**Expression of virulence genes of Listeria monocytogenes in food**

**Expression of virulence genes of *Listeria monocytogenes* in food**

Kalliopi Rantsiou[1]*, Marios Mataragas[2], Valentina Alessandria[1] and Luca Cocolin[1]

[1]Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali, Section of Agricultural Microbiology and Food Technology, Facoltà di Agraria, Università di Torino, Italy

[2]Department of Food Science and Technology, Laboratory of Food Quality Control and Hygiene, Agricultural University of Athens, Greece

Short title: *Listeria monocytogenes* virulence in food

*Author for correspondence

Di.Va.P.R.A., Università di Torino, Via Leonardo Da Vinci 44, Grugliasco, 10095 Turin, Italy. Tel.: 0039-011-6708553, Fax: 0039-011-6708549, Email: kalliopi.rantsiou@unito.it

Potential reviewers:

- Sophia Katariou, sophia_kathariou@ncsu.edu
- Steven Knabel, sjk9@psu.edu

**Abstract**

In the present study, the development of Reverse Transcription (RT)-qPCR protocols for three virulence genes (*plcA*, *iap* and *hly*) and the *sigB* stress response gene that may be applied for the determination of the virulence gene expression of *L. monocytogenes in situ* was reported. When applied to *L. monocytogenes* strains of different origin (#3, isolated from meat; NCTC 10527, a collection strain; and #162, isolated from cheese) and to four different food matrices (fermented sausage, soft cheese, UHT milk and minced meat), stored at 4 and 12°C, significant heterogeneity in their expression was recorded. By statistical analysis of the data, it was possible to determine a significant effect on the gene expression by the food matrix, especially for the strain #162. This work showed that environmental factors may influence the virulence expression of *L. monocytogenes* providing better insights to the physiology of the microorganism grown in foods.

**Practical applications**

The ability to rapidly determine the virulence potential of *Listeria monocytogenes* in food is a challenge. This study adds new information regarding the heterogeneity within this species. Moreover, it highlights the need to better understand the influence of the different food matrices on the expression of virulence and stress response genes in *L. monocytogenes*. The Reverse Transcription-quantitative PCR approach developed and exploited in this study has the potentials to be applied in the field of food safety in order to provide additional insights regarding the virulence of *L. monocytogenes*. Such information could be useful in implementing control strategies.

Key words: *Listeria monocytogenes*, virulence genes, expression, qPCR, statistical analysis

## 1. Introduction

Although *Listeria monocytogenes* food contamination rate is low, the mortality rate, caused by this microorganism is high, and as a result, it is placed on the top of the list of pathogens of concern for the public health and consequently for the food industry (Kathariou 2002). Nowadays, the changes in food processing/storage/distribution conditions, with significant expansion of the shelf-life of foods under refrigerated conditions, together with the consumer demand for ready-to-eat foods, revealed *L. monocytogenes* as an important foodborne pathogen causing severe problems. The response of the scientific community to this foodborne pathogen has been immediate and a wealth of information, regarding its physiology, ecology and molecular biology has been obtained. Of course, the molecular mechanism of virulence has attracted a lot of attention and many steps of this complex process that results in infection have been elucidated to various levels (Vazquez-Boland *et al*. 2001).

The major virulence genetic locus that has been identified is the *prfA* virulence gene cluster, consisting of the following genes: *prfA*, encoding a transcriptional regulator, *plcA* and *plcB*, encoding two phospholipases, *hly*, encoding for a hemolysin required for the lysis of host phagosomes, *mpl*, encoding for a metalloprotease involved in extracellular activation of *plcB*, and *actA*, encoding for a surface protein responsible for actin-based motility and cell-to-cell spread. In addition, various genes, spread around the genome of *L. monocytogenes*, contribute to the infection potential of the various strains (Vazquez-Boland *et al.* 2001). The *iap* gene, encoding for an invasion associated protein, has been extensively studied and is known to be an important virulence factor (Wuenscher *et al.* 1993). Apart from the *prfA*, encoding for the virulence gene(s) transcriptional activator, also *sigB*, encoding for an alternative sigma factor, plays a role in expression of virulence genes, either directly or

85 through interplay with the *prfA* gene (Nadon *et al.* 2002; Kazmierczak *et al.* 2003;
86 Chaturongakul and Boor 2006).
87 All *L. monocytogenes* strains found in foods are considered to be pathogenic;
88 however, the relative virulence of individual *L. monocytogenes* isolates can vary substantially
89 in selected animal models. The genetic basis underlying these virulence differences is not yet
90 understood. Differences in gene content exist between strains of different serovars and
91 origins. Some of these differences may be implicated in the various disease potentials of *L.*
92 *monocytogenes* strains. However, differences among strains may also be due to different gene
93 expression/regulation of the core genes of *L. monocytogenes* (Severino *et al.* 2007).
94 The ability to rapidly determine the pathogenic potential of *L. monocytogenes* strains
95 is integral to the control and prevention campaign against listeriosis (Liu *et al.* 2007).
96 Throughout the years, different approaches have been employed to assess virulence: *in vivo*
97 bioassays, *in vitro* cell assays and targeting virulence-associated proteins and genes (Liu *et al.*
98 2007). The main disadvantages of the first two are the need for laboratory animals (bioassays)
99 and the time constraints. On the other hand, targeting virulence genes gives only an indication
100 of the virulence potential of strains. Nowadays, powerful alternatives are available that can
101 assist in the definition of the virulence potential of *L. monocyotogenes* strains. Reverse
102 Transcription - quantitative PCR (RT-qPCR) is currently used as an accurate method to
103 determine changes in gene expression.
104 In this study, we employed RT-qPCR to investigate the expression profile of a stress
105 response gene (*sigB*) and three virulence genes (*plcA, iap* and *hly*), in three *L. monocytogenes*
106 strains of different origin. Our purpose was to understand whether any changes in the
107 virulence potential can be detected for the different *L. monocytogenes* strains when found in a
108 food matrix and incubated at common (4 ºC) or abuse (12 ºC) refrigeration conditions.
109

## 2. Materials and Methods

### *2.1 Bacterial strains*

For the gene expression experiments, three different *L. monocytogenes* strains were used: NCTC 10527 belonging to serotype 4b, strain #3, previously isolated from fermented sausage and belonging to serotype 3c and strain #162, previously isolated from a raw milk cheese and belonging to serotype 4c. For the determination of the specificity of the protocols, a panel of bacterial strains that belonged to species commonly found in foods was used. In addition, strains that belonged to all the *Listeria* species and different serotypes of *L. monocytogenes* were included. The bacterial strains used in the study were obtained from culture collections and were previously isolated in our laboratory (Cocolin *et al.* 2002; Cocolin *et al.* 2005).

### *2.2 Primer and probe design*

Using publicly available sequences of the virulence genes of interest in *L. monocytogenes* and other microorganisms, and the CLUSTALW (www.ebi.ac.uk/tools/clustalw2/index.html) and Amplify software (University of Wisconsin, USA) programs, we designed sets of primers and probes that theoretically were specific for *L. monocytogenes*. The sequences are reported in Table 1.

### *2.3 Optimization of quantitative PCR*

The optimization of the qPCR protocols (temperature and time of annealing and concentration of the primers and probes) for the different virulence genes was carried out using as a template DNA extracted from pure cultures of all the bacterial strains as described by Cocolin *et al.* (2005). DNA, after extraction, was quantified by measuring the absorbance at 260 nm using the NanoDrop instrument (Celbio, Milan, Italy) and diluted to 100 ng/$\mu$L.

135 The qPCR reactions were carried out using the FluoMix for probe kit from Euroclone
136 (Celbio) and the Chromo4 RealTime PCR Detection System (BioRad, Milan, Italy). The
137 optimized reaction mix (25 $\mu$L), using the TaqMan probes contained the following: 12.5 $\mu$L
138 of the 2X reaction mix, 400 nM of each primer, 250 nM of the probe and 100 ng of DNA.
139 The cycle for the gene *hly* was: 95°C for 30 s, 54°C for 30 s and 72°C for 30 s, repeated 50
140 times and with an initial denaturation at 95°C for 10 min. For the genes *sigB*, *iap* and *plcA*,
141 the annealing temperature was reduced to 50°C.
142 In order to validate the conditions of qPCR amplification for the housekeeping gene
143 and the target genes, about 80 strains of different *Listeria* and non-*Listeria* species were used
144 (see paragraph 2.1).
145
146 *2.4 Expression in situ*
147 The expression of the tested genes was studied in four types of food: fermented
148 sausage, minced meat, soft cheese and UHT milk. The pH of the fermented sausage and
149 cheese was 5.3 and 5.5, while the salt content 3.2 % (w/w) and 1 % (w/w), respectively. An
150 overnight culture of the respective *L. monocytogenes* strain was used to inoculate 10 g or mL
151 of each of the above foods. The culture was centrifuged for 5 min at 14,000 *g* and the cell
152 pellet was washed twice in salted peptone water (8 g/L NaCl, 1 g/L bacteriological peptone,
153 both from Oxoid, Milan, Italy). Then, the pellet was resuspended in the same solution and
154 used to inoculate the food samples. One mL of approximately $10^9$ colony forming units
155 (cfu)/mL was mixed into every sample and they were then incubated at 4 or 12°C for 48 h.
156 After the incubation, 40 mL of salt peptone water were added in the sample and mixing for 2
157 min was carried out. *L. monocytogenes* was counted in Palcam agar (Oxoid) and the total
158 RNA was extracted for further analysis. The 16S-23S rRNA intergenic spacer (IGS) region
159 (Table 1), used in a previous study for the detection and quantification of *L. monocytogenes*

160 in foods (Rantsiou et al., 2008), was used as housekeeping reference or 'normalizer' (gene
161 with constant expression in all samples and whose expression is not changed by the treatment
162 under investigation). In relative expression studies, the 'normalizers' are used in order to
163 normalize the quantities obtained from RT-qPCR in such a manner that the data are
164 biologically meaningful. Duplicate experiments were carried out independently and from
165 each experiment three RNA extractions were performed.
166
167 *2.5 RNA extraction from food samples*
168 For the RNA extraction, after homogenization, food samples were left to rest for 5
169 min and 1 mL was taken from the surface. The samples were treated with 1 mL of RNA*later*
170 (Ambion, Applied Biosystems, Milan, Italy) and total nucleic acids were extracted using the
171 MasterPure$^{TM}$ Complete DNA and RNA Purification kit (Epicentre, Madison, WI, USA). The
172 DNA was digested by using 2 to 4 U of the Turbo DNase (Ambion) in a final volume of 50
173 $\mu$L incubated at 37°C for 3h. The complete digestion of the DNA was confirmed by qPCR of
174 the IGS gene before any further analysis. RNA quality was checked by agarose gel
175 electrophoresis before the reverse transcription step.
176
177 *2.6 Reverse transcriptase (RT) qPCR*
178 The reverse transcription, prior to the qPCR, was carried out with the M-MLV
179 Reverse Transcriptase (Promega, Milan, Italy) following the instructions of the manufacturer
180 and using 5 $\mu$L of extracted RNA. One microliter of cDNA was used in the qPCR assay,
181 which was carried out as described above and using the FluoMix for probe kit of Euroclone
182 (Celbio) in the Chromo4 RealTime PCR Detection System (BioRad).
183
184 *2.7 Statistical analysis*

185 Quantitative data on the expression of the target genes (*plcA*, *iap*, *sigB* and *hly*) were
186 obtained by RT-qPCR according to the number of cycles required for optimal amplification-
187 generated fluorescence, in order to achieve a specific threshold detection value (i.e. the
188 threshold cycle; $C_T$ value). The relative expression ratio of the target genes were calculated
189 according to Pfaffl (2001):

$$relative\ expression\ ratio = \frac{(E_{target})^{\Delta C_{Ttarget}(control-sample)}}{(E_{ref})^{\Delta C_{Tref}(control-sample)}}$$

190

191 Therefore, according to the above equation, the relative expression ratio of the target
192 genes was determined based on the actual PCR efficiencies ($E$) and the crossing point
193 difference ($\Delta C_T$) for a treated sample versus the control. The ($E$) values were calculated for
194 each gene from the slope of a linear regression model (Pfaffl 2001). Samples of DNA were
195 diluted (100, 50, 25, 5, 1 and 0.25 ng/µl) and used to construct the standard curves. The latter
196 were constructed by plotting Ct versus log DNA. Dilutions were done three times
197 independently and loaded in single. The slope of the standard curves was used to
198 determine the reaction efficiency ($E$) as $E = 10^{(-1/slope)}$.
199 The total expression ratio of the target genes over different temperature and substrate
200 conditions for each *L. monocytogenes* strain was tested for significance by a randomization
201 test implemented in the relative expression software tool (REST) (Pfaffl *et al.* 2002). This
202 method was employed to evaluate the statistical significance of up- or down- regulation of the
203 target genes (*plcA*, *iap*, *sigB* and *hly*) after their normalization to the reference gene (IGS).
204 Control condition was considered as the growth of *L. monocytogenes* in BHI broth at 37°C
205 for 24 h. Statistical significance was considered when the $p$-value was less than or equal to
206 0.05, i.e. $p$-value $\leq 0.05$.
207
208

## 3. Results and Discussion

Based on current knowledge, any strain of *L. monocytogenes* should be considered potentially pathogenic for humans. Nevertheless, a number of observations suggest that *L. monocytogenes* virulence is heterogeneous (Severino *et al* 2007, Liu *et al* 2007, Werbrouck *et al.* 2006). Moreover, limited information is available regarding the virulence within the main medium of transmission to humans: the food. Quantitative PCR has been used for determination of the expression levels of virulence genes *in vitro* (Sue *et al.* 2004; Chaturongakul and Boor 2006; Werbrouck *et al.* 2006; Chan *et al.* 2007; McGann *et al.* 2007; Rieu *et al.* 2007). However its application for expression studies *in situ* has been very limited.

The objective of this study was to investigate whether the expression level of the selected target genes (*plcA*, *iap*, *sigB*, and *hly*) differs between food matrices (fermented sausage, minced meat, soft cheese and UHT milk) and between different strains. The current work was carried out in order to optimize a protocol that would allow studies of virulence gene expression in food samples, under standard (4 ºC) and abuse (12 ºC) temperature conditions that resemble commercial and household practices. To determine the expression *in situ*, food matrices mostly associated with *L. monocytogenes* contamination were chosen and after 48 h of incubation, *L. monocytogenes* was counted on Palcam agar and the relative expression of the virulence genes was assessed. No changes were observed in the microbial counts (data not shown).

The calculated efficiencies for the target and reference genes *plcA*, *iap*, *sigB*, *hly* and IGS, were equal to 87.7, 99.1, 100.0, 87.1 and 93.5% respectively. The DNA concentrations ($n$ = 3) ranged from 0.25 to 100 ng/µl with the linearity being from 0.93 to 0.98 (Fig. 1). Relative expression of the target genes *plcA*, *iap*, *sigB* and *hly* of the *L. monocytogenes* strains at different temperature conditions and grown on various substrates was analyzed using the

233 REST software tool and the $C_T$-values of the reference gene (IGS) were used to normalize the
234 data.

235 Strain #3, in general, did not show significant changes relative to the target genes
236 expression irrespective of the conditions (i.e. different substrates, incubation at 4 or 12 °C).
237 This fermented sausages isolate exhibited significant ($p$-value < 0.05) increase only in the *hly*
238 gene and only in the minced meat stored at 12°C as revealed by 4.26-fold up-regulation. On
239 the contrary, the *iap* gene was significantly down-regulated (-4.67) in fermented sausage
240 stored at 12°C (Figs 2a and 2b).

241 Regarding strain NCTC 10527, the reference *L. monocytogenes* strain, only few target
242 genes displayed significant increase in their expression. The *hly* gene was up-regulated in soft
243 cheese stored at 4°C and minced meat stored at 12°C, as indicated by 6.35- and 8.53-fold up-
244 regulation, respectively. The *iap* gene was up-regulated (3.71) when the strain was grown in
245 UHT milk stored at high temperature conditions (12°C). The *plcA* gene was suppressed in
246 fermented sausage (-7.95) and in soft cheese (-2.62) at high temperature conditions (12°C)
247 (Figs 2c and 2d).

248 The cheese isolate strain #162 exhibited significant increase in gene expression,
249 especially when the strain was grown in minced meat irrespective of the temperature
250 conditions (4 or 12°C). Most of the target genes (*plcA*, *sigB* and *hly*) were up-regulated. The
251 fold change of these genes were 8.97, 5.63 and 5.90, respectively, for the minced meat stored
252 at 4°C. When the *in situ* gene expression of the strain #162 was studied in the minced meat
253 stored at 12°C the corresponding fold changes were 6.49, 4.30 and 3.79, respectively. Finally,
254 significant up-regulation of the *plcA* and *sigB* genes was observed in both soft cheese and
255 UHT milk stored at high temperature conditions (12°C). On the other hand, only the *hly* gene
256 was down-regulated (-5.49) when the strain was inoculated in UHT milk stored at 4°C (Figs
257 2e and 2f). One observation that should be pointed out is that the strain #162 showed

significant increase in its gene expression when it was grown on fresh meat, a substrate different from its origin. These findings strongly suggest the need to perform gene expression experiments is real food samples instead of standard broth systems.

Previously, Palumbo *et al.* (2005) investigated the expression of genes in *L. monocytogenes* during growth on cabbage, while Rieu *et al.* (2010) studied stress and virulence genes expression of *L. monocytogenes* inoculated on parsley leaves and treated with acetic acid. Olesen *et al.* (2010) investigated the response of *L. monocytogenes* in terms of gene expression in liver patè with different salt concentrations and lastly Duodu *et al.* (2010) determined the incubation temperature effect on the expression of 4 virulence genes (*hly*, *actA*, *inlA* and *prfA*), for two *L. monocytogenes* strains inoculated in salmon. The general trend that emerged from these studies is that relative transcription of certain virulence genes is higher in laboratory broths compared to real food matrices (Olesen *et al.* 2010). Furthermore, it has been suggested that a food matrix, in particular a meat based one, may influence virulence potential of *L. monocytogenes*, possibly through down-regulation of virulence genes (Mahoney and Henriksson 2003, Olesen *et al.* 2010). O'Driscoll et al (1996) showed that acid tolerant mutants of *L. monocytogenes* had increased virulence in a mouse model with respect to the wild type. This finding may suggest that acid pre-adaptation of *L. monocytogenes*, a situation that may be encountered in a food system, may enhance its virulence. Recently, a study conducted *in vitro*, investigated the effect that adaptation of *L. monocytogenes* to acidic and osmotic stress conditions has on virulence gene transcription and on its overall virulence potential (Olesen *et al.* 2009). The results, which concerned two different strains of *L. monocytogenes*, suggest that conditions that resemble those encountered in food products, may influence the virulence potential of this microorganism.

In contrast to studies conducted previously in food matrices, where an overall reduction of virulence gene expression was noted (Olesen *et al.* 2010, Rieu *et al.* 2010), here

283 we saw an increase in expression of the *plcA*, *sigB* and *hly* genes, for strain #162 when grown
284 on minced meat. Such differences could be attributed to the strains employed in the different
285 studies. For example, Rieu et al. (2010) have used a single, culture collection strain (EGDe),
286 which has been shown to repress its virulence gene expression *in situ* (Rantsiou *et al*. 2011).
287 On the other hand, apart from strain #162, the other two strains employed did not
288 significantly modify their expression levels. Only the *hly* gene was up-regulated in all strains,
289 especially when they were grown on minced meat, while the *iap* gene, in general, did not
290 display significant changes, independently of matrix or strain considered (except for an up-
291 regulation in UHT milk and a down-regulation in fermented sausage both stored at 12°C for
292 the strain #3). Overall, we observed some level of strain heterogeneity, especially for what
293 concerns the expression of *plcA* in different food matrices. The *plcA* and *hly* genes did not
294 respond in exactly the same way although they are both part of the *prfA* regulon. It should
295 however be underlined that expression of genes within this regulon is complex (Kreft and
296 Vázquez-Boland, 2001) and that *hly* can also be transcribed via a PrfA-independent promoter
297 (Domann *et al.*, 1993). The general trend regarding the *sigB* was the upergulation. In most
298 cases, no significant differences could be observed in the expression levels of *sigB* for each
299 strain in the different matrices. This implies that the main environmental factor influencing its
300 expression could be the temperature. In fact it has been demonstrated that incubation of *L.*
301 *monocytogenes* at low temperature triggers stress response through activation of the $\sigma^B$ factor
302 (Becker *et al.* 2000). The food matrices used in the different studies could play a role in the
303 expression levels obtained and this is confirmed by our results. Each of the matrices tested
304 here present stressful environmental conditions (low pH, high osmolarity) or parameters (iron
305 presence) that have been shown to influence gene expression *in vitro* (Kreft and Vázquez-
306 Boland, 2001). This study shows that *in situ*, such parameters influence in a strain-dependent
307 manner expression of genes and even though the experiments were conducted at temperatures

below 30 ˚C, virulence genes were upregulated. These results, highlight the need to further investigate gene expression of *L. monocytogenes in situ*.

## 4. Conclusions

This study confirms previously reported cases of heterogeneity in the pathogenic potential of *L. monocytogenes* strains (Jacquet *et al.* 2002; Olesen *et al.* 2010). Considering virulence gene expression, *plcA* and *iap* showed variability and no general trend could describe the response of all three strains tested in the four food matrices. On the other hand, gene *hly*, responsible for the synthesis of listeriolysin, was upregulated in almost all cases and significant differences could be identified in the levels of upregulation, between different matrices. This result suggests that *hly* is expressed and that specific food matrices (in this case meat) favor more than others (milk, cheese and fermented sausage) such expression. Finally, the behavior of *sigB* gene confirmed its role in stress response. Apparently temperature is a strong signal that results in upregulation of this gene.

Although only three strains were used, of which two were wild isolates, in concentrations that are hardly found in naturally contaminated samples, in our opinion this study is an important contribution to the comprehension of the physiology and the virulence of the microorganism in foods.

## Acknowledgements

This study was funded by the European Commission, within the VI Framework Program, contract no 007081, 'Pathogen Combat: Control and prevention of emerging and future pathogens at cellular and molecular level throughout the food chain'.

We thank Prof. Tiziana Civera, Faculty of Veterinary Medicine, University of Turin, Italy, for kindly providing us with the two *L. monocytogenes* food isolates used in this study.

334 **References**

335 BECKER, L.A., EVANS, S.N., HUTKINS, R.W. and BENSON, A.K. 2000. Role of $\sigma^{\beta}$ in
336 adaptation of *Listeria monocytogenes* to growth at low temperature. *J. Bacteriol.* **182,** 7083-
337 7087.

338 CHAN, Y.C., BOOR, K.J. and WIEDMANN, M. 2007. $\sigma^{B}$-dependent and $\sigma^{B}$-independent
339 mechanisms contribute to transcription of *Listeria monocytogenes* cold stress genes during
340 cold shock and cold growth. *Appl. Environ. Microbiol.* **73**, 6019-6029.

341 CHATURONGAKUL, S. and BOOR, K.J. 2006. $\sigma^{B}$ activation under environmental and
342 energy stress conditions in *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **72**, 5197-5203.

343 COCOLIN, L., RANTSIOU, K., IACUMIN, L., CANTONI, C. and COMI, G. 2002. Direct
344 identification in food samples of *Listeria* spp. and *Listeria monocytogenes* by molecular
345 methods. *Appl. Environ. Microbiol.* **68**, 6273-6282.

346 COCOLIN, L., STELLA, S., NAPPI, R., BOZZETTA, E., CANTONI, C. and COMI, G.
347 2005. Analysis of PCR-based methods for characterization of *Listeria monocytogenes* strains
348 isolated from different sources. *Int. J. Food Microbiol.* **103**, 167-178.

349 DOMANN, E., WEHLAND, J., NIEBHUR, K., HAFFNER, C., LEIMEISTER-WÄCHTER,
350 M. and CHAKRABORTY, T. 1993. Detection of a *prfA*-independent promoter responsible
351 for Listeriolysin gene expression in mutant *Listeria monocytogenes* strains lacking PrfA
352 regulator. *Infect. Immun.* **61**, 3073-3075.

353 DUODU, S., HOLST-JENSEN, A., SKJERDAL, T., CAPPELIER J.M., PILET, M.F. and
354 LONCAREVIC, S. 2010. Influence of storage temperature on gene expression and virulence
355 potential of *Listeria monocytogenes* strains grown in salmon matrix. *Food Microbiol.* **27**,
356 795-801.

357 JACQUET, C., GOUIN, E., JEANNEL, D., COSSART, P. and ROCOURT, J. 2002.
358 Expression of ActA, Ami, InlB and listeriolysin O in *Listeria monocytogenes* of human and
359 food origin. *Appl. Environ. Microbiol.* **68**, 616-622.
360 KATHARIOU, S. 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety
361 perspective. *J. Food Prot.* **65**, 1811-1829.
362 KAZMIERCZAK, M.J., MITHOE, S.C., BOOR, K.J. and WIEDMANN, M. 2003. *Listeria*
363 *monocytogenes* $\sigma^B$ regulates stress response and virulence functions. *J. Bacteriol.* **185**, 5722-
364 5734.
365 KREFT, J. and VASQUEZ-BOLAND, J.A. 2001. Regulation of virulence genes in *Listeria*.
366 *Int. J. Med. Microbiol.* **291**, 145-157.
367 LIU, D., LAWRENCE, M.L., AINSWORTH, A.J. and AUSTIN F.W. 2007. Toward an
368 improved laboratory definition of *Listeria monocytogenes* virulence. *Int. J. Food Microbiol.*
369 **118**, 101-115.
370 MAHONEY, M. and HENRIKSSON, A. 2003. The effect of processed meat and meat starter
371 cultures on gastrointestinal colonization and virulence of *Listeria monocytogenes* in mice. *Int.*
372 *J. Food Microbiol*. **84**, 255-261.
373 MCGANN, P., WIEDMANN, M. and BOOR, K.J. 2007. The alternative sigma factor $\sigma^B$ and
374 the virulence gene regulator PrfA both regulate transcription of *Listeria monocytogenes*
375 internalins. *Appl. Environ. Microbiol*. **73**, 2919-2930.
376 NADON, C., BOWEN, B.M., WIEDMANN, M. and BOOR K.J. 2002. $\sigma^B$ contributes to
377 PrfA-mediated virulence in *Listeria monocytogenes*. *Infect. Immun*. **70**, 3948-3952.
378 O'DRISCOLL, B., GAHAN, C.G. AND HILL, C. 1996. Adaptive acid tolerance response in
379 *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased
380 virulence. *Appl. Envir. Microbiol.* **62**, 1693-1698.

381 OLESEN, I., THORSEN, L. and JESPERSEN, L. 2010. Relative transcription of *Listeria*
382 *monocytogenes* virulence genes in liver pâtés with varying NaCl content. *Int. J. Food*
383 *Microbiol*. ***141***, S60-S68.
384 OLESEN, I., VOGENSEN, F.K. and JESPERSEN, L. 2009. Gene transcription and virulence
385 potential of *Listeria monocytogenes* strains after exposure to acidic and NaCl stress.
386 *Foodborne Path. Dis*. **6**, 669-680.
387 PFAFFL, M.W. 2001. A new mathematical model for relative quantification in real-time RT-
388 PCR. *Nucl. Ac. Res.* **29**, 1-6.
389 PFAFFL, M.W., HORGAN, G.W. and DEMPFLE, L. 2002. Relative expression software
390 tool (REST) for group-wise comparison and statistical analysis of relative expression results
391 in real-time PCR. *Nucl. Ac. Res.* **30**, 1-10.
392 PALUMBO, J.D., KANEKO, A., NGUYEN, K.D. and GORSKI, L. 2005. Identification of
393 genes induced in *Listeria monocytogenes* during growth and attachment to cut cabbage using
394 differential display. *Appl. Environ. Microbiol*. **71**, 5236-5243.
395 RANTSIOU, K., ALESSANDRIA, V., URSO, R., DOLCI, P. and COCOLIN, L. 2008.
396 Detection, quantification and vitality of *Listeria monocytogenes* in food as determined by
397 quantitative PCR. *Int. J. Food Microbiol.* **121**, 99-105.
398 RANTSIOU, K., GREPPI, A., GAROSI, M., ACQUADRO, A., MATARAGAS, M. and
399 COCOLIN, L. 2011. Strain dependent expression of stress response and virulence genes of
400 *Listeria monocytogenes* in meat juices as determined by microarray. *Int. J. Food Microbiol.* ,
401 doi: 10.1016/j.ijfoodmicro.2011.08.009.
402 RIEU, A., GUZZO, J. and PIVETEAU, P. 2010. Sensitivity to acetic acid, ability to colonize
403 abiotic surfaces and virulence potential of *Listeria monocytogenes* EGD-e after incubation on
404 parsley leaves. *J. Appl. Microbiol.* **108**, 560-570.

RIEU, A., WEIDMANN, S., GARMYN, D., PIVETEAU, P. and GUZZO, J. 2007. *agr* system of *Listeria monocytogenes* EGD-e: role in adherence and differential expression pattern. *Appl. Environ. Microbiol*. **73**, 6125-6133.

SEVERINO, P., DUSSURGET, O., VÊNCIO, R.Z.N., DUMAS, E., GARRIDO, P., PADILLA, G., PIVETEAU, P., LEMAÎTRE, J.P., KUNST, F., GLASER, P. and BUCHRIESER, C. 2007. Comparative transcriptome analysis of *Listeria monocytogenes* strains of the two major lineages reveals differences in virulence, cell wall and stress response. *Appl. Environ. Microbiol*. **73**, 6078-6088.

SUE, D., FINK, D., WIEDMANN, M. and BOOR, K.J. 2004. $\sigma^B$-dependent gene induction and expression in *Listeria monocytogenes* during osmotic and acid stress conditions simulating the intestinal environment. *Microbiol.* **150**, 3843-3855.

VAZQUEZ-BOLAND, J.A., KUHN, M., BERCHE, B., CHAKRABORTY, T., DOMINGUEZ-BERNAL, G., GOEBEL, W., GONZALEZ-ZORN, B., WEHLAND, J. and KREFT, J. 2001. *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* **14**, 1-57.

WERBROUCK, H., GRIJSPEERDT, K., BOTTELDOORN, N., VAN PAMEL, E., RIJPENS, N., VAN DAMME, J., UYTTENDAELE, M., HERMAN, L. and VAN COILLIE, E. 2006. Differential *inlA* and *inlB* expression and interaction with human intestinal and liver cells by *Listeria monocytogenes* strains of different origins. *Appl. Environ. Microbiol*. **72**, 3862-3871.

WUENSCHER, M.D., KÖHLER, S., BUBERT, A., GERIKE, U. AND GOEBEL, W. 1993. The *iap* gene of *Listeria monocytogenes* is essential for cell viability, and its gene product, p60, has bacteriolytic activity.*J. Bacteriol.* **175**, 3491 - 3501

**Figure Legends**

**Figure 1.** Standard curves of target and reference genes used to calculate the efficiency values. Efficiency values were derived from the slope of the regression line $C_T$-values = f(log DNA). Each data point represents the average ± standard deviation of 3 replications (n = 3). (♦) *plcA* ($y$ = -3.6566 + 24.541x; $E$ = 87.7%), (■) *iap* ($y$ = -3.3440 + 23.615x; $E$ = 91.1%), (▲) *sigB* ($y$ = -3.3213 + 27.687x; $E$ = 100.0%), (●) *hly* ($y$ = -3.6753 + 24.413x; $E$ = 87.1%) and (×) IGS ($y$ = -3.4889 + 23.241x; $E$ = 93.5%).

**Figure 2.** *In situ* relative expression of the four virulence genes (*plcA*, *iap*, *sigB* and *hly*), normalized to the reference gene (IGS), in four different food matrices (S, fermented sausage; C, soft cheese; ML, UHT milk; MT, minced meat) for the *L. monocytogenes* strain #3 (a, 4°C; and b, 12°C), *L. monocytogenes* strain NCTC 10527 (c, 4°C; and d, 12°C) and *L. monocytogenes* strain #162 (e, 4°C; and f, 12°C). Gene expressions marked by an asterisk symbol are significant at $p$-value 0.05. Gradient grey (▤), *plcA*; white (☐), *iap*; dark grey (■), *sigB*; and light grey (▢), *hly*. The bars presented in the figure represent the standard deviations.

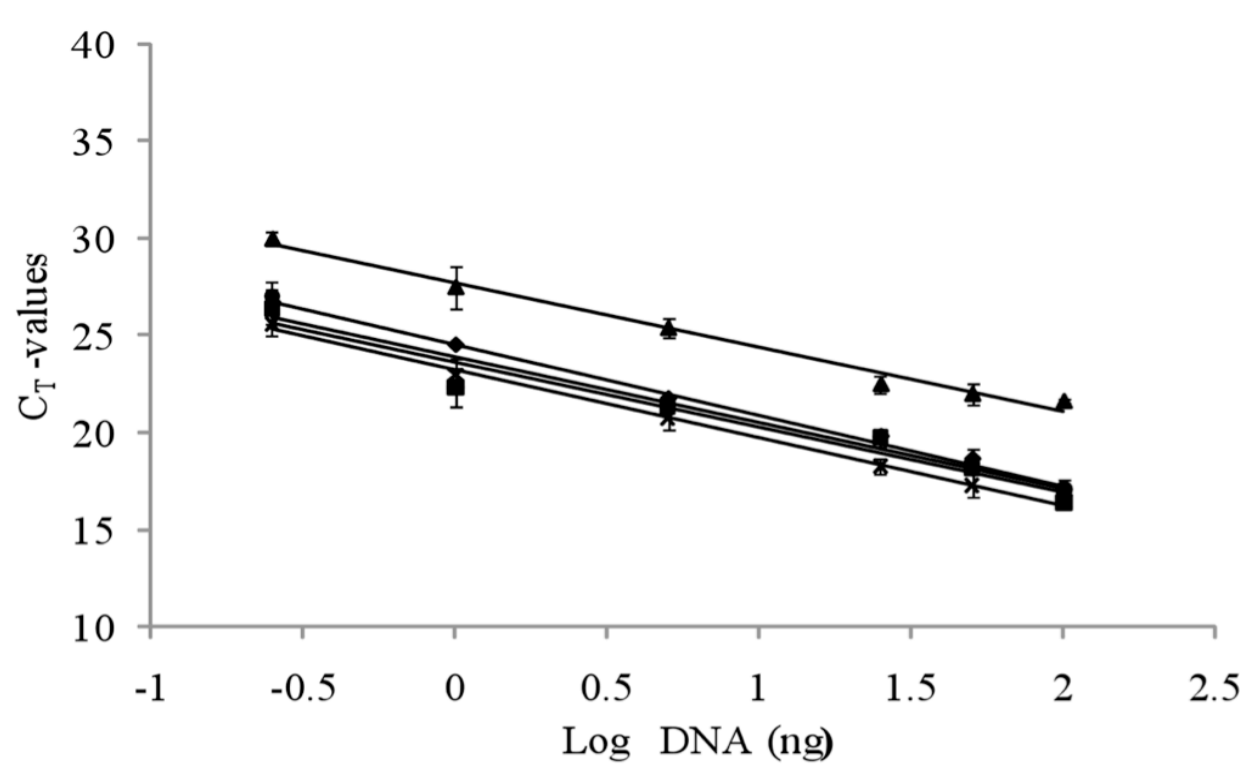
449 **Table 1.** Primers and probes used in this study.

| Target gene | Name | Sequence (5´-3´) |
|---|---|---|
| *hly* | hly_f | TACATTAGTGGAAAGATGG |
| | hly_r | ACATTCAAGCTATTATTTACA |
| | hly_probe | HEX-GAAAAATATGCTCAAGCTTATCCAAATG-Tamra |
| *sigB* | sigB_f | CCAAGAAAATGGCGATCAAGAC |
| | sigB_r | CGTTGCATCATATCTTCTAATAGCT |
| | sigB_probe | HEX-TGTTCATTACAAAAACCTAGTAGAGTCCAT-Tamra |
| *iap* | iap_f | ACAATACTAATACACCATCTAA |
| | iap_r | GAGCTTCAGCAATAATAGC |
| | iap_probe | HEX-ATGCTAATCAAGGTTCTTCCAACAATAACAG-Tamra |
| *plcA* | plcA_f | CTAGAAGCAGGAATACGGTACA |
| | plcA_r | ATTGAGTAATCGTTTCTAAT |
| | plcA_probe | HEX-AATTTATTTAAATGCATCACTTTCAGGT-Tamra |
| IGS | IGS1 | GGCCTATAGCTCAGCTGGTTA |
| | IGS2 | GCTGAGCTAAGGCCCCGTAAA |
| | probe IGS | FAM-ATAAGAAATACAAATAATCATACCCTTTTAC-Tamra |

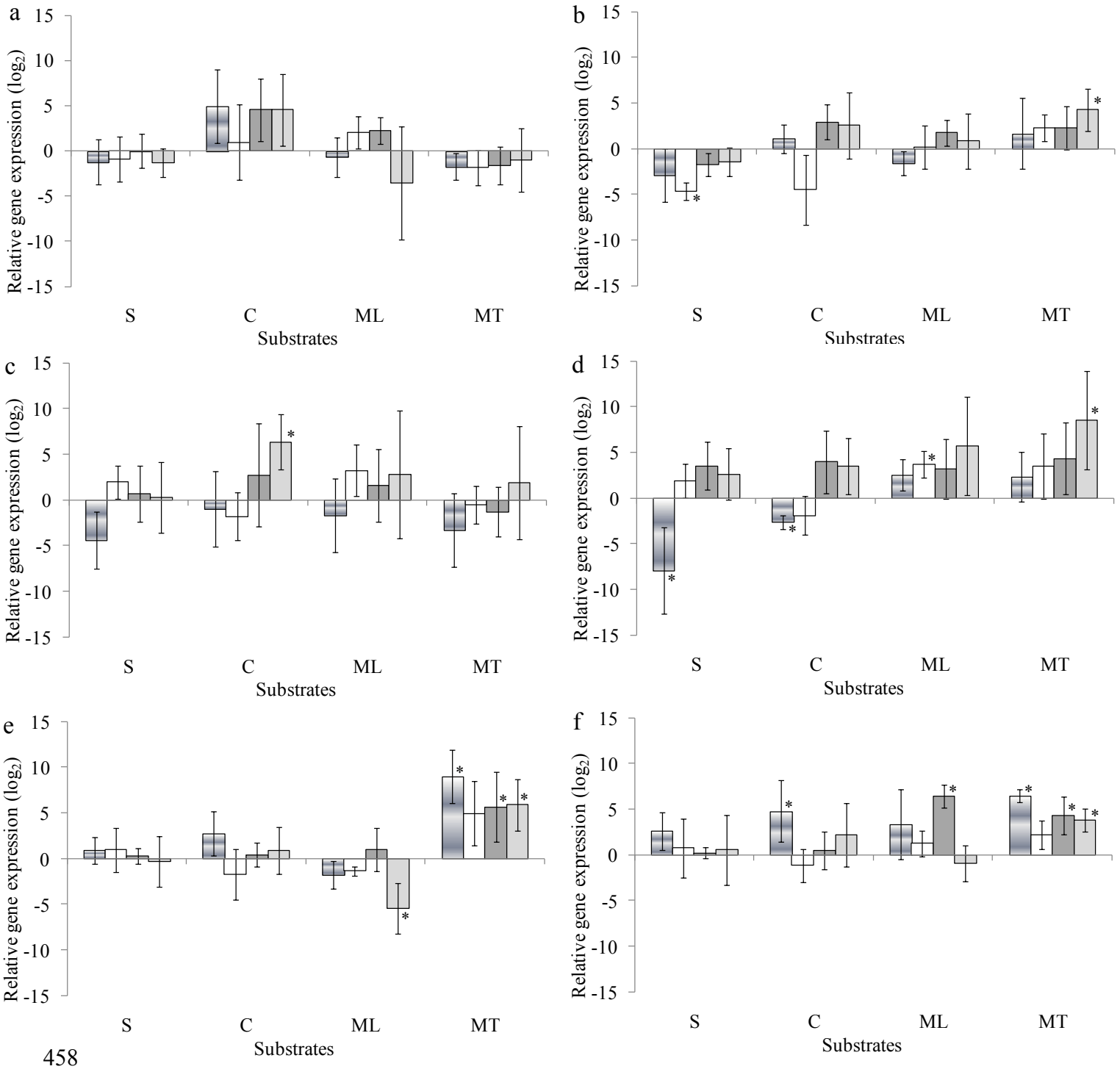
450

451

**Figure 1.**



456 **Figure 2.**
457




458

459