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**Differential gene expression profiling of *Listeria monocytogenes* in Cacciatore and Felino salami to reveal potential stress resistance biomarkers**

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## Abstract

The current study reports a) the *in situ* transcriptional profiles of *Listeria monocytogenes* in response to fermented sausage stress and b) an approach in which *in situ* RT-qPCR data have been combined with advanced statistical techniques to discover potential stress resistance or cell viability biomarkers. Gene expression profiling of the pathogen has been investigated using RT-qPCR to understand how *L. monocytogenes* responds to the conditions encountered during the fermentation and ripening of sausages. A cocktail of five *L. monocytogenes* strains was inoculated into the batter of Cacciatore and Felino sausages. The RT-qPCR data showed that the acidic and osmotic stress-related genes were up-regulated. The transcripts of the *lmo0669* gene increased during the fermentation and ripening of Cacciatore, whereas *gbuA* and *lmo1421* were up-regulated during the ripening of Felino and Cacciatore, respectively. *sigB* expression was induced in both sausages throughout the whole process. Finally, the virulence-related gene *prfA* was down-regulated during the fermentation of Cacciatore. The multivariate gene expression profiling analysis suggested that *sigB* and *lmo1421* or *sigB* and *gbuA* could be used as different types of stress resistance biomarkers to track, for example, stress resistance or cell viability in fermented sausages with short (Cacciatore) or long (Felino) maturation times, respectively.

Keywords: Fermented sausages, gene expression, *L. monocytogenes*, RT-qPCR, stress resistance biomarkers

## 1. Introduction

The widespread distribution of the food-borne pathogen *L. monocytogenes* and its adverse health effects are well known (Kathariou, 2002). It has been shown that *L. monocytogenes* can survive in stressful environments, such as low temperature, and high acidity and salt contents (Cole et al., 1990; Shabala et al., 2001). This pathogen is of great importance for the food industry due to its ability to respond to such stresses, which are highly relevant for food processes (cold, acid and salt) (Kathariou, 2002).

The production of fermented foods is greatly relied on in the hurdle technology concept (Leistner, 2000). It uses combinations of different preservation factors or techniques (temperature, redox potential, pH, water activity, preservatives, etc.), which are named hurdles, to achieve the production of safe, stable, nutritious, tasty and economical food. Fermentation has an inhibitory effect not only on spoilage microorganisms, but also on pathogenic bacteria, which might initially be present (Adams & Mitchell, 2002). Although fermented foods are generally considered as safe foods, some notable outbreaks of food-borne illness associated with fermented food have occurred (Adams & Mitchell, 2002). Several outbreaks of illness have been attributed to the consumption of fermented sausages contaminated with *Staphylococcus aureus* and *Salmonella* spp., and other pathogens, such as *Listeria monocytogenes* and *Escherichia coli* O157:H7, have been identified as causative organisms in outbreaks involving fermented products such as sausages, cheeses, and yogurt (Warburton et al., 1987; Farber & Peterkin, 1991; Beumer, 1997; Nissen & Holck, 1998). Depending on the fermentation conditions, food-borne pathogens may survive at the end of the process.

Water activity and pH constitute significant preservation factors in fermented food (Lucke, 2000). *L. monocytogenes* can trigger changes in the expression of genes relevant to the environmental stresses commonly encountered during fermented sausage manufacturing, such as low pH and water activity (Garner et al., 2006; Olesen et al., 2009; Bae et al., 2012; Walacka-Zacharska et al., 2013). Nowadays, reverse transcription quantitative polymerase chain reaction (RT-qPCR) is considered the method of choice for quantifying the expression of specific genes (Nolan et al., 2006; Desriac et al., 2012). Transcriptomic analysis, combined with predictive microbiology and/or advanced statistical techniques, has been used for the identification of the potential biomarkers involved in bacterial survival, virulence or stress resistance (den Besten et al., 2009, 2010; Ceragioli et al., 2010; Desriac et al., 2012, 2013).

Therefore, the objective of this work was to investigate the mechanism by which *L. monocytogenes* survives in food products after contamination, in particular in fermented meats. Fermented sausages constitute a complex and dynamic environment due to the changes that take place in the extrinsic (e.g. fermentation temperature) and intrinsic (e.g. pH, water activity, redox potential and strong competition for nutrients with starters) factors. Hence, two Italian fermented sausages, characterized by different maturation times, were used in these experiments to examine the gene expression of *L. monocytogenes* under such stressful conditions in order to identify the genes that allow the pathogen to cope with this environment. Furthermore, the results of the present study (gene expression) have been combined with quantitative (inactivation) data from the study of Mataragas et al. (2014) (phenotype) to identify potential stress resistance or cell viability biomarkers using advanced statistical techniques. This can be considered an interesting challenge since biomarkers,

represented by specific genes, could be used to predict the impact of several stresses on bacterial survival.

## **2. Materials and Methods**

### *2.1. Sausage manufacturing, L. monocytogenes inoculation and sampling*

The sausage manufacturing, *L. monocytogenes* inoculation and sampling procedures have been presented in detail elsewhere (Mataragas et al., 2014). In short, the batter of each fermented sausage was inoculated with a cocktail of five *L. monocytogenes* strains (final concentration  $10^5$  to  $10^6$  CFU/ g) previously isolated from minced beef meat (#5, 4b), fresh salami (#19, 1/2b) and pork meat (#36, 1/2a). The remaining two were a human clinical isolate, from a sporadic case of listeriosis (V7, not serotyped) and the reference strain EGDe (1/2a). All the strains were taken from the culture collection of the Laboratory of Agricultural Microbiology (DISAFA, Università di Torino). Two independent trials (two different batches of sausages) were carried out for each product. Two sausage samples were collected from each batch on days 0, 2, 5, 10 and 20 for Cacciatore (short maturation sausage), and on days 0, 3, 7, 10, 20, 40 for Felino (long maturation sausage) after formulation.

A 10-g sausage sample was weighed and placed into a sterile stomacher bag with 90 ml of sterile Ringer solution (quarter-strength ringer solution tablets, Oxoid, Milan, Italy). The sample was homogenized in a stomacher (BagMixer, Interscience, France) for 2 min at normal speed at room temperature. From this  $10^{-1}$  dilution, 1 ml was transferred to an Eppendorf tube, centrifuged at  $13,400 \times g$  for 1 min at  $4^\circ\text{C}$  (Eppendorf 5417R, Eppendorf, Milan, Italy) and 0.5 ml of RNAlater (Ambion, Applied Biosystems, Milan, Italy) was immediately added to the resulting pellet after

the rejection of the supernatant. Then, the samples were stored at -20°C for less than 24 h until the RNA extraction.

## 2.2. Optimization of the qPCR protocol

Altogether eighteen genes were considered for quantification (Tables 1 and 2). One gene related to general stress (*sigB*) and eleven genes relative to various stresses commonly encountered during fermented sausage production, such as acid (*lmo0669* and *lmo2434* or *gadd*) (Sue et al., 2004; Kazmierczak et al., 2006) and osmotic (*gbuA*, *gbuB*, *lmo1421*, *betL* and *opuCA*) stress (Sue et al., 2003; Cetin et al., 2004; Bae et al., 2012), and competition for nutrients (*lmo1038*, *lmo0442*, *lmo0115* and *lmo0938* associated with the uptake of different sugars) (Bae et al., 2012) were taken into consideration. A virulence-related gene was also studied (*prfA*) (Kazmierczak et al., 2006). Finally, five housekeeping genes (*rpoB*, *rplD*, *gap*, *bglA* and *tuf*) (Tasara & Stephan, 2007; Bae et al., 2012) were included as reference genes. Primer sequences and quantitative PCR (qPCR) protocols were taken from the respective literature (Sue et al., 2003; Cetin et al., 2004; Sue et al., 2004; Kazmierczak et al., 2006; Tasara & Stephan, 2007; Bae et al., 2012). The specificity of the primers used in the qPCR protocols for the different genes was tested twice using DNA, extracted from pure cultures of all the bacterial strains, i.e. the inoculated *L. monocytogenes* strains and the lactic acid bacteria (LAB) and coagulase-negative cocci (CNC) used as starters for the production of sausages, as a template. Technological microbiota, isolated from the starter culture used for the sausage production (Mataragas et al., 2014), were used as representative of the LAB and CNC.

## 2.3. DNA extraction

*L. monocytogenes* strains and starters were grown (1% inoculum, incubation at 30°C or 37°C for 24 or 48 hours) in Brain Heart Infusion (BHI) (Oxoid) and deMan, Rogosa and Sharpe (MRS) (Oxoid). After incubation, the bacterial cells were harvested at 13,400 ×g for 1 min at 4°C, washed with a sterile Ringer (Oxoid) solution and subjected to DNA extraction, as previously described (Cocolin et al., 2005). DNA from the reference *L. monocytogenes* strain EGDe was also used to estimate the PCR efficiency of each primer pair used in this study.

#### 2.4. RNA extraction

The procedure described in Rantsiou et al. (2012a) was adopted. The collected Cacciatore and Felino samples were used to extract RNA for the relative quantification of *L. monocytogenes* gene expression. After thawing the samples and rejection of the supernatant, the pellet was suspended in RNAlater (50 µl), and the suspension was treated with lysozyme (50 µl; 50 mg/ml solution) (Sigma, Milan, Italy) and proteinase K (50 µl; 25 mg/ml solution) (Sigma) for 20 min at 37°C. The MasterPure Complete DNA and RNA Purification kit (Epicentre, Madison, WI, USA) was then employed according to the manufacturer's instructions. At the end of the procedure, Turbo DNase (Ambion) was used to eliminate the DNA through enzymatic digestion. Integrity of the extracts was checked using agarose gel (1.2%) electrophoresis, and their quantity and purity were determined using a Nanodrop ND-1000 spectrophotometer (Celbio, Milan, Italy).

#### 2.5. Reverse transcription (cDNA synthesis)

Reverse transcription (RT) was performed in the same way as in Rantsiou et al. (2012a). The total RNA extracted from the sausage samples was reverse transcribed.



RT-positive (RT+) and RT-negative (RT-) reactions, containing *ca.* 0.3 µg of RNA, were performed for each sample. The RT- control was used to evaluate the possibility of contamination of the RNA preparations with genomic DNA (gDNA). The reactions also contained random hexamer primers (Promega, Milan, Italy) and a reaction solution consisting of a 5X RT buffer (Promega), a mix of dNTPs (150 µM of dTTP and 0.7 mM of dATP, dCTP and dGTP) (Promega), an M-MLV Reverse Transcriptase enzyme (Promega) and an RNase ribonuclease inhibitor (Promega), according to the manufacturer's instructions. The reverse transcriptase enzyme was omitted from the RT- control. The RT reactions were performed in a DNA Engine Peltier Thermal Cycler (BioRad, Hercules, CA, USA) using the following program: 25°C for 10 min and 42°C for 50 min. The resulting cDNA was stored at -20°C until the qPCR analysis.

## 2.6. qPCR analysis

The RT+ and RT- samples were analyzed by means of qPCR using the Chromo4 real-time PCR detection system (Bio-Rad). Samples were analyzed in a 96-well plate (Bio-Rad) for each gene of interest. The reaction mixture (final volume, 20 µl) contained 10 µl of SsoAdvanced SYBR Green Supermix (Bio-Rad), 0.8 µl of each primer (400 nM final) (Sigma), 7.4 µl of water and 1 µl of cDNA. A no template control (NTC, blank) was included in each assay. The thermo-cycling program consisted of one hold at 95°C for 2 min, followed by 40 cycles of 15 s at 95°C, 1 min at 55°C (*rpoB*, *rplD*, *gap*, *sigB*, *gbuA*, *gbuB*, *lmo1038*, *lmo0442*, *lmo0115*, *lmo0938*) or 60°C (*bglA*, *tuf*, *betL*, *opuCA*, *lmo1421*, *lmo0669*, *lmo2434*, *prfA*) and 30 s at 72°C. In order to minimize the variance introduced by the instrument between the runs (inter-runs) and

avoid the need for an inter-plate calibrator (IPC), all the samples were assayed for each gene separately in the same plate.

### *2.7. Efficiency determination of the primers*

The PCR efficiency ( $E$ ) of each primer pair was determined by means of the dilution series method using DNA (Rantsiou et al., 2012b) extracted from the reference *L. monocytogenes* strain EGDe as a template (Table 2). The  $E$  values were calculated for each gene according to Pfaffl (2001). Samples of DNA were diluted (106, 53, 26, 5, 1 and 0.26 ng/ml) and used to construct the standard curves. Dilutions were performed three times independently and loaded in single. The presence of outliers was assessed by means of the Grubbs test and linearity of the data by the CLSI EP6-A method (Anonymous, 2003) using GraphPad Prism 6.03 (GraphPad Software, Inc., San Diego, CA, USA) and Microsoft Excel 2007 (Microsoft, Redmond, WA, USA).

### *2.8. Statistical analysis*

The threshold cycle ( $C_t$ ) values from the RT-qPCR were exported to Excel for relative quantification. A pre-processing of the data (missing values and a test for outliers using Grubb's test) was performed to exclude any problematic gene and/or sample. Sometimes, amplification response curves never reach a threshold or a signal never reaches the threshold, but this can be due to the primer-dimer formation. In both cases the  $C_t$  values cannot be considered reliable. Outliers can occur by chance in any distribution, but they are often indicative either of a measurement error or that the population has a heavy-tailed distribution (Kubista et al., 2007). In either case, missing values are generated since such measurements should be removed. In order to use parametric tests for gene expression analysis such as ANOVA, an option is to

replace off-scale data with fictive  $C_t$  values (Kubista et al., 2007). Fictive  $C_t$  values are set to the highest  $C_t$  observed for a truly positive sample, which is assumed to be the level of detection (LOD), plus 1. In the current study, the cutoff was set at 35 cycles. This corresponds to assigning a concentration that is half of the LOD to the off-scale samples. This is no more erroneous than assigning a zero concentration to these samples, because there is no evidence that they are blank. It is only known that the amount of the target in these samples is lower than the detection limit (Kubista et al., 2007). Missing values generated by outlier removal with Grubb's test at 95% of confidence level can be replaced by the mean of the replicates, although no outlier was detected in the current study. However, rows (samples) or columns (genes) containing a high percentage (above 50%) of missing values were removed from the gene expression matrix without further consideration.

Finally, the genes *rpoB*, *rplD*, *gap*, *bglA*, *sigB*, *gbuA*, *gbuB*, *lmo1421*, *lmo0669* and *prfA*, and the samples from day 0, 2, 10 and 20 were selected for Cacciatore. The genes *rpoB*, *rplD*, *gap*, *bglA*, *sigB*, *gbuA*, *lmo0669* and *prfA*, and the samples from days 0, 3, 10, 20 and 40 were selected for Felino for further analysis. Sample integrity of the remaining genes was initially tested using the BestKeeper software (Pfaffl et al., 2004). An intrinsic variance (InVar) of expression was calculated and strongly deviating samples (more than/above a 3-fold over- or under-expression) were indicative of inefficient sample preparation, incomplete reverse transcription or sample degradation.

The data, after PCR efficiency correction and normalization with total RNA, were converted to relative expression and log-values (fold change) (Kubista et al., 2007) for further analysis, that is, one- and two-way ANOVA, principal component analysis (PCA) and partial least square regression (PLSR). Since the objective of the work was

to measure the gene expression in response to the fermentation and ripening processes of both sausages, relative quantification was used instead of absolute quantification (with the standard curve method). Therefore, the level of gene expression of the target genes in the treated samples (during fermentation and ripening) was compared with the level of gene expression in the untreated samples (from day 0) .

PCA was run for the samples (log-transformed data were mean-centered) and for the genes (log-transformed data were auto-scaled) (Kubista et al., 2007). PLSR was run on auto-scaled log-transformed gene expression data, and *L. monocytogenes* inactivation (log CFU/g) (Mataragas et al., 2014) was used as the response (Y-variable). The significance of linear correlation between gene expression and *L. monocytogenes* survival was evaluated using the Pearson correlation coefficient ( $r$ ) at  $P < 0.05$ . Statistical analysis was performed using the Microsoft Excel 2007, SPSS v15.0 (SPSS, Inc., Chicago, Ill., USA), GraphPad Prism 6.03 and Unscrambler X 10.0.1 (Camo Software AS, Oslo, Norway) programs. The stability of the reference genes and total RNA was assessed after PCR efficiency correction using the NormFinder and geNorm applications for Microsoft Excel 2007 (Vandesompele et al., 2002; Andersen et al., 2004). It was assumed that all the RT-qPCR gene expression measurements were comparable since the same reaction conditions were used for reverse transcription and the samples contained the same total amount of RNA (Stahlberg et al., 2004; Duquenne et al., 2010).

### **3. Results**

#### *3.1. Microbiological and physicochemical changes*

*L. monocytogenes* survived well in both sausages (inactivation less than 1 log cfu/g) (Mataragas et al., 2014). LAB increased by 2.3-2.8 and 1.6-1.9 log cfu/g within the

first days of fermentation of Cacciatore and Felino, respectively (approximately from 6.5 to 9.0 log cfu/g) and then remained unchanged. CNC showed a slight decrease over the first two days of fermentation of Cacciatore (from 6.4 to 6.0-6.1 log cfu/g) and then remained constant. However, CNC increased slightly on the third day of fermentation of Felino (from 6.1-6.4 to 6.6-6.7 log cfu/g) and then remained unchanged (Mataragas et al., 2014).

A rapid decrease in the pH was observed during the first days of fermentation for Cacciatore, from 5.7 to 4.9-4.8 on the second day of fermentation, and then it remained almost constant. Slower fermentation was observed in Felino, from 5.8-5.9 to 5.2 after three days of fermentation, and then it approached its maximum decrease (5.1) at the end of fermentation (day 7), while a slight increase (5.3) was observed at the end of the process (day 40) (Mataragas et al., 2014). Both sausages presented slow ripening. The water activity decreased constantly from 0.976-0.978 to 0.922-0.923 in Cacciatore, while the water activity in Felino started to fall from day 10 after formulation (from 0.964-0.971 to 0.928-0.936) (Mataragas et al., 2014).

### *3.2. RNA extraction from fermented sausages, primer efficiency and quality of RT-qPCR data*

In order to quantify the gene expression of *L. monocytogenes* during the manufacturing of Cacciatore and Felino, the total RNA was extracted from the respective samples. The RNA extraction yield from all the samples ( $N = 44$ ) was on average  $67.9 \pm 38.0$  ng/ $\mu$ l. Agarose gel electrophoresis showed that the RNA extracted from the samples was intact (i.e. the 16S and 23S subunits were clearly visible without signs of degradation, as described by Cocolin and Rantsiou, 2014). Initially, eighteen genes were considered, five as references and thirteen as targets.

However, after determination of the specificity, twelve genes were selected (four as references and eight as targets) (Table 1). The  $E$  values, estimated from the slope of the linear regression plot of  $C_t = f(\log\text{DNA})$  (Fig. 1), are also shown in Table 2. No outliers were detected in the data obtained for the primer efficiency determination, and the regression line was found to be linear over the tested range of the DNA concentrations. After pre-processing, no outliers were found in the gene expression data. The InVar calculation showed that all the samples were below 3-fold over- or under-expression, thus indicating that sample integrity was within an acceptable range (Table 3). Therefore, the gene expression data were considered suitable for further statistical analysis.

### 3.3. *L. monocytogenes* genes considered during sausage production

The batter of the sausages was inoculated with the cocktail of five *L. monocytogenes* strains. Therefore, the transcriptional profiles of the multiple *L. monocytogenes* strains are reported in response to the various stresses commonly encountered during sausage production. Although the different *L. monocytogenes* strains inoculated may behave differently, the cumulative result obtained in this study can be considered a good indicator to describe the behavior of *L. monocytogenes* during the manufacturing of sausages. Stress-related genes were taken into account since the objective was to investigate how *L. monocytogenes* copes with the highly stressful environment of a fermented sausage. The only virulence-related gene studied was *prfA*, which regulates the expression of many other virulence genes, and its expression could indicate the potential expression of genes associated with virulence. An optimization of the qPCR protocol was carried out to verify the specificity of the assay that included the LAB

and CNC strains used as starters in the sausage production. An amplification signal was only obtained for *L. monocytogenes*.

#### *3.4. Selection of normalizers*

The expression stability of four potential reference genes was investigated throughout the manufacturing process of both sausages. On the basis of the NormFinder and geNorm results, potential normalizers were found for the Felino sausage, but not for the Cacciatore one, where the results between NormFinder and geNorm did not coincide (data not shown). geNorm indicated that the transcriptional stability of the reference genes may differ substantially, since the *M*-value of the best pair of genes was above the limit of 1.5. According to Vandesompele et al. (2002), *M*-values below 1.5 mean that these genes present good expression stability. Therefore, normalization with the reference genes was compared to normalization with the total RNA. The results showed that normalization with the total RNA was the best choice. For samples with good quality RNA, normalization with the total amount of RNA is often as good as normalizing with a single reference gene (Kubista et al., 2006; Bergkvist et al., 2008). Hence, when the detection of appropriate reference genes for normalization purposes is difficult, the choice of using the total RNA of good quality as a normalizer can be considered a sound alternative.

#### *3.5. Differential gene expression of *L. monocytogenes* in fermented sausages*

The differential gene expression of the food-borne pathogen *L. monocytogenes* was studied in response to the stress conditions that prevailed during the manufacturing of Cacciatore and Felino. The results from the RT-qPCR revealed that the genes related to acidic and osmotic stresses were up-regulated during the process (Fig. 2). The

graph shows the changes in expression of each gene throughout the different process stages (fermentation, ripening and the end of the process) relative to the control (day 0, start of the process). Only the genes for which good quality  $C_t$  values were obtained, during the RT-qPCR experiments, were analyzed and are displayed in the graph. No  $C_t$  values were obtained for the *lmo1038* and *lmo0442* genes in Cacciatore or Felino, while a few sporadic  $C_t$  values were obtained in Felino for the *gbuB* and *lmo1421* genes. Thus, the aforementioned genes in the respective sausages were excluded from the analysis. During fermented sausage production, significant changes were observed in the expression for the *sigB* (in both sausages), *gbuA* (Felino), *lmo0669*, *lmo1421* and *prfA* (Cacciatore) genes.

One-way ANOVA was employed to compare the gene expression of each process stage against the control condition in order to reveal which changes were significant. The corrected  $P$ -value of 0.01 was used as the limit of significance because of the multiple testing, i.e. multiple genes were compared simultaneously. For the Cacciatore samples, the *sigB*, *lmo0669*, *prfA* and *lmo1421* genes showed significant expression differences ( $P < 0.01$ ). The *sigB* gene was up-regulated in all the Cacciatore manufacturing stages. Up-regulation was also observed for the *lmo1421* gene, at the end of the process, and for *lmo0669* during the fermentation and ripening processes. Down-regulation of the *prfA* gene occurred during the fermentation process (Fig. 2a). Significant differences were found for the *sigB* and *gbuA* genes for the Felino samples. Both genes were up-regulated ( $P < 0.01$ ). A significant increase in the expression of *sigB* and *gbuA* was observed during the manufacturing of Felino (all stages) and late ripening (end of process), respectively (Fig. 2b).

Two-way ANOVA was run to investigate whether the gene expression between the two types of sausages was different due to the different maturation times (factor A,



referred to as time) or to the product composition/technology (factor B, referred to as product). Only the genes common to both sausages, i.e. *sigB*, *gbuA*, *lmo0669* and *prfA*, were investigated. The results showed that, for the *sigB* and *gbuA* genes, there was a significant effect of time and product ( $P < 0.01$ ), as well as a significant interaction (time  $\times$  product) between these two variables ( $P < 0.01$ ). The interaction effect indicated that maturation time had a different effect on gene expression, depending on the product composition and fermentation technology. The *sigB* gene was over-expressed during the manufacturing of Cacciatore and Felino, but the gene depicted higher fold change values in Cacciatore, especially during fermentation and ripening (Fig. 2). The *gbuA* gene increased its expression along the Felino manufacturing process and displayed higher fold change values compared to the Cacciatore samples (Fig. 2). There was only a significant effect ( $P < 0.01$ ) of time for the *lmo0669* gene, which means that the expression of the gene increased over time, primarily during fermentation and secondarily during ripening, irrespective of the product type (product composition or fermentation technology) (Fig. 2). Finally, time and product displayed a significant effect ( $P < 0.01$ ) on *prfA* expression. By ignoring the main effect of the product, the *prfA* expression appeared to be altered during the manufacturing of sausages, and a decrease in its expression was mainly depicted at the fermentation stage. By ignoring the main effect of time, the *prfA* expression appeared to be different in Cacciatore and Felino (Fig. 2).

### *3.6. Multivariate gene expression profiling of L. monocytogenes in fermented sausages*

On the basis of the PCA results, the first two and three PCs were extracted for Cacciatore and Felino, respectively. The expression of *gbuA*, *lmo0669*, *sigB* and

*lmo1421* seemed to be important during the fermentation and ripening (samples from day 2, 3, 10 and 20) of Cacciatore (*gbuA*, *lmo0669*, *sigB* and *lmo1421*) and Felino (*sigB* and *lmo0669*), since both the samples and the genes displayed positive PC1 (Fig. 3) or PC3 (Fig. 4) values. In other words, *lmo0669* and *gbuA* were mainly expressed during the fermentation of Cacciatore (day 2) (positive PC2 values), while *sigB* and *lmo1421* were mainly associated with Cacciatore samples from day 10 (ripening) and day 20 (end of process) (negative PC2 values). In the case of Felino, *gbuA* was mainly expressed at the end of the process (day 40) (both had positive PC1 and negative PC3 values). The *gbuB* and *prfA* genes were negatively correlated to the Cacciatore samples taken during the process stage (after day 0, i.e. 2, 10 and 20), thus indicating that the expression of these genes was suppressed or remained unchanged. The *prfA* expression in the Felino samples also seemed to be suppressed or to remain unchanged (samples from day 0 and *prfA* had negative PC1 and PC3 values).

The *L. monocytogenes* gene expression (X-variables) was combined with its inactivation (Y-variable) to identify potential biomarkers. On the basis of the PLSR results, *L. monocytogenes* survival appeared to be highly related to a) *sigB*, *lmo1421*, *lmo0669* and *prfA* in the Cacciatore samples (Fig. 5a), but also to b) *sigB*, *gbuA* and *prfA* in the Felino samples (Fig. 5b). The *gbuA* and *gbuB* genes in Cacciatore, and *lmo0669* in Felino showed insignificant responses, thus indicating no substantial contribution to the model and no correlation to *L. monocytogenes* survival. On the basis of the Pearson correlation results, the expression patterns of *sigB* ( $r = 0.51$ ,  $P = 0.044$  for Cacciatore;  $r = 0.63$ ,  $P = 0.003$  for Felino), *lmo1421* ( $r = 0.57$ ,  $P = 0.020$  for Cacciatore) and *gbuA* ( $r = 0.70$ ,  $P = 0.001$  for Felino) were linearly correlated to bacterial resistance, but those of *lmo0669* ( $r = 0.01$ ,  $P = 0.986$  for Cacciatore) and *prfA* ( $r = 0.49$ ,  $P = 0.055$  for Cacciatore;  $r = -0.13$ ,  $P = 0.584$  for Felino) were not.

## 4. Discussion

### 4.1. Differential gene expression profiling of *L. monocytogenes* in fermented sausages

Fermented sausages constitute a highly stressful environment for any pathogen present in the matrix, because of changes in the extrinsic (e.g. fermentation temperature) and intrinsic (e.g. pH,  $a_w$  and competitive microbiota) factors. Such changes are a signal of the activation of general and/or stress-specific genes, which allow the pathogen to adapt to the fermented sausage environment. The main hurdles encountered in fermented sausages are the pH and  $a_w$  reduction, and the presence of competitive microbiota. Thus, general (*sigB*) and stress-specific genes (*gbuA*, *gbuB*, *lmo1421*, *lmo0669*, *betL*, *opuCA*, *lmo2434*, *lmo1038*, *lmo0442*, *lmo0115* and *lmo0938*) have been considered. The master gene, *prfA*, which regulates the expression of many virulence-related genes, was also studied to investigate the possibility of the modulation of the expression levels of virulence genes due to stress. The protocols developed by Rantsiou et al. (2012a) for the extraction of RNA have been used. As described in that study, the gene expression of *L. monocytogenes* did not change during sample preparation, thus allowing a detailed and reliable description to be obtained of its transcriptomic profile. As reported in Table 2, not all the primers selected from the literature could be used in the present study, due to the lack of specificity. In fact some of them also gave amplification signals for the LAB and CNC strains contained in the starter culture inoculated for the sausage fermentation. This could be related to the reaction conditions and the amplification cycles adopted in the study, which were not specific for *L. monocytogenes*, rather than to a lack of specificity of the primers described in the previous papers.

The qPCR results showed that the transcripts of *sigB*, associated with general stress, was up-regulated in both sausages throughout the manufacturing process, from early fermentation until late ripening (end of process). The data indicated that this stress regulator gene may be involved in the stress adaptation of *L. monocytogenes*, which is known to have contaminated fermented sausages. Sigma(B) regulon contains several stress response and virulence genes, as well as gene regulators (Kazmierczak et al., 2003). The *lmo0669* gene, which is related to acid-stress, was found to have increased transcripts, primarily during the fermentation of Cacciatore and secondly during its early ripening. Cacciatore belongs to the class of rapid fermentation sausages (fermentation lasts about 48-72 h) with a short maturation time (ripening lasts up to 20 days). A dramatic (5.7 to 4.9-4.8) and rapid (0.43 units per day) decrease in pH was observed during its fermentation (Mataragas et al., 2014). These changes may trigger the expression of the gene. Sue et al. (2004) have demonstrated that *lmo0669* is up-regulated during acid stress conditions. Furthermore, the qPCR data showed that the expression of the *prfA* gene was suppressed during this stage. The expression of stress-related genes is probably enhanced and the transcription of other non stress-related genes is repressed to facilitate *L. monocytogenes* survival in highly stressful environments. Jiang et al. (2010) found that *L. monocytogenes* strains, after exposure to the conditions that prevail in the gastrointestinal tract, enhanced the expression of stress-related genes and decreased the transcription of an adhesion-related gene in order to survive in the diverse microenvironment. The vast majority of published works, which, in most of the cases, have indicated an up-regulation of the *prfA* gene, have been conducted *in vitro* with laboratory-based media. The long-term adaptation of *L. monocytogenes* strain 4140 to acidic stress using laboratory-based media induced the transcription of genes associated with a stress response and invasion,

including *prfA*, but the strain EGD-e showed no change in the expression of the *prfA* gene (Olesen et al., 2009). On the other hand, an overall reduction in virulence gene expression was noted in studies conducted in food matrices (Olesen et al. 2010; Rieu et al. 2010). Thus, significant differences can be observed in gene expression between *in situ* and *in vitro* experiments. Rantsiou et al. (2012b) stressed the need to perform gene expression experiments in real food samples instead of standard broth systems. The general trend that has emerged from published studies is that the relative transcription of certain virulence genes is higher in laboratory broths than in real food matrices (Palumbo et al., 2005; Duodu et al., 2010; Olesen et al. 2010; Rieu et al., 2010). Furthermore, it has been suggested that a food matrix, in particular a meat-based one, may influence the virulence potential of *L. monocytogenes*, possibly through the down-regulation of virulence genes (Mahoney and Henriksson 2003; Olesen et al. 2010). Finally, the ability of *L. monocytogenes* to adapt to osmotic stress, due to  $a_w$  reduction during ripening, was found to be modulated by the expression levels of transporter genes such as *lmo1421* (Cacciatore) and *gbuA* (Felino). Both genes have been found to increase transcripts during osmotic stress (Sue et al., 2003; Bae et al., 2012). It is worth mentioning that the genes that were up-regulated during acidic (*lmo0669* in Cacciatore) and osmotic (*lmo1421* and *gbuA* in Cacciatore and Felino, respectively) stress are sigma(B)-dependent (Sue et al., 2003, 2004; Cetin et al., 2004). *gbuA* is transcribed from dual promoters, one of which is sigma(B)-dependent (Cetin et al., 2004).

Two-way ANOVA showed that *sigB* expression was higher in Cacciatore than in Felino. This could be related to the intensiveness of the stress that prevailed during the manufacturing of the sausages. The fermentation and ripening processes were more intensive in Cacciatore than in Felino, and resulted in more abrupt pH ( $0.43 \pm$

0.01 vs.  $0.22 \pm 0.01$  units per day, respectively) and  $a_w$  ( $0.0030 \pm 0.0001$  vs.  $0.0010 \pm 0.0001$  units per day, respectively) reductions (Mataragas et al., 2014). The analysis also showed that the depicted *lmo0669* gene increased transcripts, irrespective of the product type, although this increase was not found to be significant in Felino by means of one-way ANOVA. This result could be due to the milder conditions prevailing in Felino, in which the *lmo0669* expression was significantly lower than that of Cacciatore, or other genes could be involved in acid stress adaptation such as the *gadCB* operon, which encodes a glutamate/gamma-aminobutyrate antiporter and a glutamate decarboxylase, respectively, or the *lmo2434* or *gadD* gene, which encodes a putative glutamate decarboxylase (Wemekamp-Kamphuis et al., 2004), none of which were studied in this work. The latter gene (*lmo2434* or *gadD*) was excluded during the optimization of the qPCR protocol. *In vitro* reverse transcriptase PCR experiments have indicated that the transcription of all three known compatible solute uptake systems (*opuC*, *betL* and *gbu*), as well as a gene that is predicted to encode a compatible solute transporter subunit (*lmo1421*) is induced in response to elevated osmolarity (Fraser et al., 2003).

#### *4.2. Multivariate gene expression profiling of L. monocytogenes in fermented sausages*

Although parametric tests, such as one- and two-way ANOVA, can provide an indication of what differences are significant between two or more conditions, when multiple samples, each containing the expression of multiple genes, are analyzed, the proper way is to use multivariate methods (Kubista et al., 2006; Bergkvist et al., 2008) since gene expressions tend to be correlated (violation of the parametric test assumption of independence). Multivariate gene expression profiling through PCA

was run to classify the samples and genes, and to investigate the relationships between the variables. PCA confirmed the ANOVA results, but also revealed that *gbuA* in Cacciatore and *lmo0669* in Felino could play a role in the osmotic and acidic stress adaptation of *L. monocytogenes*, respectively. This was not identified by means of ANOVA.

PLSR was used to combine gene expression with bacterial survival (phenotype) to identify potential biomarkers and/or for prediction purposes (Desriac et al., 2014).

However, the objective of using PLSR in the present study was to identify any potential biomarkers of bacterial survival rather than to develop a predictive model.

On the basis of the PCA results, although the *gbuA* gene seemed to contribute to osmotic stress resistance during the fermentation of Cacciatore, the expression of the *lmo1421* gene was probably more important, as indicated by the PLSR analysis. On the basis of the PLSR results, the *gbuA* gene explained less than 50% of the variation in the data and its correlation to *L. monocytogenes* inactivation was rather limited.

Another finding of PCA was the potential role of *lmo0669* expression in *L. monocytogenes* acid resistance in both sausages. Although *lmo0669* is significant, this gene did not show a linear correlation (Cacciatore) or even showed no correlation at all (Felino) to bacterial survival, according to PLSR. The gene depicted a transient up-regulation of its expression at the early stages of sausage manufacturing in Cacciatore and then this expression gradually reduced. Desriac et al. (2013) also found biomarkers that showed a transiently up-regulated gene expression linked to increased resistance over time, which were identified as long-acting biomarkers. The authors underlined the importance of also considering non-linear correlations, particularly when focusing on the transcriptional level, in order to find relevant biomarkers.

The PLSR analysis showed that it is possible to track resistance ( $k_{max} = 0.04 \ln$  CFU/g/day) (Mataragas et al., 2014) in rapid fermented sausages with a short maturation time (Cacciatore) (*sigB* and *lmo1421*) or higher resistance ( $k_{max} = 0.02 \ln$  CFU/g/day) (Mataragas et al., 2014) in slow fermented sausages with a long maturation time (Felino) (*sigB* and *gbuA*). In both cases, the up-regulation of *sigB*, which is involved in the general stress response, was observed. This increase seems to be associated with an early stress response during the fermentation and maintenance of such an expression throughout the whole process. A gene involved in osmotic stress adaptation (*lmo1421* or *gbuA*), which was different for each product, was also identified. Furthermore, *prfA* down-regulation and *lmo0669* up-regulation could be considered as additional biomarkers of cell viability in fermented sausages, as indicated by the multivariate gene expression profiling, but further studies should be performed to confirm this since the Pearson correlation showed no significant linear correlation between *prfA* or *lmo0669* and *L. monocytogenes* survival.

## 5. Conclusions

The environmental conditions that prevail during sausage manufacturing may stimulate the expression of general and/or stress-specific genes and the intensiveness of these stresses may have an impact on their expression (fold change). The results of this study could help to extend the use of the identified biomarkers to other similar products and/or stresses, and rationalize a decision of developing a predictive model for bacterial resistance. In this context, further investigation using a larger number of target genes and/or other bacterial physiological states are required to accurately predict *L. monocytogenes* resistance, although the current work provides information that the most relevant genes that reflect specific bacterial resistance could be selected



and then combined for use as a predictive tool. Furthermore, additional studies will help to elucidate the role of the *lmo0669* and *prfA* genes as potential biomarkers, and also whether other genes that are parts of the glutamate decarboxylase acid resistance system (GAD) of *L. monocytogenes* are involved in the acid adaptation of the pathogen present in slow fermentation and long maturation time sausages. Finally, this study reports the *in situ* transcriptional profiles of *L. monocytogenes* in response to fermented sausage stress, and thus contributes to a better understanding of the stress adaptation of the pathogen.

## **6. Acknowledgements**

The present work was supported by the FP7-People-2011-CIG (LisGenOmics) project through the granting of a Marie Curie scholarship (Marie Curie - Career Integration Grant) to M. Mataragas (Grant Agreement no PCIG09-GA-2011-293406). The authors would also like to acknowledge the contribution of two Erasmus students, Maria Kypriadi and Anastasia Aletra, who performed a part of the experiments for their BSc thesis.

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**Figure legends.**

**Fig. 1.** A representative standard curve of the *lmo0669* gene. The slope of the regression line ( $y = -3.3622x + 31.829$ ) was used to determine the efficiency ( $E = 10^{(-1/\text{slope})} = 98\%$ ) of each primer pair. The solid line is the regression line; the dashed lines are the 95% confidence bands of the regression line; the solid circles are the observed data.

**Fig. 2.** Fold change of *L. monocytogenes* genes during the manufacturing of a) Cacciatore and b) Felino sausages relative to the control (day 0, start of process and immediately after inoculation). White bar, fermentation; bar with squares, ripening; and bar with horizontal lines, end of process. The error bars represent the standard deviation. The asterisk indicates that expression is significantly ( $P < 0.01$ ) different between that stage and the control.

**Fig. 3.** PCA classification of the a) Cacciatore samples and b) *L. monocytogenes* genes for which their expression was measured.

**Fig. 4.** PCA classification of the a) Felino samples and b) *L. monocytogenes* genes for which their expression was measured.

**Fig. 5.** PLSR correlation loading plots based on the measured variables as predictors (X) and *L. monocytogenes* inactivation as the response variable (Y) for the a) Cacciatore and b) Felino sausages during their manufacturing. The outer and inner ellipses show 100% and 50% of the explained variance, respectively.

**Table 1**

Function of the reference and target genes of *L. monocytogenes* considered in the challenge tests with Cacciatore and Felino sausages.

Genes	Function	Stress related
<i>rpoB</i> <sup>a</sup>	DNA-directed RNA polymerase subunit beta	Reference gene
<i>rplD</i>	50S ribosomal protein L4	Reference gene
<i>Gap</i>	highly similar to glyceraldehyde-3-phosphate dehydrogenase	Reference gene
<i>bgIA</i>	6-phospho-beta-glucosidase	Reference gene
<i>Tuf</i>	highly similar to translation elongation factor EF-Tu	Reference gene
<i>sigB</i>	RNA polymerase sigma factor SigB	Regulation of virulence and stress-response genes/Target gene
<i>gbuA</i>	very similar to glycine betaine ABC transporter (ATP-binding protein)	Adaptation (osmotic stress)/Target gene
<i>gbuB</i>	very similar to glycine betaine ABC transporters (permease)	Adaptation (osmotic stress)/Target gene
<i>lmo1421</i>	similar to glycine betaine/carnitine/choline ABC transporter, ATP-binding protein	Adaptation (osmotic stress)/Target gene
<i>lmo0669</i>	similar to oxidoreductase	Adaptation (acid stress)/Target gene
<i>prfA</i>	listeriolysin positive regulatory protein	Regulation of virulence genes/Target gene
<i>lmo1038</i>	PTS system encoding enzyme II cytoplasmic subunits for the transport of major carbon sources	Glucose uptake/Target gene
<i>lmo0442</i>	PTS system encoding enzyme II cytoplasmic subunits for the transport of major carbon sources	Fructose uptake/Target gene
<i>lmo0115</i>	PTS system encoding enzyme II cytoplasmic subunits for the transport of major carbon sources	Mannose uptake/Target gene
<i>lmo0938</i>	PTS system encoding enzyme II cytoplasmic subunits for the transport of major carbon sources	Cellobiose uptake/Target gene
<i>betL</i>	glycine betaine transporter	Adaptation (osmotic stress)/Target gene

<i>opuCA</i>	similar to glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)	Adaptation (osmotic stress)/Target gene
<i>lmo2434</i> or <i>gadD</i>	highly similar to glutamate decarboxylases	Adaptation (acid stress)/Target gene

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<sup>a</sup> The genes in bold were selected for further analysis on the basis of the results of the optimization of the qPCR protocol using *L. monocytogenes* strains, *Lb. sakei* and *S. xylosus*.

**Table 2**

Primer pairs along with their efficiency values for the reference and target genes of *L.*

*monocytogenes* considered in the challenge tests with Cacciatore and Felino sausages.

Genes	Primers <sup>a</sup>	Reference	<i>E</i> <sup>b</sup>	<i>R</i> <sup>2</sup>
Housekeeping				
<i>rpoB</i>	F:AATCGGGGACAATGACT R:GTGTGCGGAAACCTAC	Tasara & Stephan, 2007	84	0.94
<i>rplD</i>	F:GTATTCGGCCCAACAC R:AGCACCTCCTCTACTT	Tasara & Stephan, 2007	97	0.95
<i>gap</i>	F:ACCAGTGTAAGCGTGAA R:TCACAGCGCAAGACAAA	Tasara & Stephan, 2007	99	0.94
<i>bglA</i>	F:GCCTACTTTTTATGGGGTGGAG R:CGATTAAATACGGTGCGGACATA	Tasara & Stephan, 2007	ND	ND
Target				
<i>sigB</i>	F:TCATCGGTGTCACGGAAGAA R:TGACGTTGGATTCTAGACAC	Bae et al., 2012	97	0.93
<i>gbuA</i>	F:TTGAAAAAGATGGTCCTCG R:ATCTTCGGTTACAGCAATCG	Bae et al., 2012	96	0.92
<i>gbuB</i>	F:TGGTATTTGGATGGCGAA R:CAATTACGACCATGGAAAGT	Bae et al., 2012	94	0.94
<i>lmo1421</i>	F:CCACTGACAACCTGGAACCATTATA R:GAAAGAGCGCAATTTGTTGTA AAA	Sue et al., 2003	93	0.90
<i>lmo0669</i>	F:TCAAGCTATCAAGGCGCTAATAAA R:CCGACCAATTCCGGAGTCT	Sue et al., 2004	98	0.95
<i>prfA</i>	F:CAATGGGATCCACAAGAATATTGTAT R:AATAAAGCCAGACATTATAACGAAAGC	Kazmierczak et al., 2006	92	0.93
<i>lmo1038</i>	F:GGCTTAGAAACCGTATCCTT R:CCTGCTTCTGCCTTAGTTAC	Bae et al., 2012	ND	ND
<i>lmo0442</i>	F:GAAGAAATGGCAGAAATG R:GTCAGAATCAGTAATCGCCA	Bae et al., 2012	ND	ND

<sup>a</sup> F, forward; R, reverse

<sup>b</sup> *E*, efficiency of the primer pair estimated from the standard curves using the  $10^{(-$

$1/\text{slope})$  equation. An *E* value equal to 1.84 means 84% PCR efficiency. The

amplification efficiency was not determined for the *bglA*, *lmo1038* and *lmo0442*

genes because no gene results were found for the Cacciatore and/or Felino samples,

i.e. no *C<sub>t</sub>* values were obtained.

Table 3

Calculation of the intrinsic variance (InVar) of expression of the remaining genes.

Trials	InVar ( $\pm$ 3-fold) <sup>a</sup>					
	S0 <sup>b</sup>	S2 <sup>b</sup>	S3 <sup>b</sup>	S10 <sup>b</sup>	S20 <sup>b</sup>	S40 <sup>b</sup>
<i>Cacciatore</i>						
Trial #1	0.13	0.02		0.02	0.24	
Trial #2	0.15	0.06		0.05	0.12	
Trial #3	0.13	0.04		0.26	0.04	
Trial #4	0.13	0.04		0.01	0.15	
<i>Felino</i>						
Trial #1	0.01		0.14	0.01	0.25	0.24
Trial #2	0.04		0.13	0.10	0.24	0.04
Trial #3	0.01		0.12	0.05	0.24	0.10
Trial #4	0.03		0.13	0.12	0.24	0.13

<sup>a</sup> Strongly deviating samples ( more than a 3-fold over- or under-expression) are indicative of inefficient sample preparation, incomplete reverse transcription or sample degradation

<sup>b</sup> Each symbol stands for the day for which the samples were taken. The samples were taken at 0, 2, 10 and 20 days after formulation for Cacciatore and at 0, 3, 10, 20 and 40 days after formulation for Felino.

Fig. 1

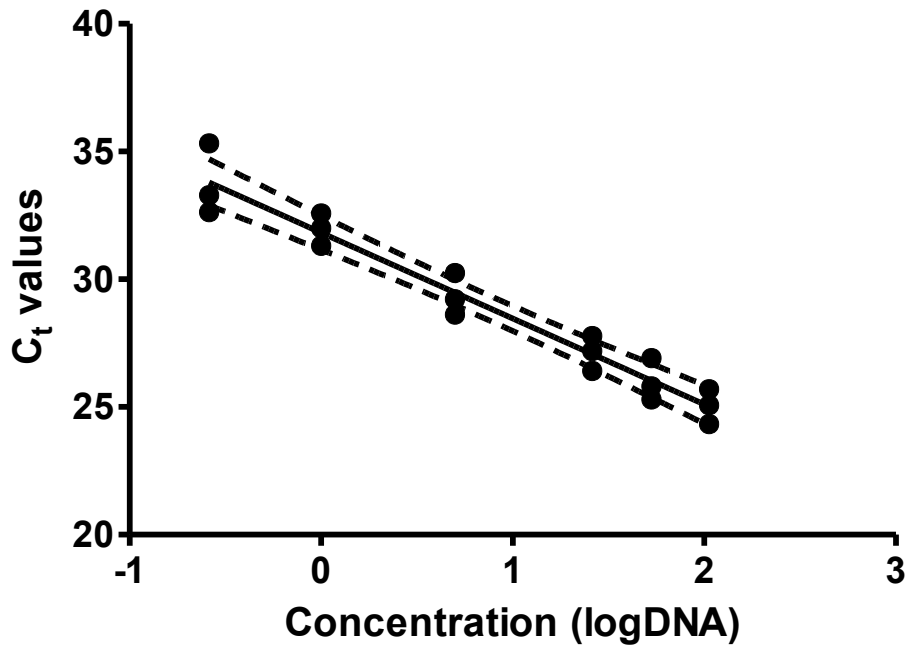




Fig. 2

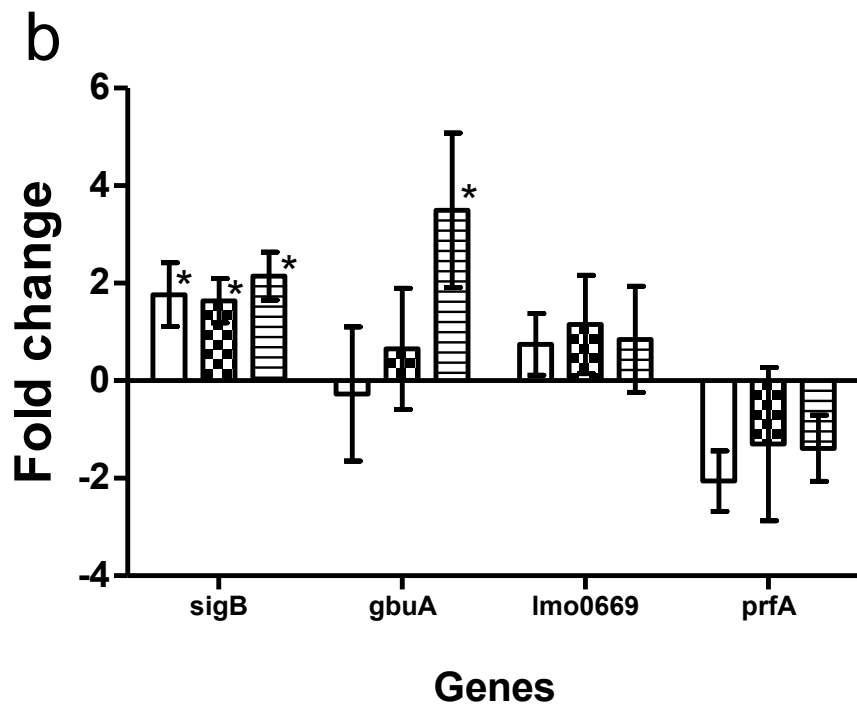
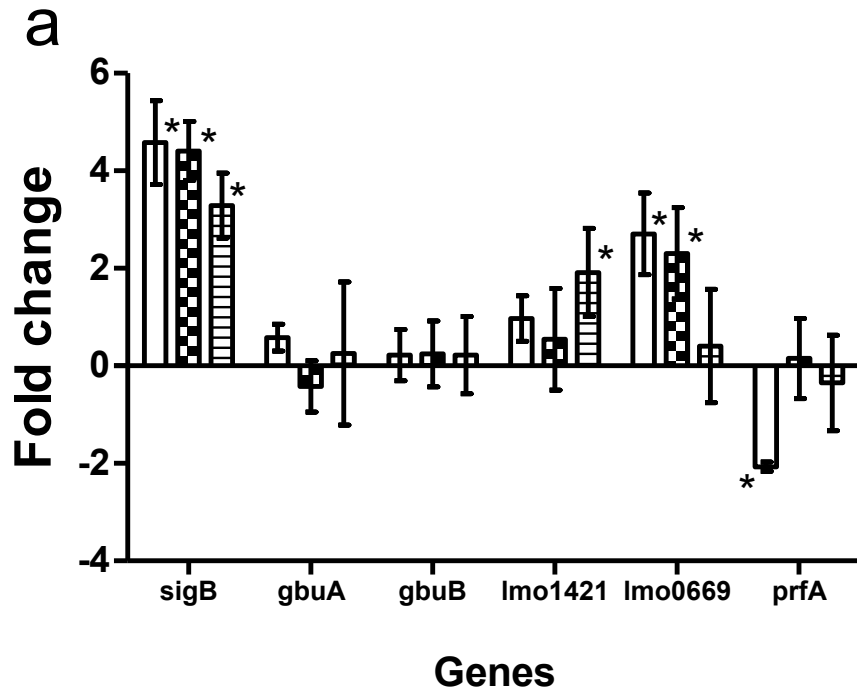


Fig. 3

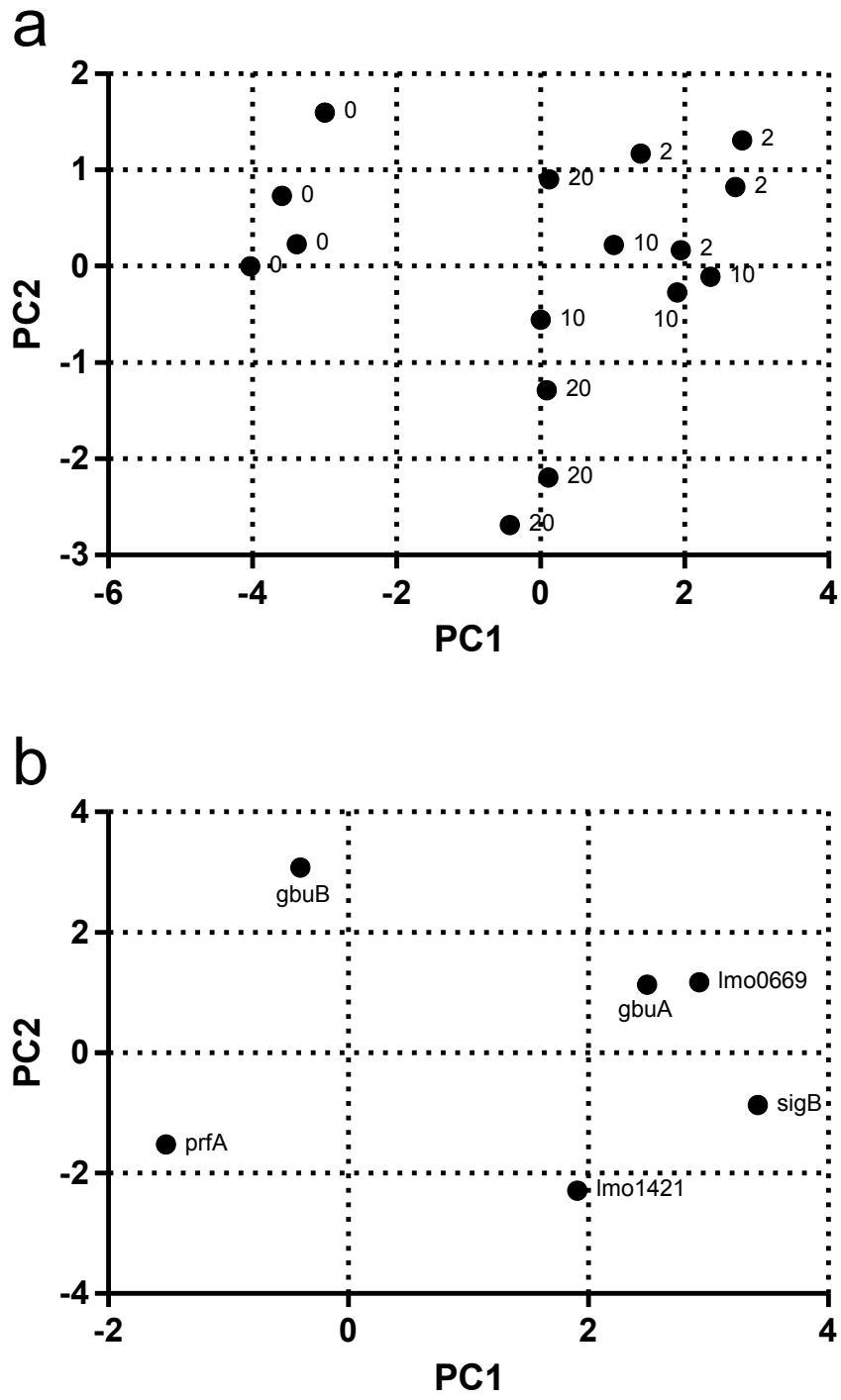


Fig. 4

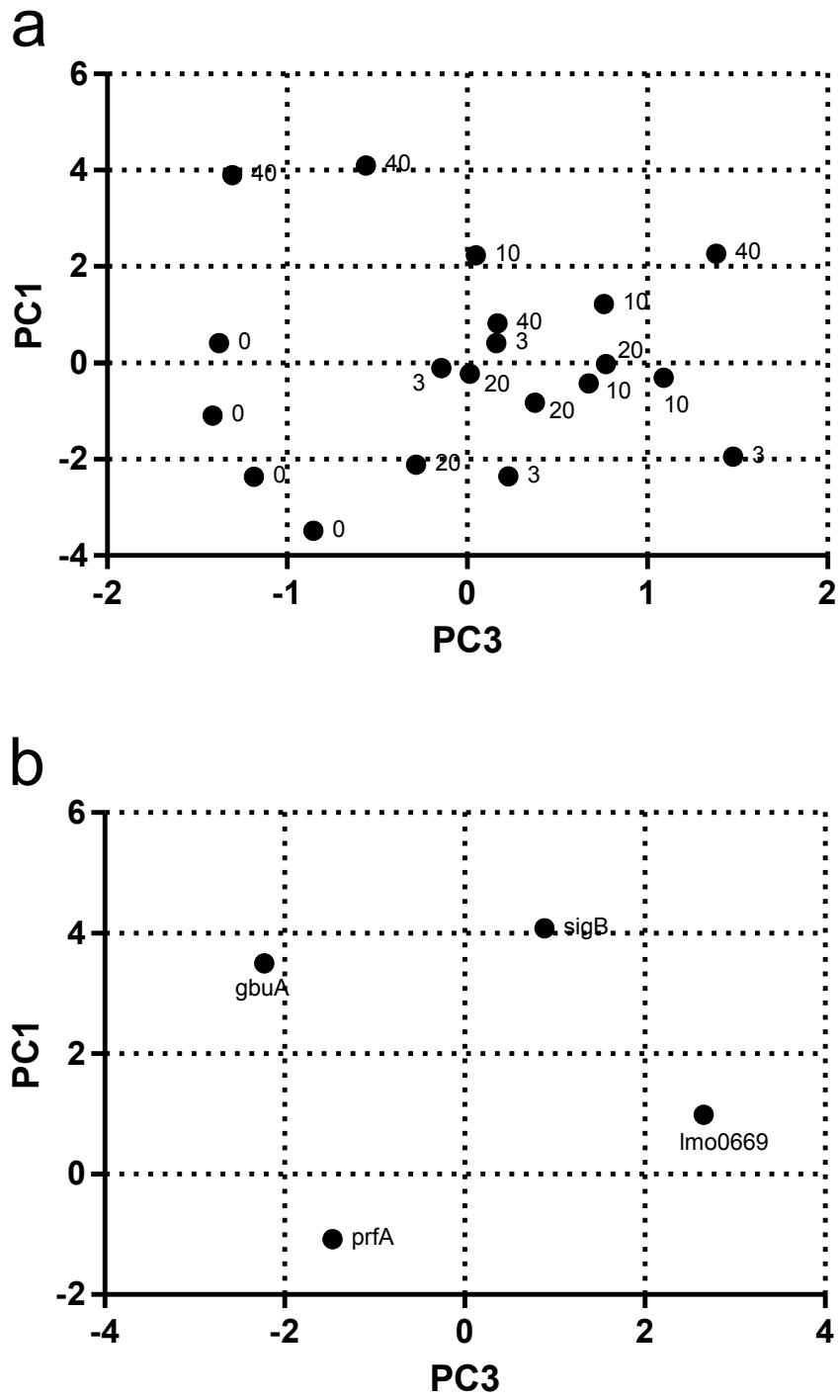


Fig. 5

