Strain dependent expression of stress response and virulence genes of Listeria monocytogenes in meat juices as determined by microarray

(Article begins on next page)

23 April 2024

Strain dependent expression of stress response and virulence genes of *Listeria monocytogenes* in meat juices as determined by microarray

Kalliopi Rantsiou[1]*, Anna Greppi[1], Matteo Garosi[1], Alberto Acquadro[2], Marios Mataragas[3] 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]Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali, Section of Agricultural Genetics, Facoltà di Agraria, Università di Torino, Italy

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

Short title: *In situ* virulence by microarray

*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**

Abstract

A subgenomic array, encompassing 54 probes targeting genes responsible for virulence, adhesion and stress response in *Listeria monocytogenes*, was used in order to study their expression in food systems. RNA extracted from *L. monocytogenes* inoculated in BHI and *in situ* (i.e. in minced meat and fermented sausage juices) and incubated at 4 °C, was hybridized on the array and the results obtained were compared in order to understand the effect that the food juice has on the expression. Three different strains of *L. monocytogenes* were tested, in order to determine the effect of the strain provenience. As determined by cluster analysis, each strain behaved in a different way when inoculated in food juices. The goal was to respond to acidic and osmotic stresses encountered in the food, particularly in the fermented sausage juice. No differences in the expression profile between the three strains were observed, when they were inoculated in BHI. On the other hand, in the meat and sausage juices, the *iap*, *gadC* and *gadE* genes, together with different internalin encoding genes, were significantly differentially expressed in the three strains.

1. Introduction

Listeria monocytogenes is one of the main safety concerns of the food industry, legal authorities and consumers. Due to its ubiquitous nature, it can contaminate a wide range of foodstuffs and it is commonly associated with ready to eat commodities (Anonymous, 2009). Its biofilm forming capacity has been recognized as an important risk factor since it can lead to establishment in food plants and cause cross-contamination of otherwise safe products (Pan et al., 2006, Alessandria et al., 2010). Unlike other foodborne pathogens, *L. monocytogenes* is an intracellular pathogen with a complex virulence mechanism, which has been extensively studied both *in vitro* and *in vivo*. Intense research carried out during the last decade, including genome and expression studies, have made *L. monocytogenes* an emerging model in prokaryotic transcriptomics (Cossart and Archambaud, 2009).

Food is the vehicle through which *L. monocytogenes* enters the human body where, under certain circumstances, it elicits disease. Recent findings have shown that the 'history' of *L. monocytogenes* cells, may influence their virulence potential. It was shown that long-term adaptation to acidic and NaCl stress (like the ones commonly encountered in foods) increased expression of virulence genes and improved adhesion and invasion to Caco-2 cells (Olesen et al., 2009). Furthermore, it has been suggested that a food matrix, in particular a meat based, may influence virulence potential of *L. monocytogenes*, possibly through down-regulation of virulence genes (Mahoney and Henriksson, 2003, Olesen et al., 2010). These findings suggest that environmental conditions that *L. monocytogenes* may encounter in foods could influence its virulence potential. Furthermore, it is expected that modulation of expression of stress response genes, under certain food conditions, may enhance survival and directly or indirectly virulence. *In situ* studies, using real food matrices, provide valuable data and will complement available information from *in vitro* and *in vivo* experiments, regarding virulence and survival potential.

L. monocytogenes gene expression *in situ*, i.e. in a food matrix, has been studied so far using both reverse transcription quantitative PCR (RT-qPCR) and microarrays (Olesen et al, 2010; Duodu et al., 2010; Liu and Ream, 2008; Rieu et al., 2010). These are the two experimental approaches most commonly employed for transcription analyses. Generally, it is recognized that RT-qPCR is the appropriate method when one needs to study a moderate number of genes in a number of samples that ranges from small to hundreds. On the contrary, microarrays offer the possibility for whole genome discovery experiments in small number of samples (VanGuilder et al, 2008). More function-focused subgenomic-arrays, that target specific cellular functions, for example virulence regulons for pathogens or metabolic regulons of interest for technologically important microorganisms, allow application in a larger number of samples and facilitate interpretation of the data obtained.

The purpose of this study was to investigate the effect of two food juices (minced meat and fermented sausages) on the expression of genes involved in virulence, adherence and stress response of *L. monocytogenes*. A subgenomic-array was employed, with 54 spots targeting such genes, and it was tested using three different strains of *L. monocytogenes*, one laboratory strain and two strains previously isolated from foods. In this way, possible differences in expression profiles between strains could also be determined. This is the first time that a subgenomic-array, targeting genes that directly or indirectly influence virulence, is applied to investigate *L. monocytogenes* transcription in food model systems.

2. Materials and methods

2.1. Strains

In this study, 3 strains of *L. monocytogenes* were employed: the sequenced strain EGDe, belonging to serotype 1/2a and two strains, previously isolated from food matrices and

identified as *L. monocytogenes* by 16S rRNA sequencing. These were: strain #3, serotype 3c, isolated from fermented sausage and strain #12, serotype 4b, isolated from milk. Strains were propagated in Brain Heart Infusion, BHI (Oxoid, Milan, Italy) broth at 37 °C.

2.2. Artificial inoculation and experimental conditions

Two different food matrices, namely fermented sausage (FS) and minced meat (MM), were chosen, in order to determine the gene expression profile of *L. monocytogenes* and to compare it with that exhibited in BHI. To avoid cross hybridizations by the resident microbiota of the food matrices considered in this study, a sterile juice was prepared from each of the two matrices as follows. One hundred g of fermented sausage or minced meat were mixed with 100 ml of Ringer's solution (Oxoid) in a stomacher bag and homogenized for 3 minutes in a BagMixer® 400 (Interscience, Paris, France). The supernatant was carefully collected, centrifuged for 20 min at 6000xg and at 4 °C. The supernatant was then filtered through a 0.2 μm filter. The resultant food juice (about 100 ml final volume) was tested for sterility by plating on BHI and stored at 4 °C until its use. For the inoculation of the two different types of food juice and of the BHI broth, an overnight culture in BHI of each strain was used. One ml of culture was centrifuged for 5 minutes at 13.400xg in an Eppendorf 5417R refrigerated centrifuge (Eppendorf, Milan, Italy). The pellet was washed with 1 ml Ringer's Solution and centrifuged as above. The pellet obtained was re-suspended in 1 ml of food juice or BHI and inoculated in 6 ml of the same food juice or BHI, respectively. In this way, a final concentration of about 10^7 - 10^8 colony forming units (cfu)/ml was reached. The inoculated juices and the BHI were incubated at 4 °C. Samples were collected at 30 minutes and at 48 hours for RNA extraction and virulence gene expression analysis and a *L. monocytogenes* count in BHI was also performed. Three independent biological replicates were performed for each food juice and for the BHI.

2.3. RNA extraction

At each time point, a 1 ml sample was collected for RNA extraction. Furthermore, 1 ml of the culture grown in BHI overnight at 37°C, used to inoculate the food juices, was also analyzed. The sample was centrifuged for 30 seconds, the supernatant was removed and 200 μl of RNA*later* (Ambion, Applied Biosystems, Milan, Italy) were immediately added to the pellet to prevent alteration of the gene expression profile, as recommended by the manufacturers. After 5 minutes, RNA was extracted from the pellet using the MasterPure™ Complete DNA and RNA Purification kit (Epicentre, Madison, WI, USA). The instructions of the manufacturer were followed, after a 20 min treatment at 37°C with proteinase K (Sigma, Milan, Italy) (addition of 50 μl of a 25 mg/ml solution) and lysozyme (Sigma) (addition of 50 μl of a 50 mg/ml solution). DNA was eliminated from the preparation with an enzymatic digestion, using the Turbo DNase (Ambion). The RNA was quantified using the Nanodrop ND-1000 spectrophotometer (Celbio, Milan, Italy) and its quality checked by agarose gel electrophoresis.

2.4. Reverse transcription (RT)

For each sample, two RT reactions were performed, each with approximately 1 μg of RNA. The M-MLV Reverse Transcriptase (Promega, Milan, Italy) enzyme was used, together with random hexamers (Promega), following the instructions of the manufacturer. The reaction contained 150 μM of Biotin-11-dUTP (Fermentas, M-Medical, Milan, Italy), 150 μM of dTTP and 0.7 mM of each of dATP, dCTP and dGTP. The RNasin ribonuclease inhibitor (Promega) was also added, according to the suggestions of the manufacturer. The RT reaction was carried out at 42 °C for 1 hour and it was stopped by inactivation of the enzyme. The two RT reactions of each sample were pooled together and the cDNA was precipitated following

standard methodology (Sambrook et al., 1989). The pellet was re-suspended directly in 350 μl of the microarray hybridization buffer Q.Hyb (QInstruments, Jena, Germany).

2.5. Microarray hybridization and development

The SureArray for *L. monocytogenes* (Congen, Berlin, Germany), developed within the 6th FP integrated project Pathogen Combat, was employed for the study of gene expression. The SureArray is a subgenomic array, consisting of 54 spots for genes involved in virulence, stress response and adhesion and it was designed based on the complete genome sequences of *L. monocytogenes* EGDe and F2365 strains. Spots for positive, negative and hybridization controls are also present. Each slide has 2 areas for hybridization of 2 different samples and in each area, the 54 gene spots and controls are repeated 4 times. Hybridization was performed at 30 °C for 18 hours in an Eppendorf Thermomixer comfort (Eppendorf). For the development of the slides, the Silverquant detection kit (Eppendorf) was used, essentially as described by the manufacturer. After the silver staining, the slides were scanned with the Silverquant scanner (Eppendorf). The images obtained, were processed and the spots quantified using the Silverquant analysis software (Eppendorf). Afterwards, the Micro Array Data Analyser (MADA) program, developed by the Max Planck Institute (http://www.mpi-bremen.de), was used for the processing of the spot intensities obtained.

2.6. Statistical analysis of data

Quantile normalization was used on the $\log_2$ intensities of each sample hybridized as results from different hybridizations need to be compared against each other in order to determine a meaningful estimate of the level of differential expression in a given gene (Bolstad et al., 2003). Statistical analysis of the microarray data was performed using the Excel add-in software BRB-Array Tools (Simon et al., 2007). Genes that were differentially expressed among two or more classes (e.g. differences between *L. monocytogenes* strains) were

identified, using a random-variance t-test (Wright and Simon, 2003). Genes were considered statistically significant if their p-value was less than 0.05. Moreover, the software Genesis (Sturn et al., 2002) available at

http://genome.tugraz.at/genesisclient/genesisclient_description.shtml, was used for clustering analysis of the gene expression data, employing the Pearson correlation. For this purpose, normalization was performed towards *in vitro* control conditions, namely BHI cultures at 37°C incubated overnight, or at 4°C for 30 min and 48 h.

3. Results and discussion

The elucidation of the molecular mechanism of virulence of *L. monocytogenes* and the genes involved in this process have been the focus of research for the last two decades. Now a wealth of information is available regarding these two aspects, mainly from studies conducted *in vitro* and *in vivo*, using laboratory strains (Kreft and Vázquez-Boland, 2001; Loh et al., 2006; Johansson et al, 2002; Chaturongakul et al., 2008). This information has led to an in-depth understanding of the physiology and characteristics of *L. monocytogenes* that makes it an important foodborne pathogen (Gandhi and Chikindas, 2007; Kathariou, 2002). Recently, efforts are being made to enrich current knowledge conducting studies *in situ*. This is commonly done by artificially inoculating food with high numbers of *L. monocytogenes* and following expression of selected genes of interest by RT-qPCR as well as performing experiments that determine virulence potential *in vitro* (for example adhesion, biofilm formation, invasion) and *in vivo* (for example, mouse infection) (Olesen et al, 2010; Duodu et al., 2010; Rieu et al., 2010).

In this study we employed a subgenomic array, targeting genes directly or indirectly involved in virulence and overall survival of *L. monocytogenes* under stress conditions. Strain EGDe, of which the whole genome sequence was used to design the probes of the array, as well as two

food isolates were used. The specificity of the array was tested using DNA extracted from the three strains used in the study (data not presented). A protocol for the preparation of a sterile juice, starting from the two food matrices used in this study, minced meat and fermented sausage, was developed in order to avoid interference by the resident microbiota. This approach has been previously adopted in order to circumvent problems of sensitivity in the downstream processes (Duodu et al., 2010, Ligowaska et al., 2011).

By the counts performed after 30 minutes and 48 hours incubation at 4°C in the food juices, as well as in BHI, it was determined that *L. monocytogenes* strains maintained a load of 10^7 - 10^8 cfu/ml, apart from strain #12 that showed a reduction of about 1 log at 48 h, when inoculated in fermented sausage juice (Table 1).

Cluster analysis of the data was performed in order to obtain a global view of the gene expression profiles of the three strains in the conditions tested. The gene expression data were validated by using Reverse Transcription quantitative PCR (RT-qPCR) protocols, previously developed (Alessandria et al., 2010) on a set of genes included in the array (data not shown).

The global analyses of the expression profiles of the 54 genes included in the subarray are presented in Figures 1 and 2. In Figure 1, the results obtained for the three strains of *L. monocytogenes* are reported, focusing on the effect of the two different food juices and of the time of incubation. A strain-specific clustering of the profiles was observed both at 30 min (Fig. 1A) and at 48 h (Fig. 1B). While at 30 min this was more evident for strain#12, at 48 h each of the three strains formed separate clusters. It is worth noticing that at 30 min most genes were either upregulated or remained constant, apart from strain EGDe in fermented sausage juice, for which several genes were downregulated. This trend changed considerably when the data obtained at 48 h were analyzed, showing a general downregulation of most of the genes. It can be speculated that the strains responded very fast and individually to the

new environment with a general upregulation trend. In Figure 2 the expression profiles of the three strains grown in all the experimental conditions tested are reported. This comparison provides information regarding the effect of the incubation temperature (4°C) as well as the influence of the growth condition (*in vitro*, i.e. BHI, and *in situ*, i.e. meat and fermented sausage juice) and time (30 min and 48 h). Two main observations can be made. The first concerns the general upregulation of most of the genes considered in the array, independently of the strain and growth condition. From studies conducted so far *in vitro*, it was demonstrated that virulence genes in *L. monocytogenes* are regulated in a temperature-dependent manner. More specifically it is hypothesized that temperature may play a role of a sensor, which triggers virulence only when *L. monocytogenes* is found within the host (de las Heras et al., 2011). Our findings, however, suggest that virulence gene expression may take place also at food storage resembling conditions. This aspect requires further *in situ* investigation to improve our knowledge regarding the physiology of *L. monocytogenes* in foods, in order to develop more accurate risk assessment approaches. The second evidence confirms the findings related to the strain-dependent response, since also in this case a strain-specific clustering was obtained. As shown in Figure 2, the three *L. monocytogenes* grouped in separate clusters including, for each strain, all the conditions tested in this study.

The array hybridization data obtained for the three strains in the conditions tested (BHI and two food juices incubated at 4 °C for 30 min and 48h) were also statistically elaborated in order to answer two questions: (i) for each strain, are there differences in the expression profile between the different substrates inoculated? and (ii) for each substrate, are there differences in the expression between the three different strains? Table 2 shows the behavior of the three different *L. monocytogenes* strains. Genes that were differentially expressed in the three substrates tested are presented. The strain that appears to be most influenced by the substrate, as indicated by the number of genes with differential expression, is the EGDe. On

the other hand, strain #12 showed the lowest number of differentially expressed genes. These trends were evident both at 30 min and at 48 h of incubation. For all three strains, the number of genes differentially expressed was lower at 48 h. Gene *proA*, encoding a γ- glutamyl phosphate reductase which makes part of the proline biosynthesis pathway, was significantly upregulated in meat or sausage or both, when compared to BHI, for all three genes. Proline has been shown to play a role in the survival of *L. monocytogenes* in high osmolarity environments (Sleator et al., 2001), while its effect on the virulence of *Staphylococcus aureus* and *Escherichia coli* has been demonstrated (Bae and Miller, 1992, Culham et al., 1992, Schawn et al. 1998). Gene *sod*, encoding the superoxide dismutase, responsible for oxygen detoxification, was downregulated in the meat environment for strains EGDe and #12, and the difference in the expression was significant when compared to the sausage environment, in which this gene was upregulated compared to the BHI substrate. Other genes that showed some similarities in their expression pattern between strains were: *cysK* (encoding a protein highly similar to a cysteine kinase), which was downregulated in the sausage juice for strains #3 and #12 and for strain EGDe had lower expression in sausage with respect to meat; *gadC*, a gene that is part of the glutamate decarboxylase (GAD) acid resistance system of *L. monocytogenes* (Cotter et al, 2001a, Cotter et al. 2001b), which was significantly upregulated for strains #3 and EGDe; *dat*, encoding a D-amino acid aminotransferase, which was upregulated in meat and sausage for strain #3 and in meat for the EGDe strain and finally, gene *hly*, a virulence gene encoding for listeriolysin, that was upregulated in sausage for strain #3 and in meat and sausage for the EGDe. Taken as a whole, these results suggest that some differences exist in the behavior of the 3 strains in the different substrates, i.e. each strain responds to the substrates tested in a different way. However, it seems that all three *L. monocytogenes* strains modulate (upregulate) the expression of genes that may confer protection and increase survival under stressful conditions encountered in meat, but most

importantly, in sausage. With a measured pH of 5.3 and salt concentration of 3.2 % (w/w) (data not shown), the fermented sausage matrix puts on *L. monocytogenes* a significant acidic and salt stress.

Table 3 presents the results of the comparison of the expression profile of the three *L. monocytogenes* strains, in each of the 3 substrates tested. Apparently, little or no difference in the expression profile was observed between the 3 strains when they were inoculated in BHI. On the contrary, when a food juice was inoculated, namely minced meat or sausage, then a number of genes showed significant differential expression between the strains. More specifically, 7 genes were differentially expressed when the food juice considered was minced meat while a maximum of 19 genes were differentially expressed when a fermented sausage was considered. These results suggest that when different *L. monocytogenes* strains are inoculated in the laboratory medium BHI and incubated at 4 °C, they behave, in terms of expression of the genes present in the array, in a similar way while when they are inoculated in a food juice, they behave differently. This is an interesting observation and further emphasizes the need to conduct studies *in situ* conditions. Moreover, another parameter that needs to be taken into consideration, in studies regarding survival and virulence, is the provenience of the strain to be used.

The single gene, which was differentially expressed in all the conditions tested, was the *iap* gene, encoding the invasion-associated protein p60. Furthermore, in all conditions tested, strain #12 had the lowest level of expression while strain #3 had the highest level (down-regulation and up-regulation respectively, compared to the EGDe strain). This gene is essential for cell viability and at the same time it is considered an important virulence factor of *L. monocytogenes* (Wuenscher et al., 1993).

Other genes, for which expression was affected in a similar way in different conditions, were the *gadE* and *gadC*. Both genes are linked to the glutamate decarboxylase activity, encoding

putative antiporters, and are therefore believed to play an important role in the Acid Tolerance Response (ATR) (Cotter et al., 2001a, Davis et al., 1996). Gene *gadC* showed significant differential expression in minced meat juice after 48 h and in sausage juice after 30 min. In both cases, strain EGDe had the lowest expression while strain #3 had the highest. Strain #3 had the highest expression also for the *gadE* gene in the sausage juice. Finally, different virulence genes, encoding proteins that are included in the family of the internalins, were differentially expressed in the sausage after 30 min of incubation and in the minced meat after 48 h. Internalins are a family of proteins, associated with the cell surface of *L. monocytogenes* and involved with adhesion and internalization by epithelial cells (Cabanes et al., 2002,) or are as up to date of unknown function but determined to be involved in virulence (Bierne and Cossart, 2007). No general trend of expression for the three strains could be deduced for these genes. Recently, it was shown that differential expression of internalin genes in *L. monocytogenes* 10403S is temperature dependent (McGann et al., 2007). Based on the results obtained here, we cannot exclude the influence of the food juice, but also of the strain provenience on the expression of these apparently versatile genes with still unknown physiological functions.

In summary, from the comparison of the expression results for the three strains, some differences were observed. These interested mainly genes involved in the ATR, the *iap* virulence gene and different genes encoding for internalins. The differences were observed when the strains were inoculated in a food juice, but not in BHI. Furthermore, at least for the ATR and the *iap* genes, a specific trend could be inferred: strain #3, a wild strain previously isolated from meat, had the highest level of expression. It seems that these genes are expressed in a strain-dependent manner in food juices.

In conclusion, the subgenomic array, targeting genes for virulence, adhesion and stress response of *L. monocytogenes*, was successfully applied *in situ*, in order to study gene

transcription. Our findings are in support of the hypothesis that there is variation in the virulence and stress response potential for different strains of *L. monocytogenes* and that the food matrix is an important environmental parameter, affecting this potential.

References

Alessandria V., Rantsiou K., Cocolin L., 2010. Virulence gene expression in *Listeria monocytogenes* strains isolated from different sources. ISOPOL XVII: International Symposium on Problems of Listeriosis, Porto, P, May 5-8.

Alessandria, V., Rantsiou, K., Dolci, P., Cocolin, L., 2010. Molecular methods to assess Listeria monocytogenes route of contamination in a dairy processing plant. International Journal of Food Microbiology 141, S156-S162.

Anonymous, 2009. The community report on trends and sources of zoonoses and zoonotic agents on the European Union in 2007. The EFSA Journal 223.

Bae, J.-H., Miller, K. J., 1992. Identification of two proline transport systems in *Staphylococcus aureus* and their possible roles in osmoregulation. Applied and Environmental Microbiology 58, 471–475.

Bierne, H., Cossart, P., 2007. *Listeria monocytogenes* surface proteins: from genome predictions to function. Microbiology and Molecular Biology Reviews 71, 377-397.

Bolstad, B.M., Irizarry, R.A., Astrand, M., Speed, T.P., 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19, 185-193.

Cabanes, D., Dehoux, P., Dussurget, O., Frangeul, L., Cossart, P., 2002. Surface proteins and the pathogenic potential of *Listeria monocytogenes*. Trends in Microbiology 10, 238-245.

Chaturongakul, S., Raengpradub, S., Wiedmann, M., Boor, K. J., 2008. Modulation of stress and virulence in *Listeria monocytogenes*. Trends in Microbiology 16, 388-396.

Cossart, P., Archambaud, C., 2009. The bacterial pathogen *Listeria monocytogenes*: an emerging model in prokaryotic transcriptomics. Journal of Biology 8, 107.

Cotter, P.D., Gahan , C., G., M., Hill, C., 2001a. A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. Molecular Microbiology 40, 465-475.

Cotter, P.D., O'Reilly, K., Hill, C., 2001b. Role of the glutamate decarboxylase acid resistance system in the survival of *Listeria monocytogenes* LO28 in low pH foods. Journal of Food Protection 64, 1362-1368.

Culham, D. E., Dalgado, C., Gyles, C. L., Mamelak, D., Maclellan, S., Wood, J. M., 1998. Osmoregulatory transporter ProP influences colonization of the urinary tract by *Escherichia coli*. Microbiology 144, 91–102.

Davis, M. J., Coote, P. J., O'Byrne, C. P., 1996. Acid tolerance in *Listeria monocytogenes:* the adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. Microbiology 142, 2975-2982.

de las Heras, A., Cain, R.J., Bielecka, M.K., Vazquez-Boland, J.A., 2011. Regulation of *Listeria* virulence: PrfA master and commander. Current Opinions in Microbiology 14, 118-127.

Duodu, S., Holst-Jensen, A., Skjerdal, T., Cappelier J.M., Pilet, M.F., Loncarevic, S., 2010. Influence of storage temperature on gene expression and virulence potential of *Listeria monocytogenes* strains grown in salmon matrix. Food Microbiology 27, 795-801.

Gandhi, M., Chikindas, M. L., 2007. *Listeria*: a foodborne pathogen that knows how to survive. International Journal of Food Microbiology 113, 1-15.

Johansson, J., Mandin P., Renzoni, A., Chiaruttini, C., Springer, M., 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.

Kreft, J., Vázquez-Boland, J. A., 2001. Regulation of virulence genes in *Listeria*. International Journal of Medical Microbiology 291, 145-157.

Ligowska, M., Thorup Cohn, M., Stabler, R.A., Wren, B. W., Brøndsted, L., 2011. Effect of

chicken meat environment on gene expression of *Campylobacter jejuni* and its relevance to survival in food, International Journal of Food Microbiology 145, S111-S115.

Liu, Y., 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.

Loh, E., Gripenland, J., Johansson, J., 2006. Control of *Listeria monocytogenes* virulence by 5'-untranslated RNA. Trends in Microbiology 14, 294-298.

Mahoney, M., Henriksson, A., 2003. The effect of processed meat and meat starter cultures on gastrointestinal colonization and virulence of *Listeria monocytogenes* in mice. International Journal of Food Microbiology 84, 255-261.

McGann, P., Ivanek, R., Wiedmann, M., Boor, K. J., 2007. Temperature-dependent expression of *Listeria monocytogenes* Internalin and Internalin-like genes suggests functional diversity of these proteins among the Listeriae. Applied and Environmental Microbiology 73, 2806-2814.

Olesen, I., Thorsen, L., Jespersen, L., 2010. Relative transcription of *Listeria monocytogenes* virulence genes in liver pâtés with varying NaCl content. International Journal of Food Microbiology 141, S60-S68.

Olesen, I., Vogensen, F.K., 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.

Pan, Y., Breidt Jr., F., Kathariou, S., 2006. Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. Applied and Environmental Microbiology 72, 7711–7717.

Rieu, A., Guzzo, J., Piveteau P., 2010. Sensitivity to acetic acid, ability to colonize abiotic surfaces and virulence potential of *Listeria monocytogenes* EGD-e after incubation on parsley leaves. Journal of Applied Microbiology 108, 560-570.

Sambrook, J., Fritsch, E. F., Maniatis, T., 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Springs Harbor Laboratory Press, Cold Springs Harbor, N.Y.

Schwan, W. R., Coulter, S. N., Ng, E. Y. W., Langhorne, M. H., Ritchie, H. D., Brody, L. L., Westbrock-Wadman, S., Bayer, A. S., Folger, K. R., Stover, C. K., 1998. Identification and characterisation of the PutP proline permease that contributes to *in vivo* survival of *Staphylococcus aureus* in animal models. Infection and Immunity 66, 567–572.

Simon, R., Lam, A., Li, M.C., Ngan, M., Menenzes, S., Zhao, Y., 2007. Analysis of gene expression data using BRB-Array Tools. Cancer Informatics 3, 11-17.

Sleator, R.D., Gahan, C. G., Hill, C., 2001. Identification and disruption of the *proBA* locus in *Listeria monocytogenes*: role of proline biosynthesis in salt tolerance and murine infection. Applied and Environmental Microbiology 67, 2571-2577.

Sturn, A., Quackenbush, J., Trajanoski, Z., 2002. Genesis: cluster analysis of microarray data. Bioinformatics 18, 207-208.

VanGuilder, H.J., Vrana, K.E., Freeman, W.M., 2008. Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques 44, 619-626.

Vazquez-Boland, J.A., Kuhn, M., Berche, B., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J., Kreft, J., 2001. *Listeria* pathogenesis and molecular virulence determinants. Clinical Microbiology Reviews 14, 1-57.

Wright, G.W., Simon, R., 2003. A random variance model for detection of differential gene expression in small microarray experiments. Bioinformatics 19, 2448-2455.

Wuenscher, M.D., Kohler, S., Bubert, A., Gerike, U., Goebel, W., 1993. The *iap* gene of *Listeria monocytogenes* is essential for cell viability, and its gene product, p60, has

bacteriolytic activity. Journal of Bacteriology 175, 3491-3501.

Table 1. Counts (expressed as colony forming units/ml) of *L. monocytogenes* in the different substrates used in this study. Data presented are the means (± standard deviation) of the three replicates.

	EGD-e	#3	#12
BHI overnight 37°C	3.8 x 10^8 (0.21)	5.6 x 10^8 (0.08)	3.9 x 10^8 (0.12)
BHI, 4°C, 30 min	4.5 x 10^7 (0.24)	7.8 x 10^7 (0.03)	4.7 x 10^7 (0.06)
BHI, 4°C, 48 h	2.2 x 10^8 (0.11)	2.9 x 10^8 (0.13)	4.1 x 10^8 (0.21)
Meat juice, 30 min	8.2 x 10^7 (0.07)	5.3 x 10^7 (0.24)	5.6 x 10^7 (0.31)
Meat juice, 48 h	3.9 x 10^7 (0.24)	7.2 x 10^7 (0.22)	8.3 x 10^7 (0.07)
Fermented sausage juice, 30 min	8.5 x 10^7 (0.11)	5.5 x 10^7 (0.31)	6.4 x 10^7 (0.09)
Fermented sausage juice, 48 h	7.3 x 10^7 (0.15)	6.2 x 10^7 (0.16)	3.9 x 10^6 (0.41)

Table 2. Genes differentially expressed in the three substrates, individually inoculated with *L. monocytogenes* strains #3, #12 and EGDe, and stored at 4°C for 30 min and 48 h.

Experimental condition	Gene	Geometric mean of intensities in class 1[a]	Geometric mean of intensities in class 2[a]	Geometric mean of intensities in class 3[a]	p-value[b]	Pairwise significant[c]
Strain #3, 30 min	*proA*	34.20	162.01	96.66	0.008	(1<2), (1<3)
	dat	69.59	172.00	160.24	0.018	(1<2), (1<3)
	cysK	111.13	192.42	58.38	0.042	(3<2)
	svpA	65.73	49.36	197.46	0.045	(1<3), (2<3)
Strain #3, 48 h	*gadC*	84.93	63.64	170.54	0.007	(1<3), (2<3)
	proA	31.69	167.18	73.99	0.018	(1<2)
	hly	39.98	124.70	189.42	0.049	(1<3)
Strain #12, 30 min	*cysK*	140.01	168.30	66.67	0.004	(3<1), (3<2)
	proA	49.26	179.80	106.83	0.013	(1<2), (1<3)
	bvrB	191.79	67.93	120.76	0.039	(2<1)
Strain #12, 48 h	*proA*	39.39	199.68	124.46	0.009	(1<2), (1<3)
	sod	100.23	39.11	225.09	0.038	(2<3)
	gadC	26.95	74.94	226.07	0.002	(1<2), (1<3), (2<3)
EGDe, 30 min	*inlA*	189.04	38.03	66.17	0.005	(2<1), (3<1)
	sod	137.35	21.34	156.10	0.005	(2<1), (2<3)
	cysK	41.06	184.83	133.63	0.026	(1<2), (1<3)
	gadA	70.05	59.45	241.43	0.026	(1<3), (2<3)
	sigB	159.81	69.37	29.59	0.034	(3<1)
	proA	40.88	225.28	63.42	0.046	(1<2)
EGDe, 48 h	*inlA*	145.78	29.66	68.70	0.005	(2<1), (3<1), (2<3)
	sod	85.80	27.05	186.35	0.006	(2<1), (2<3)
	hly	43.49	119.77	243.15	0.007	(1<2), (1<3)
	dat	46.37	190.87	125.46	0.026	(1<2)
	proA	35.95	193.33	79.40	0.046	(1<2)

[a] Class 1, BHI; Class 2, minced meat (MM); Class 3, fermented sausage (FS).
[b] Significant at 0.05 level of the t-test.
[c] The 'Pairwise significant' column displays pairs of classes with significantly different gene expression at 95% confidence level or $\alpha = 0.05$. The first number in the brackets indicates the class in which the specific gene is down-regulated when compared to the class indicated with the second number in the brackets. For example, the first row indicates that in *L. monocytogenes* strain #3 at 30min, the *proA* gene was significantly down-regulated in the BHI (class 1) compared to MM (class 2) and FS (class 3), while between class 2 (MM) and class 3 (FS) there was no significant difference in the expression of the specific gene.

Table 3. Genes differentially expressed between the three *L. monocytogenes* strains inoculated in BHI, MM and FS and stored at 4ºC for 30 min and 48 h.

Experimental condition	Gene	Geometric mean of intensities in class 1[a]	Geometric mean of intensities in class 2[a]	Geometric mean of intensities in class 3[a]	p-value[b]	Pairwise significant[c]
BHI, 30 min	*gbuB*	130.86	20.98	66.69	0.014	(2<1), (2<3)
	iap	153.52	108.26	207.41	0.022	(2<3)
BHI, 48 h	no significant differences in gene expression between classes were found					
MM, 30 min	*iap*	153.99	93.71	165.99	0.001	(2<1), (2<3)
	hly	36.08	14.61	12.06	0.003	(2<1), (3<1)
	proA	52.78	88.62	44.65	0.018	(1<2), (3<2)
	ami	55.96	44.62	75.86	0.021	(2<3)
	rpoB	23.70	23.28	54.14	0.038	(1<3), (2<3)
	gadE	16.52	43.64	26.12	0.040	(1<2)
	arpJ	24.22	15.75	11.81	0.047	(3<1)
MM, 48 h	*betL*	142.89	202.51	116.74	<0.001	(1<2), (3<1), (3<2)
	ctsR	182.11	268.72	171.27	<0.001	(1<2), (3<2)
	iap	190.41	97.06	210.66	0.001	(2<1), (2<3)
	rpoB	37.62	18.30	63.60	0.001	(2<1), (1<3), (2<3)
	gadC	12.39	63.18	65.59	0.003	(1<2), (1<3)
	inlA	68.01	128.76	28.19	0.006	(3<1), (3<2)
	inlB	74.70	109.66	42.52	0.007	(3<1), (3<2)
FS, 30 min	*inlG*	74.19	151.08	67.37	<0.001	(1<2), (3<2)
	inlA	150.56	51.88	98.04	<0.001	(2<1), (3<1), (2<3)
	dal	142.43	47.08	120.23	0.004	(2<1), (2<3)
	inlC	39.67	75.90	99.55	0.007	(1<2), (1<3)
	inlE	48.64	76.06	30.93	0.007	(1<2), (3<1), (3<2)
	bsh	172.43	83.47	154.21	0.011	(2<1), (2<3)
	inlH	28.67	135.95	46.70	0.013	(1<2), (3<2)
	gadA	43.64	83.82	47.10	0.016	(1<2), (3<2)
	proA	49.68	52.47	26.64	0.016	(3<1), (3<2)
	gadE	41.01	32.27	55.43	0.023	(2<3)
	opuCA	24.82	11.05	58.61	0.024	(2<3)
	gadC	22.14	68.98	82.07	0.024	(1<2), (1<3)
	hly	72.82	17.24	18.92	0.029	(2<1), (3<1)
	proC	27.91	59.93	44.50	0.033	(1<2)
	proB	26.93	40.53	11.38	0.034	(3<2)
	sod	121.98	204.10	99.39	0.034	(1<2), (3<2)
	bvrC	38.29	145.68	95.75	0.042	(1<2)
	iap	116.02	102.15	160.33	0.047	(2<3)
	fri	101.97	174.41	142.63	0.047	(1<2)

Table 3 (continued)

Experimental condition	Gene	Geometric mean of intensities in class 1[a]	Geometric mean of intensities in class 2[a]	Geometric mean of intensities in class 3[a]	p-value [b]	Pairwise significant[c]
FS, 48 h	*dal*	188.43	38.83	84.74	0.001	(2<1), (3<1), (2<3)
	gadE	14.22	16.90	35.19	0.003	(1<3), (2<3)
	iap	126.46	94.25	184.36	0.003	(2<1), (1<3), (2<3)
	dat	48.07	159.17	40.46	0.006	(1<2), (3<2)
	clpE	298.74	88.19	277.42	0.008	(2<1), (2<3)
	ami	24.73	17.28	39.34	0.022	(2<3)
	actA	165.73	100.24	81.56	0.027	(2<1), (3<1)
	cysK	36.93	33.79	16.60	0.031	(3<1), (3<2)

[a] Class 1, strain EGDe; Class 2, strain #12; Class 3, strain #3.

[b] Significant at 0.05 level of the t-test.

[c] The 'Pairwise significant' column displays pairs of classes with significantly different gene expression at 95% confidence level or $\alpha = 0.05$. The first number in the brackets indicates the class in which the specific gene is down-regulated when compared to the class indicated with the second number in the brackets.

For example, the first row indicates that in the BHI at 30 min, the *gbuB* gene was significantly down-regulated in the *L. monocytogenes* strain #12 (class 2) compared to *L. monocytogenes* strain EGDe (class 1) and *L. monocytogenes* strain #3 (class 3), while between EGDe (class 1) and #3 (class 3) there was no significant difference in the expression of the specific gene.

Figure legends

Figure 1. Cluster analysis of the gene expression data for the three strains of *L. monocytogenes* incubated in food juices for 30 min (panel 1A) and for 48 h (panel 1B) at 4°C. Data presented were normalized towards *in vitro* control conditions, namely BHI at 4°C for 30 min (panel 1A) or 48 h (panel 1B). Variation in the color indicates level of expression as compared to the normalizing condition, i.e. green, downregulation; red, upregulation.

Figure 2. Cluster analysis of the gene expression data for the three strains of *L. monocytogenes* incubated in food juices and BHI for 30 min and for 48 h at 4°C. Data presented were normalized towards an *in vitro* control condition, namely BHI at 37°C for an overnight period. Variation in the color indicates level of expression as compared to the normalizing condition, i.e. green, downregulation; red, upregulation.
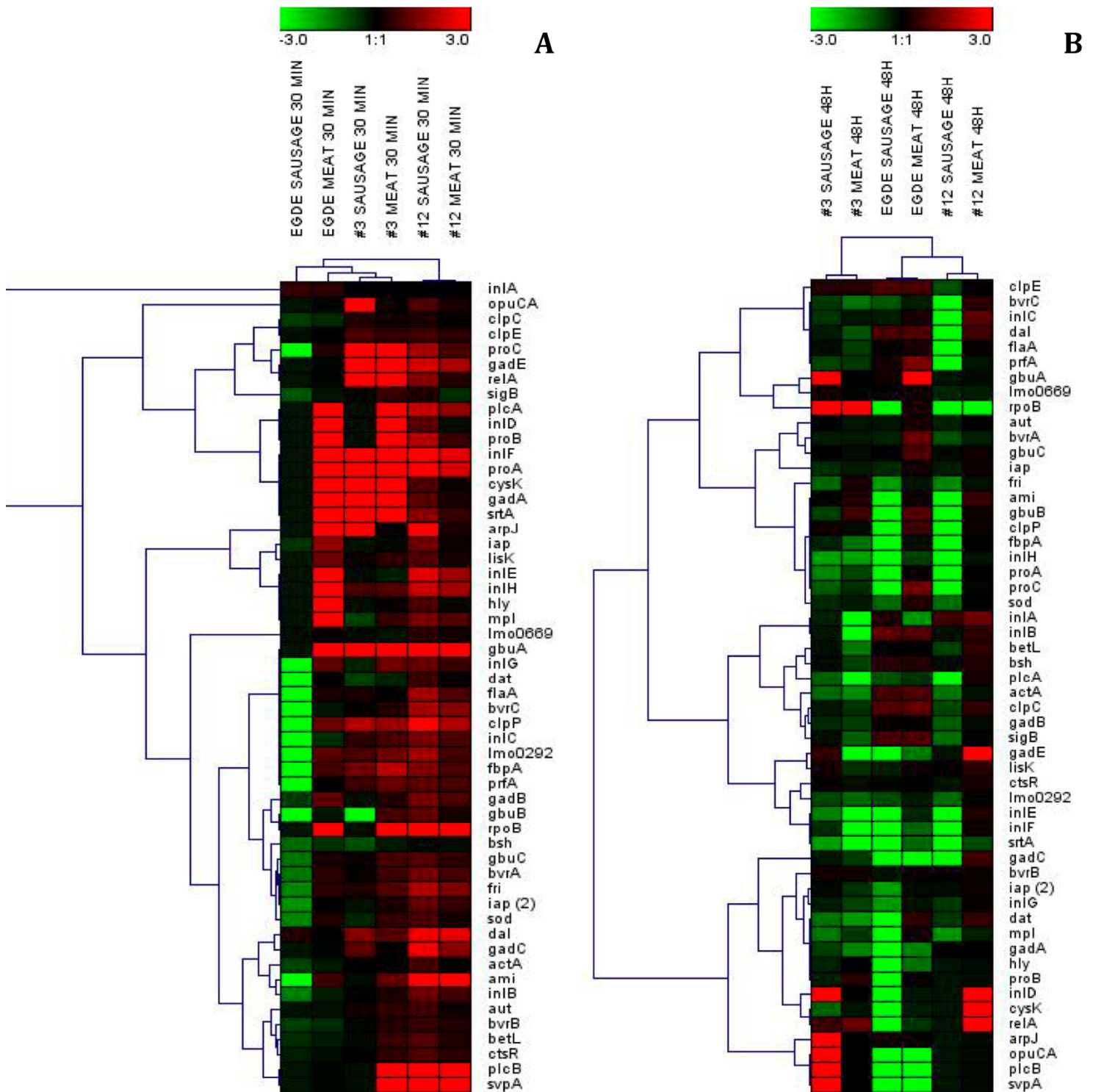
Figure 1

Figure 2