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Expression of virulence genes of *Listeria monocytogenes* in food

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Short title: *Listeria monocytogenes* virulence in food

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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
through interplay with the prfA gene (Nadon et al. 2002; Kazmierczak et al. 2003; Chaturongakul and Boor 2006).

All L. monocytogenes strains found in foods are considered to be pathogenic; however, the relative virulence of individual L. monocytogenes isolates can vary substantially in selected animal models. The genetic basis underlying these virulence differences is not yet understood. Differences in gene content exist between strains of different serovars and origins. Some of these differences may be implicated in the various disease potentials of L. monocytogenes strains. However, differences among strains may also be due to different gene expression/regulation of the core genes of L. monocytogenes (Severino et al. 2007).

The ability to rapidly determine the pathogenic potential of L. monocytogenes strains is integral to the control and prevention campaign against listeriosis (Liu et al. 2007). Throughout the years, different approaches have been employed to assess virulence: in vivo bioassays, in vitro cell assays and targeting virulence-associated proteins and genes (Liu et al. 2007). The main disadvantages of the first two are the need for laboratory animals (bioassays) and the time constraints. On the other hand, targeting virulence genes gives only an indication of the virulence potential of strains. Nowadays, powerful alternatives are available that can assist in the definition of the virulence potential of L. monocytogenes strains. Reverse Transcription - quantitative PCR (RT-qPCR) is currently used as an accurate method to determine changes in gene expression.

In this study, we employed RT-qPCR to investigate the expression profile of a stress response gene (sigB) and three virulence genes (plcA, iap and hly), in three L. monocytogenes strains of different origin. Our purpose was to understand whether any changes in the virulence potential can be detected for the different L. monocytogenes strains when found in a food matrix and incubated at common (4 ºC) or abuse (12 ºC) refrigeration conditions.
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/µL.
The qPCR reactions were carried out using the FluoMix for probe kit from Euroclone (Celbio) and the Chromo4 RealTime PCR Detection System (BioRad, Milan, Italy). The optimized reaction mix (25 µL), using the TaqMan probes contained the following: 12.5 µL of the 2X reaction mix, 400 nM of each primer, 250 nM of the probe and 100 ng of DNA.

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 times and with an initial denaturation at 95°C for 10 min. For the genes sigB, iap and plcA, the annealing temperature was reduced to 50°C.

In order to validate the conditions of qPCR amplification for the housekeeping gene and the target genes, about 80 strains of different Listeria and non-Listeria species were used (see paragraph 2.1).

2.4 Expression in situ

The expression of the tested genes was studied in four types of food: fermented sausage, minced meat, soft cheese and UHT milk. The pH of the fermented sausage and cheese was 5.3 and 5.5, while the salt content 3.2 % (w/w) and 1 % (w/w), respectively. An overnight culture of the respective L. monocytogenes strain was used to inoculate 10 g or mL of each of the above foods. The culture was centrifuged for 5 min at 14,000 g and the cell pellet was washed twice in salted peptone water (8 g/L NaCl, 1 g/L bacteriological peptone, both from Oxoid, Milan, Italy). Then, the pellet was resuspended in the same solution and used to inoculate the food samples. One mL of approximately 10⁹ colony forming units (cfu)/mL was mixed into every sample and they were then incubated at 4 or 12°C for 48 h.

After the incubation, 40 mL of salt peptone water were added in the sample and mixing for 2 min was carried out. L. monocytogenes was counted in Palcam agar (Oxoid) and the total RNA was extracted for further analysis. The 16S-23S rRNA intergenic spacer (IGS) region (Table 1), used in a previous study for the detection and quantification of L. monocytogenes...
in foods (Rantsiou et al., 2008), was used as housekeeping reference or ‘normalizer’ (gene with constant expression in all samples and whose expression is not changed by the treatment under investigation). In relative expression studies, the ‘normalizers’ are used in order to normalize the quantities obtained from RT-qPCR in such a manner that the data are biologically meaningful. Duplicate experiments were carried out independently and from each experiment three RNA extractions were performed.

2.5 RNA extraction from food samples

For the RNA extraction, after homogenization, food samples were left to rest for 5 min and 1 mL was taken from the surface. The samples were treated with 1 mL of RNAlater (Ambion, Applied Biosystems, Milan, Italy) and total nucleic acids were extracted using the MasterPure™ Complete DNA and RNA Purification kit (Epicentre, Madison, WI, USA). The DNA was digested by using 2 to 4 U of the Turbo DNase (Ambion) in a final volume of 50 µL incubated at 37°C for 3h. The complete digestion of the DNA was confirmed by qPCR of the IGS gene before any further analysis. RNA quality was checked by agarose gel electrophoresis before the reverse transcription step.

2.6 Reverse transcriptase (RT) qPCR

The reverse transcription, prior to the qPCR, was carried out with the M-MLV Reverse Transcriptase (Promega, Milan, Italy) following the instructions of the manufacturer and using 5 µL of extracted RNA. One microliter of cDNA was used in the qPCR assay, which was carried out as described above and using the FluoMix for probe kit of Euroclone (Celbio) in the Chromo4 RealTime PCR Detection System (BioRad).

2.7 Statistical analysis
Quantitative data on the expression of the target genes (\textit{plcA, iap, sigB and hly}) were obtained by RT-qPCR according to the number of cycles required for optimal amplification-generated fluorescence, in order to achieve a specific threshold detection value (i.e. the threshold cycle; \textit{C\textsubscript{T}} value). The relative expression ratio of the target genes were calculated according to Pfaffl (2001):

$$\text{relative expression ratio} = \frac{(E\textsubscript{target})^{\Delta C\textsubscript{T target} (control-sample)}}{(E\textsubscript{ref})^{\Delta C\textsubscript{T ref} (control-sample)}}$$

Therefore, according to the above equation, the relative expression ratio of the target genes was determined based on the actual PCR efficiencies (\textit{E}) and the crossing point difference (\textit{\Delta C\textsubscript{T}}) for a treated sample versus the control. The (\textit{E}) values were calculated for each gene from the slope of a linear regression model (Pfaffl 2001). Samples of DNA were diluted (100, 50, 25, 5, 1 and 0.25 ng/µl) and used to construct the standard curves. The latter were constructed by plotting \textit{Ct} versus log DNA. Dilutions were done three times independently and loaded in single. The slope of the standard curves was used to determine the reaction efficiency (\textit{E}) as $E = 10^{-1/(\text{slope})}$.

The total expression ratio of the target genes over different temperature and substrate conditions for each \textit{L. monocytogenes} strain was tested for significance by a randomization test implemented in the relative expression software tool (REST) (Pfaffl \textit{et al.} 2002). This method was employed to evaluate the statistical significance of up- or down-regulation of the target genes (\textit{plcA, iap, sigB and hly}) after their normalization to the reference gene (IGS). Control condition was considered as the growth of \textit{L. monocytogenes} in BHI broth at 37°C for 24 h. Statistical significance was considered when the \textit{p}-value was less than or equal to 0.05, i.e. \textit{p}-value $\leq$ 0.05.
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 °C) and abuse (12 °C) 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
REST software tool and the $C_T$-values of the reference gene (IGS) were used to normalize the data.

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

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

The cheese isolate strain #162 exhibited significant increase in gene expression, especially when the strain was grown in minced meat irrespective of the temperature conditions (4 or 12°C). Most of the target genes ($plcA$, $sigB$ and $hly$) were up-regulated. The fold change of these genes were 8.97, 5.63 and 5.90, respectively, for the minced meat stored at 4°C. When the in situ gene expression of the strain #162 was studied in the minced meat stored at 12°C the corresponding fold changes were 6.49, 4.30 and 3.79, respectively. Finally, significant up-regulation of the $plcA$ and $sigB$ genes was observed in both soft cheese and UHT milk stored at high temperature conditions (12°C). On the other hand, only the $hly$ gene was down-regulated (-5.49) when the strain was inoculated in UHT milk stored at 4°C (Figs 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
we saw an increase in expression of the \textit{plcA}, \textit{sigB} and \textit{hly} genes, for strain #162 when grown on minced meat. Such differences could be attributed to the strains employed in the different studies. For example, Rieu et al. (2010) have used a single, culture collection strain (EGDe), which has been shown to repress its virulence gene expression \textit{in situ} (Rantsiou \textit{et al.} 2011). On the other hand, apart from strain #162, the other two strains employed did not significantly modify their expression levels. Only the \textit{hly} gene was up-regulated in all strains, especially when they were grown on minced meat, while the \textit{iap} gene, in general, did not display significant changes, independently of matrix or strain considered (except for an up-regulation in UHT milk and a down-regulation in fermented sausage both stored at 12°C for the strain #3). Overall, we observed some level of strain heterogeneity, especially for what concerns the expression of \textit{plcA} in different food matrices. The \textit{plcA} and \textit{hly} genes did not respond in exactly the same way although they are both part of the \textit{prfA} regulon. It should however be underlined that expression of genes within this regulon is complex (Kreft and Vázquez-Boland, 2001) and that \textit{hly} can also be transcribed via a PrfA-independent promoter (Domann \textit{et al.}, 1993). The general trend regarding the \textit{sigB} was the upregulation. In most cases, no significant differences could be observed in the expression levels of \textit{sigB} for each strain in the different matrices. This implies that the main environmental factor influencing its expression could be the temperature. In fact it has been demonstrated that incubation of \textit{L. monocytogenes} at low temperature triggers stress response through activation of the \textit{σB} factor (Becker \textit{et al.} 2000). The food matrices used in the different studies could play a role in the expression levels obtained and this is confirmed by our results. Each of the matrices tested here present stressful environmental conditions (low pH, high osmolarity) or parameters (iron presence) that have been shown to influence gene expression \textit{in vitro} (Kreft and Vázquez-Boland, 2001). This study shows that \textit{in situ}, such parameters influence in a strain-dependent 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.

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DOMINGUEZ-BERNAL, G., GOEBEL, W., GONZALEZ-ZORN, B., WEHLAND, J. and


WERBROUCK, H., GRIJSPEERDT, K., BOTTELDOORN, N., VAN PAMEL, E.,


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

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<th>Target gene</th>
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</table>
Figure 1.
Figure 2.

Relative gene expression (log_2)

Substrates

S C ML MT