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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.

1. Introduction

The widespread distribution of the food-borne pathogen *Listeria 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 and 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 and 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 *L. 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 and Peterkin, 1991; Beumer, 2001; Nissen and Holck, 1998). Depending on the fermentation conditions, food-borne pathogens may survive at the end of the process.

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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. (submitted for publication) (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., submitted for publication). In short, the batter of each fermented sausage was inoculated with a cocktail of five *L. monocytogenes* strains (final concentration 10^5–10^6 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 × *g* for 1 min at 4 °C (Eppendorf 5417R, Eppendorf, Milan, Italy) and 0.5 ml of RNA*later* (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*)

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
rpoB[a]	DNA-directed RNA polymerase subunit beta	Reference gene
rplD	50S ribosomal protein L4	Reference gene
Gap	Highly similar to glyceraldehyde-3-phosphate dehydrogenase	Reference gene
bglA	6-phospho-beta-glucosidase	Reference gene
	Highly similar to translation elongation factor EF-Tu	Reference gene
sigB	RNA polymerase sigma factor SigB	Regulation of virulence and stress-response genes/Target gene
gbuA	Very similar to glycine betaine ABC transporter (ATP-binding protein)	Adaptation (osmotic stress)/Target gene
gbuB	Very similar to glycine betaine ABC transporters (permease)	Adaptation (osmotic stress)/Target gene
lmo1421	Similar to glycine betaine/carnitine/choline ABC transporter, ATP-binding protein	Adaptation (osmotic stress)/Target gene
lmo0669	Similar to oxidoreductase	Adaptation (acid stress)/Target gene
prfA	Listeriolysin positive regulatory protein	Regulation of virulence genes/Target gene
lmo1038	PTS system encoding enzyme II cytoplasmic subunits for the transport of major carbon sources	Glucose uptake/Target gene
lmo0442	PTS system encoding enzyme II cytoplasmic subunits for the transport of major carbon sources	Fructose uptake/Target gene
lmo0115	PTS system encoding enzyme II cytoplasmic subunits for the transport of major carbon sources	Mannose uptake/Target gene
lmo0938	PTS system encoding enzyme II cytoplasmic subunits for the transport of major carbon sources	Cellobiose uptake/Target gene
betL	Glycine betaine transporter	Adaptation (osmotic stress)/Target gene
opuCA	Similar to glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)	Adaptation (osmotic stress)/Target gene
lmo2434 or *gadD*	Highly similar to glutamate decarboxylases	Adaptation (acid stress)/Target gene

[a] 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*.