 4 and 12 °C. Two strains (70 and EGDe) responded to this temperature shift with an upregulation of the *sigB* gene. At 12 °C, these two strains showed a

significantly different expression level with respect to the rest of the strains that did not increase *sigB* transcription.

Regarding the source of isolation of the strains analysed, the two meat strains (3 and 36) have similar behaviour in the expression of genes *sigB* and *iap*, while for gene *plcA,* a higher expression was detected for stain 36. Regarding the three strains from cheese (18, 70 and 162), they showed no significant differences for genes *iap*, *hly* and *plcA*. On the other hand, gene *sigB* was more expressed in strain 70 with the highest value of expression. Therefore, as in the case of *in vitro* condition, also from cheese analysis results, it appears clear how the source does not constitute a determinant factor in the virulence gene expression.

Most of the *L. monocytogenes* outbreaks are caused by serotypes 1/2a, 1/2b, 1/2c and 4b (Jacquet *et al.* 2002; Kathariou 2002). Other serotypes (especially 4a and 4c), commonly isolated from animal, food or environment are seldom associated with human *L. monocytogenes* infections. However, from our results, it is possible to notice how the serotypes are not correlated with the virulence expression. As also reported in other studies, not all virulent strains maintain their infectious ability under environmental stress, and the presence of low virulence *L. monocytogenes* can be found in different kind of foods, but as observed by Roche *et al.* (2009), no specific link can be identified. Strain 36, serotype 1/2a, shows a high value of expression for the *plcA* gene. However, at the same time, the other genes tested for this strain were poorly expressed. Still, strains belonging to the same serotype behave differently as also reported by Werbrouck *et al.* (2006). As also shown by Duodu *et al.* (2010), it is possible that the same strains are able to conserve their infectious ability under environmental stress conditions in food products. At the same time, it can happen that low virulent strains become highly virulent in food, in so doing representing a risk for food safety.

In a food matrix as soft cheese, it is necessary to consider that there are factors influencing the virulence, as the pH and the salt concentration as reported in previous studies (Datta and Kothary 1993; O'Driscoll *et al.* 1996). In the cheese used in this work, the pH was 5.5 with a salt content of about 1% (w/w). As shown by Olesen *et al.* (2009), the transcription of virulence and stress response genes can be induced from an acid (pH 5.5) and osmotic (4.5% w/v NaCl) shock.

CONCLUSION

The obtained results showed different levels of expression in the various strains, underlining the different pathogenic potentials among different isolates. These differences could not be associated neither with the origin of the strains nor with their serotype. Based on our current knowledge, it is not possible to explain these differences. *L. monocytogenes* is an important foodborne pathogen in the dairy sector, and it is often isolated from fresh soft cheeses, where the pH, salt and water activity conditions may not kill it or limit its growth. In this study, it has been demonstrated that the strains tested behave differently in terms of expression of virulence genes, and this aspect should be taken into consideration to assess the risk associated with the consumption of dairy products. However, it should be underlined that the incidence of the disease caused by this micro-organism depends on different factors including also the infective dose and the immunity of the host. Further study of the pathogenic potential of this foodborne pathogen, as affected by the surrounding conditions and as influenced by the food, is needed to provide new insights for the understanding of its behaviour.

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REFERENCES

Avery S M and Buncic S (1997) Differences in pathogenicity for chick embryos and growth kinetics at 37 degrees C between clinical and meat isolates of *Listeria monocytogenes* previously stored at 4 degrees C. *International Journal of Food Microbiology* **34** 319–327.

Becker L A, Cetin M S, Hutkins R W and Benson A K (1998) Identification of gene encoding the alternative sigma factor σ^{β} from *Listeria monocytogenes* and its role in osmotolerance. *Journal of Bacteriology* **180** 4547–4554.

Becker L A, Evans S N, Hutkins R W and Benson A K (2000) Role of σ^{β} in adaptation of *Listeria monocytogenes* to growth at low temperature. *Journal of Bacteriology* **182** 7083–7087.

Cetin M S, Zhang C, Hutkins R W and Benson A K (2004) Regulation of transcription of compatible solute transporters by the general sigma factor, σ^{β}, in *Listeria monoytogenes*. *Journal of Bacteriology* **186** 794–802.

Chan Y C, Boor K J and Wiedmann M (2007) σ^{B} -dependent and σ^{B} -independent mechanisms contribuite to transcription of *L. monocytogenes* cold stress genes during cold shock and cold growth. *Applied and Environmental Microbiology* **73** 6019–6029.

Conte M O, Petrone G, Di Biase A M, Ammendolia M G, Superti F and Seganti L (2000) Acid tolerance in *Listeria monocytogenes* influences invasiveness of enterocyte-like cells and macrophages-like cells. *Microbial Pathogenesis* **29** 137–144.

Cossart P and Toledo-Arana A (2008) *Listeria monocytogenes*, a unique model in infection biology: an overview. *Microbiology Infection* **10** 1041–1050.

Datta A R and Kothary M H (1993) Effect of glucose, growth temperature, and pH on listeriolysisn O production in *Listeria monocytogenes*. *Applied and Environmental Microbiology* **59** 3495–3497.

Duodu S, Jensen A, Skjerdal T, Cappelier J, Pilet M and Loncarevic S (2010) Influence of storage temperature on gene expression and

virulence potential of *Listeria monocytogenes* strains grown in a salmon matrix. *Food Microbiology* **27** 795–801.

Gardan R, Cossart P and Labadie J (2003) The European Listeria Genome Consortium, Identification of *Listeria monocytogenes* genes involved in salt and alkaline-pH tolerance. *Applied and Environmental Microbiology* **69** 3137–3143.

Jacquet C, Gouin E, Jeannel D, Cossart P and Rocourt J (2002) Expression of ActA, Ami, InlB and lysteriolysin O in *Listeria monocytogenes* of human and food origin. *Applied and Environmental Microbiology* **68** 616–622.

Johansson J, Mandin P, Renzoni A, Chiaruttini C, Springer M and Cossart P (2002) An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. *Cell* **110** 551–561.

Kathariou S (2002) *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *Journal of Food Protection* **65** 1811–1829.

Leimeister-Wachter M, Domann E and Chakraborty T (1992) The expression of virulence genes in *Listeria monocytogenes* is thermoregulated. *Journal of Bacteriology* **174** 947–952.

Liu Y and Ream A (2008) Gene expression profiling of *Listeria monocytogenes* strain F2365 during growth in ultrahigh-temperature-processed skim milk. *Applied and Environmental Microbiology* **74** 6859–6866.

Liu S, Graham J E, Bigelow L, Morse P D and Wilkinson B J (2002) Identification of *Listeria monocytogenes* genes expressed in response to growth at low temperature. *Applied and Environmental Microbiology* **68** 1697–1705.

Liu D, Lawrence M, Ainsworth J and Austin F (2007) Toward an improved laboratory definition of *Listeria monocytogenes* virulence. Review. *International Journal of Food Microbiology* **118** 101–115.

Livak K J and Schmittgen T D (2001) Analysis of relative gene expression using real-time quantitative PCR and the 2 (-Delta Delta C(T)) method. *Methods* **25** 402–408.

McGann P, Wiedmann M and Boor K J (2007) The alternative sigma factor σB and the virulence gene regulator prfA both regulate transcription of *Listeria monocytogenes* internalins. *Applied and Environmental Microbiology* **73** 2919–2930.

Miner M D, Port G C and Greitag N E (2007) Regulation of *Listeria monocytogenes* virulence genes. In: *Listeria monocytogenes pathogenesis and host responses*, pp. 139–158. Golfine H, Shen H, eds. New York, USA: Springer.

O'Driscoll B, Gahan C G M and Hill C (1996) Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid tolerant mutant which demonsstrates increased virulence. *Applied and Environmental Microbiology* **62** 1693–1698.

Olesen I, Vogensen F V and Jespersen L (2009) Gene transcription and virulence potential of *Listeria monocytogenes* strains after exposure to acidic and NaCl stress. *Foodborne Pathogens and Disease* **6** 669–680.

Olesen I, Thorsen L and Jespersen L (2010) Relative transcription of *Listeria monocytogenes* virulence genes in liver pâtés with varying NaCl content. *International Journal of Food Microbiology* **31** 60–68.

Rantsiou K, Alessandria V, Urso R, Dolci P and Cocolin L (2008) Detection, quantification and vitality of *Listeria monocytogenes* in food as determined by quantitative PCR. *International Journal of Food Microbiology* **121** 99–105.

Roche S M, Kerouanton A, Minet J, Le Monnier A, Brisabois A and Velge P (2009) Prevalence of low-virulence *Listeria monocytogenes* strains from different foods and envrironments. *International Journal of Food Microbiology* **130** 151–155.

Sauders B D and Wiedmann M (2008) Ecology of *Listeria monocytogenes* in Natural Environment. In *Listeria, listeriosis, and Food Safety 3rd*, pp 21–54. Ryser E T, Marth E H, eds. New York: CRC press.

Schuchat A, Swaminathan B and Broome C (1991) Epidemiology of human listerosis. *Clinical Microbiology Reviews* **4** 169–183.

Werbrouck H, Grijspeerdt K, Botteldoorn N, Van PE, Rijpens N, Van D J, Uyttendaele M, Herman L and Van C E (2006) Differential inlA and inlB expression and interaction with human intestinal and liver cells by *Listeria monocytogenes* strains of different origins. *Applied and Environmental Microbiology* **72** 3862–3871.