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ORIGINAL ARTICLE

Adaptation of *Listeria monocytogenes* in a simulated cheese medium: effects on virulence using the *Galleria mellonella* infection model

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Significance and Impact of the Study: In this study, the impact of adaptation to low pH and salt in a cheese-based medium on *L. monocytogenes* virulence was tested using the Wax Moth *G. mellonella* model. This model allowed the differentiation of the virulence potential between the *L. monocytogenes* strains. The effect of adaptation on virulence is strain dependent. The *G. mellonella* model revealed to be a prompt method to test food-related factors on *L. monocytogenes* virulence.

Keywords
adaptation, cheese, insect model, *Listeria monocytogenes*, virulence.

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Abstract

The aim of this study was to evaluate the effect of the acid and salt adaptation in a cheese-based medium on the virulence potential of *Listeria monocytogenes* strains isolated from cheese and dairy processing environment using the *Galleria mellonella* model. Four *L. monocytogenes* strains were exposed to a cheese-based medium in conditions of induction of an acid tolerance response and osmotolerance response (pH 5·5 and 3·5% w/v NaCl) and injected in *G. mellonella* insects. The survival of insects and the *L. monocytogenes* growth kinetics in insects were evaluated. The gene expression of *hly*, *actA* and *inlA* genes was determined by real-time PCR. The adapted cells of two dairy strains showed reduced insect mortality ($P < 0{\cdot}05$) in comparison with nonadapted cells. *Listeria monocytogenes* Scott A was the least virulent, whereas the cheese isolate C882 caused the highest insect mortality, and no differences ($P > 0{\cdot}05$) was found between adapted and nonadapted cells. The gene expression results evidenced an overexpression of virulence genes in cheese-based medium, but not in simulated insect-induced conditions. Our results suggest that adaptation to low pH and salt in a cheese-based medium can affect the virulence of *L. monocytogenes*, but this effect is strain dependent.

Introduction

The Gram-positive *Listeria monocytogenes* is the etiologic agent of listeriosis, a foodborne illness, with a high mortality rate (20–30%) (Swaminathan and Gerner-Smidt 2007). Dairy products are the most implicated food, and soft cheese has special relevance, in particular those produced from raw milk. Recently, a few outbreaks related to cheese produced from pasteurized milk were also reported (Koch *et al.* 2010).

Once the pathogen is introduced in the processing environment, the food product is at risk of contamination, and *L. monocytogenes* has outstanding abilities to overcome the most hurdle treatments (Gandhi and Chikindas 2007). During cheese processing, *L. monocytogenes* is exposed to several challenges, namely low pH due to the presence of organic acids, mainly lactic acid, high salt content (15–20% (w/v) NaCl) when cheeses are immersed in brine solution and owing the presence of lactic acid

bacteria can experience oxidative stress due to the release of H_2O_2 (Zalán *et al.* 2005; Pintado *et al.* 2005). When exposed to these challenges, *L. monocytogenes* is capable, as many other bacteria, to mount tolerance responses and its acid tolerance response (ATR) and osmotolerance response (OTR) are well known (O'Driscoll *et al.* 1996; Faleiro *et al.* 2003; Adrião *et al.* 2008). However, the impact of this ability on virulence has received limited attention (O'Driscoll *et al.* 1996; Conte *et al.* 2000; Garner *et al.* 2006). *Listeria* spp. virulence is mainly evaluated using animal models (Lecuit 2007), although the murine model is well established, it is not the most appropriate due to improper interaction between the virulence factor InlA and the E-cadherin of mice (Lecuit 2007). Joyce and Gahan (2010) and Mukherjee *et al.* (2010) evaluated the larvae model *Galleria mellonella* for studying *L. monocytogenes* virulence. This model also has the advantage that larvae can be incubated at 37°C to simulate human temperature. Joyce and Gahan (2010) showed that the virulence factor listeriolysin influences the mortality of the larvae infected with *L. monocytogenes*.

This study aimed to evaluate the effect of induction of an ATR and OTR in a cheese-based medium of dairy and cheese *L. monocytogenes* isolates on virulence using the *G. mellonella* model.

Results and discussion

Effect of adaptation to low pH and salt in *Galleria mellonella* mortality is strain dependent

As others, we established pH 5·5 as an inducer pH value of ATR in *L. monocytogenes* (Davies *et al.* 1996; Faleiro *et al.* 2003), which results in acquisition of protection when exposed to more lethal low pH values or even gain protection to different stress conditions (e.g. heat, ethanol, H_2O_2) (Faleiro *et al.* 2003; Koutsoumanis *et al.* 2003), that is, a cross-protection event occurs. The previous exposure of *Listeria* cells to sublethal salt concentration (e.g. 3·5% w/v NaCl) also can result in protection when challenged with lethal salt concentrations known as OTR or also acquire protection to other stresses as observed in response to pH (Lou and Yousef 1997; Faleiro *et al.* 2003). The sublethal pH values (pH 5·5) and salt concentration (3·5% w/v NaCl) can be found in cheeses produced in Portugal (Faleiro *et al.* 2003; Pintado *et al.* 2005). The ATR and OTR in *L. monocytogenes* are strain dependent (Faleiro *et al.* 2003; Adrião *et al.* 2008) and can constitute an advantage to those strains that colonize the cheese processing environment and will pass into the final product exhibiting defence mechanisms that will contribute to improve the infectious nature of this foodborne pathogen. In this study, two *L. monocytogenes* strains isolated from dairy processing environment (A9 and T8), a cheese isolate (C882) and *L. monocytogenes* Scott A were exposed to a cheese-based medium at conditions of induction of an ATR and OTR (pH 5·5 and 3·5% w/v NaCl), and the impact of this exposure on virulence using the *G. mellonella* model was estimated.

Differences in the ability of the tested *L. monocytogenes* strains to cause the death of the Greater Wax Moth larvae were observed (Fig. 1), and the impact of a previous adaptation to low pH and sublethal salt concentration on the mortality of insects was strain dependent (Fig. 1). *Listeria monocytogenes* Scott A and A9 were the less virulent strains in contrast to C882 that caused a decrease of 50% of survival of insects after 24 h. T8 strain showed a higher virulence in comparison with Scott A and A9 but was less virulent than the C882 strain. Adapted T8 and A9 cells (pH 5·5 and 3·5% w/v NaCl) showed a lower mortality of insects ($P > 0{\cdot}05$) in comparison with the nonadapted cells (pH 7·0 no salt added) (Fig. 1). The lower decrease ($P < 0{\cdot}05$) in mortality of insects was observed after two days of injection with adapted T8 cells (56·66 $\pm$ 5·77%) in comparison with the nonadapted cells for which only 13·33 $\pm$ 5·77% of insects were alive. However, after three days, no significantly differences ($P > 0{\cdot}05$) in mortality of insects were observed between adapted and nonadapted T8 cells. A lower decrease ($P < 0{\cdot}05$) in mortality of insects injected with adapted A9 cells was also observed after two days of injection. However, the survival of insects injected with adapted A9 cells remained higher ($P < 0{\cdot}05$) in comparison with nonadapted cells until after 5 days (Fig. 1). The survival of larvae injected with PBS (control) remained at 100% until the end of the assay.

The studies of Conte *et al.* (2000, 2002) evidenced that *L. monocytogenes* acid-adapted cells were better suited to invade Caco-2 cells and to grow inside macrophages in comparison with nonadapted cells. Garner *et al.* (2006) also found that *L. monocytogenes* 10403S cells exposed to salt and acid increased the capacity to invade Caco-2 cells, but its ability to overcome the gastric stress was affected. In our study, the effect of acid and salt adaptation of *L. monocytogenes* strains in a cheese-based medium on the mortality of the Greater Wax Moth was strain dependent. The adapted cells of the dairy isolate A9 showed a reduced infectious ability in comparison with the nonadapted cells, and the adapted cells of the other dairy isolate (T8) also seemed to have a diminished infectious capacity at 48 h but recovered their mortality capacity after that. In contrast, adapted C882 and nonadapted cells were equally able to cause insect death at 24 h. C882 strain in a murine model showed the highest virulence when compared to clinical and cheese isolates (Faleiro 2000), and this behaviour was also shown in our study

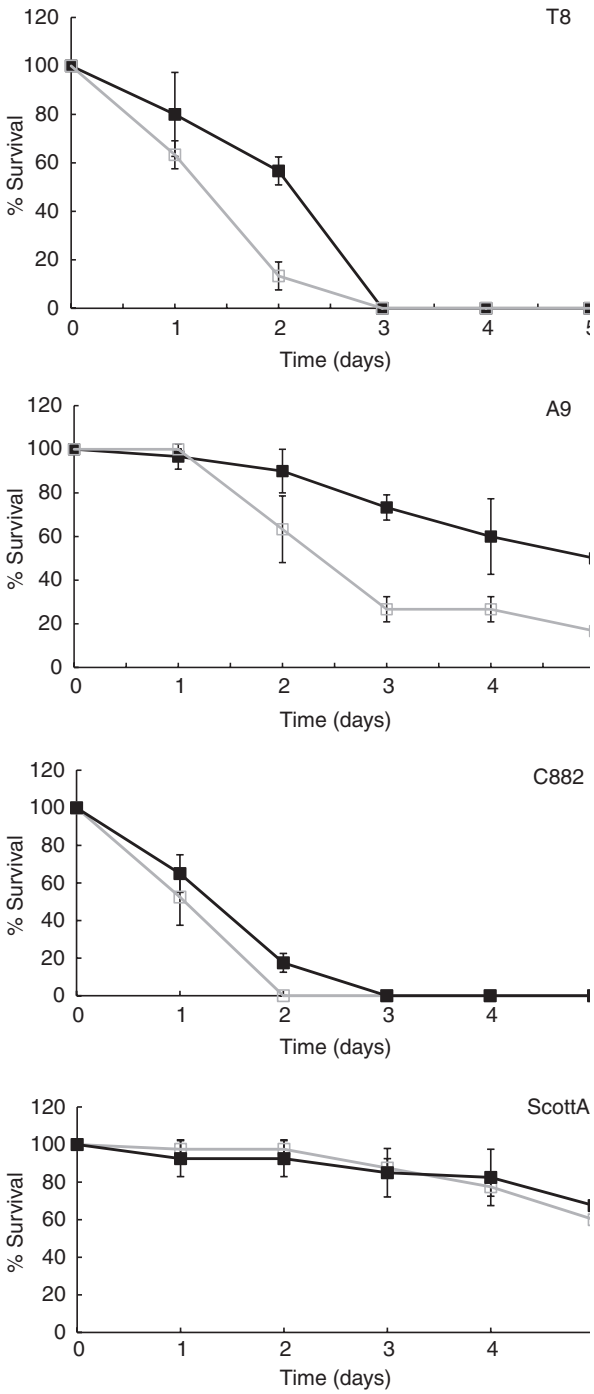

Figure 1 Survival of insects after injection with (■) adapted (pH 5·5 and 3·5% w/v NaCl) and (□) nonadapted cells (pH 7·0, no salt added) of *Listeria monocytogenes* strains in a cheese simulated medium. Data are the mean of four independent experiments. Error bars represent the standard deviation.

using the *G. mellonella* model. The Scott A strain was the least virulent, and no differences were found between adapted and nonadapted cells.

Adaptation does not influence the growth kinetics of *Listeria monocytogenes* strains in *Galleria mellonella*, but growth differs between strains

To verify whether a previous adaptation influences the listerial growth in insects, the bacterial cell numbers after infection were determined. For this, infected *G. mellonella* larvae were sacrificed, and the bacterial cells were recovered and plated in an appropriate medium to determine the bacterial numbers. No statistically differences ($P > 0{\cdot}05$) were observed between the growth of adapted and nonadapted cells postinfection (Fig. 2). Overall, the number of the *L. monocytogenes* cells had undergone a slight decrease during the first 2 h and afterwards remained stable until the end of sampling (Fig. 2). For T8 strain, no loss of viability was observed during the 24 h sampling.

Joyce and Gahan (2010) reported an initial decrease in the *Listeria* numbers after 2 h postinfection of insects remaining stable after that, and our findings of the growth kinetics of the tested *L. monocytogenes* in *G. mellonella* are analogous to those reported by Joyce and Gahan (2010). The growth kinetics of A9 strain was similar to C882, the highest virulent strain suggesting that the lowest virulence of adapted cells is more complex and not only dependent on bacterial numbers.

Induction of virulence gene expression by adaptation is strain dependent

Virulence of *L. monocytogenes* is reached by expression of well-known genes, among them are the ones that codify the internalins A and B (*inlA* and *inlB*) that are crucial in the entry host cell process. To escape from the vacuole, *Listeria* cells fall back on a pore-forming cytolysin, the listeriolysin LLO (*hly*), and with the involvement of the surface protein ActA (*actA*), the intracellular invasion can occur (Cossart and Toledo-Arana 2008). In the infection process of *L. monocytogenes* in insects, the participation of LLO occurs at two levels: one is that allows listerial growth, and the second is due to its action as a toxic factor to insects at high bacterial numbers (Joyce and Gahan 2010). To elucidate the effect of cheese-based medium adaptation in the gene expression of virulence genes that could compromise the pathogenesis of the *L. monocytogenes* strains, the gene expression of *hly*, *actA* and *inlA* genes was determined by real-time PCR.

The analysis of the relative gene expression showed that all three genes were overexpressed in *L. monocytogenes* adapted A9 and Scott A cells (pH 5·5 and 3·5% w/v NaCl) in comparison with nonadapted cells (pH 7·0 and no salt added) (Fig. 3). Adapted T8 cells overexpressed

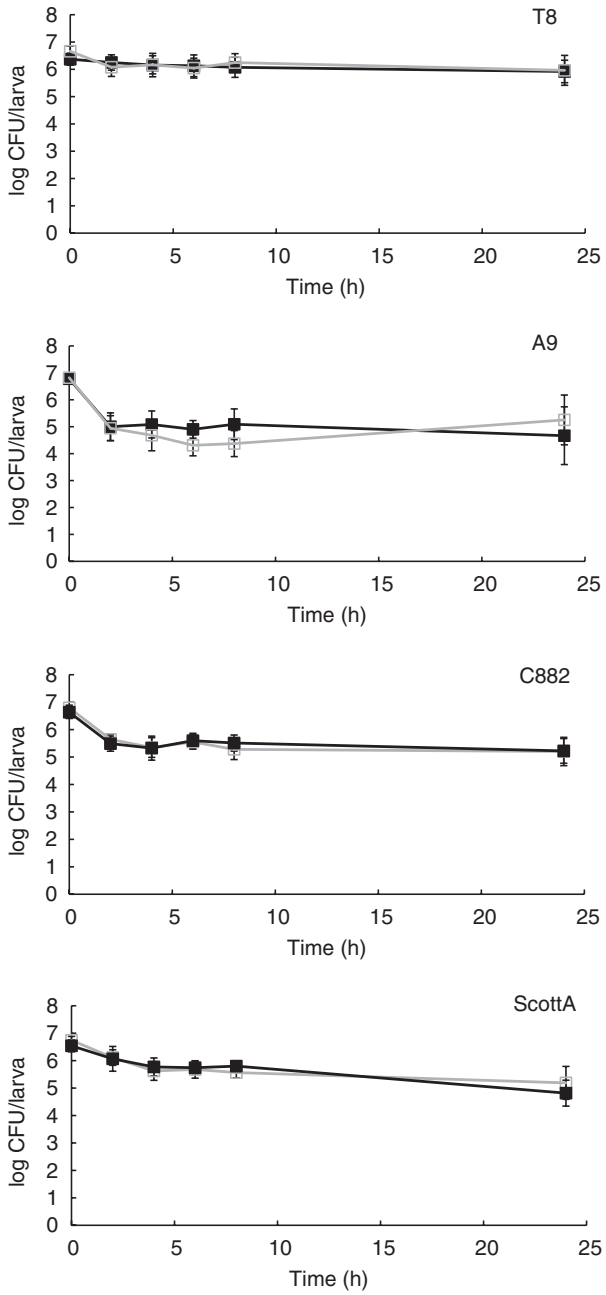

Figure 2 The growth kinetics of (■) adapted (pH 5·5 and 3·5% w/v NaCl) and (□) nonadapted (pH 7·0, no salt added) cells of *Listeria monocytogenes* strains in *Galleria mellonella* after 2, 4, 6, 8 and 24 h postinfection. Data are the mean of five insects. Error bars represent the standard deviation.

hly and *actA*, but *inlA* gene was down-regulated. In contrast, adapted C882 cells showed a down-regulation of all three genes (Fig. 3).

Our results show that the impact of the adaptation on the expression of the virulence genes is strain dependent. Moreover, the pattern of the gene expression did not correlated with the virulence potential of the *L. monocytogenes* strains. The lowest virulent strains, A9 and Scott A, overexpressed the virulence genes after being adapted in the cheese-based medium in opposition to C882, the highest virulent strain. To clarify the differences in virulence between the *Listeria* strains, the expression of *hly* was evaluated under insect-induced conditions.

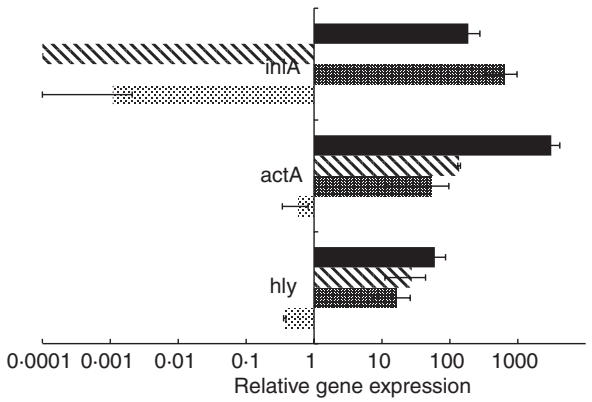

Figure 3 Relative gene expression of *hly*, *actA* and *inlA* genes determined by real-time PCR in adapted cells (pH 5·5 and 3·5% w/v NaCl) in comparison with nonadapted cells (pH 7·0, no salt added) of *Listeria monocytogenes* strains in a cheese simulated medium. Data are the mean of three replicates, and the error bars represent the standard deviation (■) A9; (▧) T8; (▩) ScottA; (⁘) C882.

Expression of listeriolysin under simulated insect conditions

The *hly* expression of adapted C882 and A9 cells representing a higher and a lower virulent strain, respectively, was evaluated in Schneider's medium supplemented with *G. mellonella* insect larvae homogenate (induced insect host) and in Schneider's medium (noninduced insect host). No overexpression of *hly* ($P > 0{\cdot}05$) was observed in adapted C882 and A9 cells either after 2 h induction or after 6 h in comparison with nonadapted cells (Fig. 4a,b). However, the *hly* expression after 6 h was higher in conditions of induction or noninduction in adapted A9 cells in comparison with adapted C882 cells (Fig. 4b). It was also observed that nonadapted A9 cells in noninduced conditions showed a higher *hly* expression in comparison with nonadapted C882 cells (Fig. 4b). Our results evidenced that besides the recognized role of *hly* in *L. monocytogenes* virulence in the *G. mellonella* model, the lower insect mortality shown by adapted A9 cells is not associated with a lower *hly* expression rather suggests that adapted A9 cells may have other impaired virulence factors. For instance, McLaughlin *et al.* (2012) reported that the Fur-regulated virulence factor A (FrvA) is crucial for *L. monocytogenes*

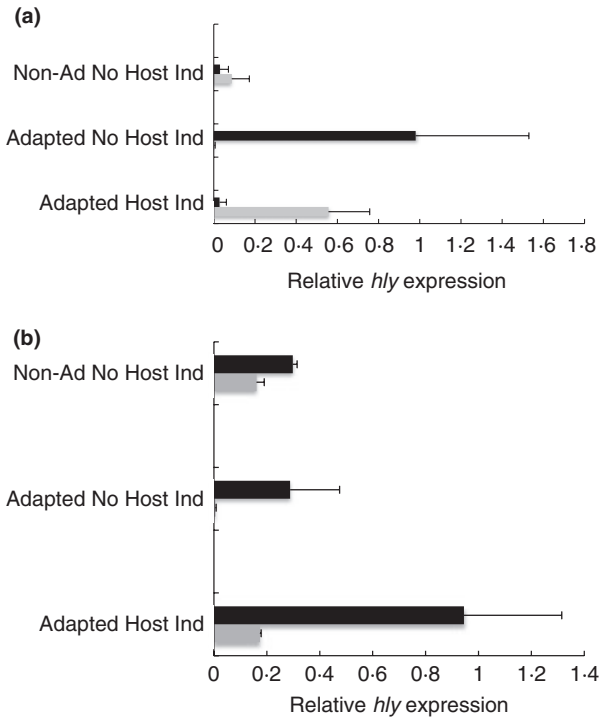

Figure 4 Relative gene expression of *hly* determined by real-time PCR in adapted cells (pH 5·5 and 3·5% w/v NaCl) in comparison with non-adapted cells (pH 7·0, no salt added) of *Listeria monocytogenes* strains in insect-induced conditions after (a) 2 h and (b) 6 h. For control, the relative expression of *hly* in adapted and nonadapted cells in Schneider's medium (no host induced) was determined. Data are the mean of three replicates, and the error bars represent the standard deviation. (■) A9; (▒) C882.

virulence both in a murine and in the *G. mellonella* model of infection. Our results suggest that the impact of adaptation to low pH and salt in *L. monocytogenes* virulence occurs at a more extended level, which needs further investigation.

In conclusion, this study shows that the use of *G. mellonella* model can be useful to investigate the impact of food-matrix- and food associated factors, such as low pH and salt, on *L. monocytogenes* virulence and promptly discriminate the isolates.

Materials and methods

Bacterial strains and growth conditions

The components of the cheese-based medium (Kagkli *et al.* 2006) were purchased from Sigma-Aldrich (Madrid, Spain) or Merck (Darmstadt, Germany). Tryptic soy broth (TSB) was purchased from Oxoid (Basingstoke, Hampshire, UK) and for solid media, agar (nº1, Oxoid) was added at 1·5% w/v. Four *L. monocytogenes* strains were used in this study and are listed in Table 1. Bacteria were stored in TSB supplemented with 25% v/v glycerol at −80°C and, when necessary, recovered in TSB and maintained in Tryptic soy agar (TSA) at 4°C. Prior to use, bacteria were transferred to fresh TSA plates and incubated at 30°C for 24 h.

Adaptation to low pH and salt in cheese-based medium

Cells were grown in TSB overnight at 30°C with agitation (120 rev min^{-1}) and collected by centrifugation (1575 ***g***, 10 min, 4°C). For adaptation to sublethal conditions, cells (10^8 CFU ml^{-1}) were exposed to pH 5·5 and 3·5% w/v NaCl in a cheese-based medium (Kagkli *et al.* 2006) with the following composition per litre: amicase 15 g, sodium lactate 38 ml, yeast extract 3 g, $CaCl_2$ 0·1 g, $MgSO_4$ 1·02 g, KH_2PO_4 6·8 g, methionine 6 g and lactose 2·1 g. The medium was modified by addition of 0·2% (w/v) glucose to provide the minimum glucose concentration to the growth of *L. monocytogenes* strains (Trivett and Meyer 1971). Nonadapted cells were transferred to simulated cheese medium with pH 7·0 and no NaCl added. The incubation was performed at 20°C for 2 h with low agitation (60 rev min^{-1}). The viability after adaptation was determined by Miles and Misra method (Miles *et al.* 1938). Three biological experiments were carried out.

Galleria mellonella L. *(Lepidoptera: Pyralidae) infection.* Virulence of *L. monocytogenes* strains was determined using larvae of the Greater Wax Moth *G. mellonella* with weights between 250 and 350 mg. Larvae of *G. mellonella* were reared during the first and second instars with pollen and wax and subsequently using an artificial diet made with a mixture of glycerol, honey, water, dry dog food and wheat bran. All developmental stages of the insects were carried out in the dark at 30°C. Each *L. monocytogenes* cheese-based medium culture (10^8 CFU ml^{-1}) was centrifuged at 2790 ***g*** for 5 min at 4°C. The bacterial cells were resuspended in 1 ml of sterile PBS. Ten larvae were infected with 10 μl of the initial suspension (10^6 CFU) by injection on the second right proleg using a 50-μl microsyringe (Sigma). Before infection, larvae were superficially disinfected with ethanol at 70% v/v. Larvae were placed in sterile Petri dishes at 37°C and death checked every 24 h for 5 days. Death was considered when no response to touch was observed.

Determination of bacterial numbers in *Galleria mellonella*

The growth of *L. monocytogenes* strains in the larvae was determined after 2, 4, 6, 8 and 24 h after injection. At each time point, five larvae were anesthetized for 1 min in ethanol at 70% (v/v) and killed in 5 ml of sterile PBS. From the previous homogenate, serial dilutions were made in

Table 1 *Listeria monocytogenes* strains

Strain	Serovar	Origin	Source
C882	4b	Portuguese cheese (cow's milk, cured, pH 6·0, 2·4%, w/v NaCl)	INETI-DTIA (Faleiro *et al.* 2003)
A9	1/2b or 3b	Portuguese dairy processing environment	Chambel *et al.* (2007), Adrião *et al.* (2008)
T8	4b	Portuguese dairy processing environment	Chambel *et al.* (2007), Adrião *et al.* (2008)
Scott A	4b	Epidemic strain, human isolate	McLauchlin, J. Food Safety Laboratory, Div. Gastrointestinal Infections, London, UK

INETI-DTIA-Instituto Nacional de Engenharia e Tecnologia Industrial-Dept. Tecnologia das Industrias Alimentares, Lisbon, Portugal.

sterile peptone water and plated on Palcam agar (Biokar Diagnosis, Beauvais, France). Incubation was performed at 37°C, and viability was determined after 48 h.

Analysis of Listeria monocytogenes virulence genes expression

RNA extraction. After 2 h adaptation in cheese-based medium (pH 5·5 and 3·5% w/v NaCl) and at nonadaptation conditions (pH 7·0 and no NaCl added), 2 ml of each *L. monocytogenes* culture was centrifuged at 2790 ***g*** for 5 min at 4°C, and the pellet was washed with sterile PBS. RNA extraction was performed using the Promega kit *SV RNA isolation* and quantified on the Nanodrop 2000c (Thermo Scientific, Waltham, MA, USA) spectrophotometer.

Synthesis of cDNA and real-time PCR

Synthesis of cDNA was performed with the *iScript™ cDNA synthesis* kit (Bio-Rad, Hercule, USA) following the manufacture instructions. For a 20-μl reaction volume were added 4 μl of the *iScript reaction mix* (5x), 1 μl of *iScript reverse transcriptase* and 1 μg of extracted RNA. The total volume was adjusted with sterile water free of nucleases. The reaction occurred by sequential incubation at 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. The relative gene expression of *hly* (*lmo0202*), *actA* (*lmo0204*) and *inlA* (*lmo0433*) genes was determined by the ΔC_T method using the *rpsP* (*lmo 1796*) gene in the data normalization. The primers used for gene expression are indicated in Table S1. For the real-time PCR, the *IQ SYBR Green supermix* (Bio-Rad) was used and carried out in the *icycler iQ multicolor real-time PCR detection system* (Bio-Rad). Each reaction was carried out in triplicate, and mean values of relative gene expression of each gene were calculated. The efficiency of the used primers was similar and reached 90%.

Simulated larvae-induced conditions

Exposure of *L. monocytogenes* to simulated insect-induced conditions was carried out as described by Münch *et al.* (2008). For this determination, a *G. mellonella* larvae suspension in Schneider's medium (Sigma, Madrid, Spain) was prepared. The suspension was obtained by surface-sterilized larva (45–55 mg) in a 70% v/v ethanol bath followed by sterile water passage. The insect was sacrificed by removing the head. The insect was crushed in 20 ml of Schneider's medium and homogenized in an agitator at 180 rev min^{-1} during 20 min. Tissue debris was eliminated by centrifugation at 4690 ***g*** at 4°C during 20 min.

The insect suspension was inoculated with 10^8–10^9 CFU ml^{-1} of adapted and nonadapted C882 and A9 cells in cheese-based medium. The viability of the cultures, RNA extraction and cDNA synthesis were determined as above.

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Conflict of interests

The authors report no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers used in real-time PCR.