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How temperature influences the development of lactococcosis? Transcriptomic and immunoproteomic *in vitro* approaches

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27 **Short running title:** Water temperature, a factor involved in lactococcosis**Original**
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30 **How temperature influences the development of lactococcosis?**
31 **Transcriptomic and immunoproteomic *in vitro* approaches**

32 **Abstract**

33 *Lactococcus garvieae* is the etiological agent of lactococcosis, a hemorrhagic septicemia that
34 affects marine and freshwater fish, with special incidence and economic relevance in farmed
35 rainbow trout. Water temperature is one of the most important predisposing factors in the
36 development of lactococcosis outbreaks. Lactococcosis in trout usually occur when water
37 temperatures rise to about 18°C, while fish carriers remain asymptomatic at temperatures
38 below 13°C. The aim of this work is to analyze the differences in the complete transcriptome
39 response of *L. garvieae* grown at 18°C and at 13°C, and to identify the immunogenic proteins
40 expressed by this bacterium at 18°C. Our results show that water temperature influences the
41 expression of *L. garvieae* genes involved in the lysis of part of the bacterial cell population
42 and in the cold response bacterial adaptation. Moreover, the surface immunogenic protein
43 profile at 18°C suggests an important role of the lysozyme-like enzyme, WxL surface proteins
44 and some putative moonlighting proteins (proteins with more than one function, usually
45 associated with different cellular locations) as virulence factors in *L. garvieae*. The results of
46 this study could provide insights into the understanding of the virulence mechanisms of *L.*
47 *garvieae* in fish.

48 **Keywords:** *Lactococcus garvieae*, water temperature, immunogenic proteins, surface proteins,
49 virulence.

50 **Introduction**

51 Lactococcosis is a hemorrhagic septicemia that affects different marine and
52 freshwater fish species, but with special prevalence and economic relevance in farmed
53 rainbow trout *Oncorhynchus mykiss* (Walbaum). Since its description in the early 90's

(Doménech *et al.* 1993) lactococcosis turned out to be one of the most important fish diseases in Europe (Eldar *et al.* 1996; Algöet *et al.* 2009), as well as in other countries around the world (Eldar *et al.* 1999; Evans, Klesius & Shoemaker 2009; Karsidani *et al.* 2010). The causative agent of lactococcosis is *Lactococcus garvieae*, a ubiquitous and widely distributed Gram-positive bacterium. *L. garvieae* has also been isolated from cows and water buffalos with subclinical mastitis and pigs with pneumonia (Collins *et al.* 1983; Teixeira *et al.* 1996; Tejedor *et al.* 2011). In humans, it has been associated with a variety of infections, being considered an emerging opportunistic and potentially zoonotic pathogen (Russo *et al.* 2012; Reguera-Brito *et al.* 2016; Gibello *et al.* 2016 in press). In addition to its relevance as a pathogen, *L. garvieae* can also be isolated from rivers and sewage waters (Aguado-Urda *et al.* 2010), and from different foods such as vegetables, meat, raw milk and dairy products (Ferrario *et al.* 2013; Gibello *et al.* 2016). The wide distribution of *L. garvieae* is likely related to its ability to adapt and survive in many environmental conditions including a wide range of pH (4.5 to 9.6), salinity concentrations (0 to 6.5%), nutrient sources and temperatures (from 10°C to 45°C) (Kusuda *et al.* 1991).

Epidemiological data indicate that water temperature is one of the most important predisposing factors in the development of lactococcosis (Eldar & Guittino, 1999; Vendrell *et al.* 2006, Algöet *et al.* 2009). Lactococcosis outbreaks usually occur when water temperatures rise to about 18°C (Vendrell *et al.* 2006; Algöet *et al.* 2009), while fish carriers remain asymptomatic at temperatures below 13°C, temperatures within the optimal range for rainbow trout farming. The understanding of virulence gene expression and its regulation in response to temperature in fish pathogenic bacteria is essential to enable a rational approach to the problem of the bacterial fish diseases affecting the aquaculture industry (Guijarro *et al.* 2015). In a previous study, we described that *L. garvieae* responds globally to temperature, and interestingly, some of the genes that were modulated encoded proteins associated with the pathogenesis of different bacterial pathogens (Aguado-Urda *et al.* 2013).

The data generated from the sequencing of genomes of several *L. garvieae* strains (<http://www.ncbi.nlm.nih.gov/genome/genomes/699>) have contributed to the identification of different genes potentially implicated in its virulence (Morita *et al.* 2011; Miyauchi *et al.* 2012). Also, as virulence factors are usually strongly immunogenic (Chitlaru *et al.* 2007; Yadav *et al.* 2016), the knowledge of immunogenic antigens of *L. garvieae* might contribute to explain the differences in virulence among strains and these molecules could be used as

potential markers for developing effective vaccines against this pathogen (Shin *et al.* 2007 & 2009). In this sense, temperature must be taken in consideration, as it can affect the *L. garvieae* antigenic profiles obtained due to the changes in gene expression (Aguado-Urda *et al.* 2013).

In the present work the strain *L. garvieae* 8831, isolated from diseased rainbow trout suffering lactococcosis (Aguado-Urda *et al.* 2011) was used to i) investigate the differences in complete transcriptome expression of *L. garvieae* at 18°C with respect to 13°C by microarray expression analysis and ii) identify immunogenic proteins expressed by this bacterium at 18°C using an immunoproteomic approach. The results of this study could provide insights into the understanding of the virulence mechanisms of *L. garvieae* in fish.

Materials and methods

Microarray analysis

RNA extraction and purification

L. garvieae 8831 (Lg8831) was grown aerobically in BHI broth (bioMérieux, Marcy l'Etoile, France) at 13°C, 18°C and 29°C and harvested at the mid-log phase (OD₆₀₀ ~ 0.9) for RNA extraction. Total RNA from Lg8831 was isolated from three independent samples (biological replicates) of each temperature condition as described by Aguado-Urda *et al.* (2013). The quality and concentration of RNA was determined by using the RNA 6000 NanoKit on the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) at the Genomics Unit facilities (Parque Científico de Madrid). Only high-quality RNA samples (RIN > 9) were used for the next step.

Microarray experimental design

A dual-color reference design experiment using a DNA microarray containing 60-mer oligonucleotide specific probes for the 1992 ORFs of the Lg8831 genome (Aguado-Urda *et al.* 2011) was performed as previously described (Aguado-Urda *et al.* 2013). Interrogated samples of Lg8831 were grown in triplicate at 13°C and 18°C, and RNA was extracted. These RNAs were independently labeled with Cy-5. A common reference RNA obtained from Lg8831 grown at 29°C was quantified and labeled with Cy-3 to be used as reference for all the hybridizations.

cDNA synthesis, labeling of total RNA, hybridization and scanning

A total of 5 µg of total RNA of each sample replicate was reverse transcribed and labeled by using the FairPlay III Labeling Kit (Agilent Technologies) as described by Aguado-Urda *et al.* (2013). The yield and the specific activity of each labeling reaction were determined in a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Inc., Rockland, DE). The hybridization and washing steps were carried out according to the Agilent's "Two Color Microarray-based Prokaryote Analysis" protocol. Image acquisition and scanning was performed on an Agilent G2565AA scanner according to the manufacturer's instructions (GE2_107_Sep09).

Data analysis

Image quantification was carried out using Agilent's Feature Extraction Software v 8.5.1.1. Background-subtracted data were log-transformed, LOWESS-normalized, and analyzed for the detection of differentially expressed genes using BRB-ArrayTools Software developed by Dr. Richard Simon and the BRB-ArrayTools Development Team (Simon *et al.* 2007). Triplicate probes on the microarray were averaged for the analyses. A multivariate permutation test was used to identify the differentially expressed genes keeping the false discovery rate (FDR) at 10% with a 90% confidence (Simon *et al.* 2003), and in addition a pairwise t-test with $p=0.05$. A further filtering step was carried out by selecting among SAM results only those whose expression ratio between the analyzed conditions was ≥ 2 . Throughout this article, the term "up-regulated genes" has been used to indicate those genes that were found differentially overexpressed at one temperature relative to the other.

Microarray accession numbers

The data presented in this publication have been deposited in the Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE40357.

Validation of microarray data by reverse-transcription quantitative real-time PCR (RT-qPCR)

To validate the results obtained during the microarray analysis, RT-qPCR analysis was performed. A total of 4 genes were selected for the validation process. These genes were

selected by taking the top two expressed and top two repressed genes at each comparison between the two temperatures. The *gyrA* gene was selected as an endogenous control because it did not present variation in its expression throughout the experiment. The RT-qPCR primers were designed using Primer Express software version 2.0 (Applied Biosystems Technologies, Paisley, UK) based on the analysis of the sequences from the 4 genes of Lg8813 strain: phosphate ABC transporter substrate-binding protein, 1-phosphofructokinase, 1,4-beta-N-acetylmuramidase, glutamine synthetase (Supplementary Fig. 1A). Triplicate assays utilizing the same three independent RNA samples used for the microarrays hybridization were used for the RT-qPCR validation as described by Aguado-Urda *et al.* (2013).

Immunoproteomics

Extraction of whole cell bacterial proteins and membrane proteins

Lg8831 was grown aerobically at 18°C in BHI broth (bioMérieux, Marcy l'Etoile, France). Upon reaching logarithmic phase ($OD_{600} \sim 0.9$), the bacteria were harvested for protein extraction as described by Reguera-Brito *et al.* (2014).

To obtain the membrane protein fraction Lg8831 was collected at an $OD_{600} \sim 1.7$. Pellet was washed twice with 0.1 M Tris-HCl, pH 7.5, and resuspended in 1 M Tris-HCl, pH 7.5 with 0.1% (v/v) protease inhibitor cocktail (Sigma-Aldrich), 2 mg mL⁻¹ lysozyme, 1 mM final concentration of PMSF, and 400 U mutanolysin. After 3 h at 37°C, supernatants were collected by centrifugation (5500 rpm, 10 min at 4°C) and proteins were precipitated with trichloroacetic acid at a final concentration of 10%, collected by centrifugation (13000 rpm for 10 min at 4°C), and washed with ice-cold acetone overnight. The dried pellet was resuspended in ReadyPrep Rehydration buffer (Bio-Rad, Hercules, CA).

Two-dimensional electrophoresis (2-DE) and immunoblotting

The protein concentration of each sample was quantified using the Bradford method (Bio-Rad DC Protein Assay Reagent, Bio-Rad, Hercules, CA) according to the manufacturer's protocol and BSA was used as standard protein. Proteins were separated on high-resolution 2D electrophoresis gels, using IPG strips of 11 cm, pH 4-7 and pH 3-10, and Criterion TGX gels for the second dimension of electrophoresis. Triple replicated analytical gels of each biological sample were run in parallel, analyzed and compared after staining with

Bio-Safe Coomassie (Bio-Rad) and were further visualized using the Gel Doc EZ imaging system.

One Coomassie-stained gel was transferred onto a PVDF membrane and was immunoblotted using anti-Lg8831 rabbit sera as the primary antibody at 1:750 dilution in PBST. The membrane was incubated with peroxidase-conjugated goat anti-rabbit IgG (1:3000; Bio-Rad) as the secondary antibody. The immunoreactive spots were visualized using 4-chloro-1-naphtol (HRP Color Development Reagent, Bio-Rad). Reactive proteins were excised from the Coomassie-stained gels and the selection of correct immunoreactive proteins were facilitated by using the post-staining western blotting approach described previously (Reguera-Brito *et al.* 2014).

MALDI-TOF/TOF MS analyses

Immunoreactive spots were manually excised from the Coomassie-stained gels. Proteins selected for analysis were in-gel reduced with 10 mM dithioerythritol in 25 mM ammonium bicarbonate (AmB) for 30 min at 56°C and subsequently alkylated with 55 mM iodoacetamide in AmB for 15 min in the dark. Finally, samples were digested with 12.5 ng μL^{-1} sequencing grade trypsin (Roche Molecular Biochemicals) in AmB overnight at 37°C. After digestion, the supernatant was collected and 1 μL was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.5 μL of a 3 mg mL^{-1} of α -cyano-4-hydroxy-cinnamic acid matrix (Sigma-Aldrich) in 50% acetonitrile were added to the dried peptide digested spots and allowed again to air-dry at room temperature. MALDI-TOF MS analyses were performed in a 4800 Plus Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, MDS Sciex, Toronto, Canada) at the Genomics and Proteomics Center, Complutense University of Madrid.

For protein identification, NCBI nr (40910947 sequences; 14639572021 residues) with no taxonomy restriction was searched using MASCOT 2.3 (www.matrixscience.com) through the Global Protein Server v 3.6 from ABSciex. In all protein identification, the probability scores were greater than the score fixed by MASCOT as significant with a p-value minor than 0.05.

Bacterial cell viability tests

In order to test how the lysozyme/mutanolysin treatment affect the viability of bacterial cells, samples of 100 μ L of bacterial suspension in 1 M Tris-HCl, pH 7.5 with 0.1% (v/v) protease inhibitor cocktail and lysozyme/mutanolysin were collected each 30 min, plated on BHI agar medium and incubated at 37°C.

To probe the impact of temperature on the lytic gene expression of the putative prophage (union of contigs 19 and 12), liquid cultures of Lg8831 on BHI at 29°C were serially diluted (decimal dilutions) and samples of 100 μ L were plated by triplicate on BHI agar media and incubated at 29°C, 13°C and 18°C. Virions could not be detected in the supernatant of the cells grown at 18°C using mitomycin C at concentrations between 50 and 250 μ g mL⁻¹.

Results and Discussion

Global expression patterns of Lg8831 at 18°C and 13°C

Changes in environmental factors such as temperature can have significant impacts on the physiology of fish including its immune system (Rebl *et al.* 2013; Makrinos & Bowden, 2016). Temperature is one of the most predisposing factors in the development of lactococcosis, and outbreaks usually occur when water temperature is above 18°C (Vendrell *et al.* 2006; Algöet *et al.* 2009). In previous studies we described that temperature can modulate gene expression of *L. garvieae* (Aguado-Urda *et al.* 2013). Based on these data, we used whole-genome DNA microarrays to obtain a comprehensive overview of the transcriptome of Lg8831 growing at 18°C and further compared it to that observed at 13°C. Under the conditions described for the microarray data analysis in the global comparative analysis at both 18°C and 13°C, 67 genes were up-regulated at 18°C and 65 genes were up-regulated at 13°C (Supplementary Table 1; Fig. 1). These genes represent only the 4.1% of the Lg8831 genome (Aguado-Urda *et al.* 2011), which indicates that the variations between the transcriptomes of this strain at both temperatures are minimal. The list of genes differentially regulated at 18°C in comparison with 13°C is shown in Table 1.

To validate the microarray expression profiling results, four differentially expressed genes were selected to perform quantitative RT-qPCR experiments with the same RNA samples used in the microarray experiments. The strong positive correlation between the data obtained by both techniques allowed us to validate the microarray results ($r = 0.9498$) (Supplementary Fig. 1B).

None of the genes previously described as putative virulence factors for *L. garvieae*, such as hemolysins (LCGL_0323), adhesion proteins (LCGL_1585, LCGL_1330) or iron uptake proteins (Miyauchi *et al.* 2012; Ture & Altinok, 2016; Gibello *et al.* 2016), showed significant variations in the transcriptome of Lg8831 at 18°C in comparison with 13°C (Table 1 and Supplementary Table 1). The up-regulated genes observed at 18°C included mainly two operons, one is formed by genes 20-24 of the contig 1, encoding hypothetical proteins, and the other includes some phage-associated genes of the contig 19. Most of the up-regulated hypothetical proteins had no conserved domains, which made difficult to speculate about their functions. Further examination of the phage-associated genes with PHAST (PHAge Search Tool) Web server (<http://phast.wishartlab.com/>) revealed that most of them were involved in the lytic cycle of a putative prophage, located in the union of contig 19 and 12 of Lg8831 genome (NZ_AFCD000000000). In particular, a gene encoding for a phage lysozyme protein (contig19, gen 25; LCGT_0295) was 7.38 fold regulated (Table 1). None of the transcriptional regulators previously described to be involved in *L. garvieae* survival in fish hosts (Menendez *et al.* 2007) were found significantly regulated at 18°C in this strain.

Most of the up-regulated genes at 13°C (down-regulated at 18°C in Table 1) were involved in bacterial cold-stress responses and we didn't observe significant transcriptional variations on any of the genes involved or related with the virulence. The majority (39.3%) of these up-regulated genes belong to the group of ABC transporters, which have been related with the uptake of cryoprotectants under cold temperature conditions (Shivaji & Prakash, 2010, Hoffmann & Bremer, 2011, Aguado-Urda *et al.* 2013). In particular, many of the phosphate ABC transporter genes (*pstABCS*) involved in the capture of periplasmic inorganic phosphate into the cytosol, and the *phoU* gene encoding a regulatory protein for the phosphate transport system, that are top up-regulated at 13°C were also up-regulated at 18°C vs 37°C (Table 1) (Aguado-Urda *et al.* 2013). Likewise, other genes such as *potD*, *fruB* and *fruR*, previously described as differentially expressed during the cold-stress response of *L. garvieae* showed stronger up-regulation at 13°C (Table 1). These results support the observed cold-stress response of Lg8831 at low temperatures, indicating that the expression of these genes increases according to the decrease of temperature (Aguado-Urda *et al.* 2013).

Immunoproteome analysis/Immunoblot analysis of *L. garvieae* proteins

Previous proteomic and immunoproteomic studies in *L. garvieae* have used whole cell lysates to explore the antigenic proteins that could be involved or related with its pathogenesis

(Shin *et al.* 2007 & 2009). However, none of these studies has specifically analyzed surface immunoreactive proteins, despite surface proteins usually have a role as virulence factors in pathogenic bacteria, acting as adhesins for host bacterial adhesion, host cell invasion, immune evasion and cell surface receptors for collagen, fibronectin or plasminogen (Bierne & Cossart, 2007; Löfling *et al.* 2011). Moreover, all previous immunoproteomic studies of *L. garvieae* were performed at 30°C, and so the current study is the first approach to investigate the immunogenic proteins of Lg8831 at 18°C, temperature at which lactococcosis outbreaks usually occurs.

Identification of the immunoreactive spots representative of the whole cell lysates of Lg8831 growing at 18°C is shown in Table 2 and Supplementary Fig. 2. Most of them were cytosolic proteins associated with the transport and metabolism of carbohydrates and amino acids. Proteins such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), pyruvate kinase (PyK), inosine 5'-monophosphate dehydrogenase (IMD), arginine deiminase or ornithine carbamoyltransferase have been described as immunogenic proteins in other strains of *L. garvieae* grown at 30°C (Shin *et al.* 2007 & 2009) and in other bacterial pathogens such as *Streptococcus suis* (Wu, Zhang & Lu 2008) or *Streptococcus pyogenes* (Cole *et al.* 2005). Also, Glc24, a protein involved in the virulence of *Enterococcus faecalis* (Nannini *et al.* 2005; Teng, Nannini & Murray 2005) and in the response to different stress conditions in several *Enterococcus* species (Stoyanov *et al.* 2010; Choudhury *et al.* 2011), has been previously described as immunoreactive in a human strain of *L. garvieae* (Reguera-Brito *et al.* 2014).

The analysis of the immunogenic proteins of the surface protein-enriched fraction of Lg8831 at 18°C is shown in Fig. 2A and Table 2B. Of particular significance is the protein gi|515938667 containing a WxL domain, which is characteristic of proteins that are non-covalently anchored to peptidoglycan of the cell wall (Galloway-Peña *et al.* 2015) and play a role in carbon source acquisition or adhesion to the host cells (Siezen *et al.* 2006). Also, the protein gi|518123066 seems to be a lytic enzyme and is codified by a gene (gen 25 of contig19) previously identified among those up-regulated at 18°C in comparison to 13°C and 37°C (Table 1). This gene is located into a genetic cluster involved in the lytic cycle of a putative prophage (union of contigs 19 and 12). The pattern of immunogenic proteins on the surface of *L. garvieae* grown at 13°C differs from the obtained at 18°C (Table 3, Fig. 2B). Only one protein, the chaperone DnaK, appears as immunogenic on the surface at both temperatures. DnaK chaperone (Hsp70 family) is a main protein involved in tolerance to

environmental stresses and assist in correct folding/assembly of proteins involved in diverse cellular processes. This protein has been located at the surface of many bacteria contributing to the pathogenicity of these species (Henderson, Elaine & Coates 2006; Vanghele & Ganea, 2010). Of special interest was the identification of several typically cytosolic proteins such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate mutase (PGM) and phosphoglycerate kinase (PGK) at the surface of *L. garvieae* grown at 18°C. The identification of these proteins in the surface fraction has been previously described in other pathogens as moonlighting proteins (Kainulainen & Korhonen, 2014; Amblee & Jeffery, 2015). Moonlighting proteins are multifunctional proteins that perform two or more biochemical functions that are not due to gene fusions, multiple splice variants, proteolytic fragments, or promiscuous enzyme activities, and are usually found at different cellular locations (Kainulainen & Korhonen, 2014; Jeffery, 2015). Some of these moonlighting proteins are highly conserved proteins associated with the glycolytic pathway, chaperones and cytosolic enzymes. One of the patterns that aid in the identification of moonlighting proteins is that their “moonlighting” molecular weight and pI differ from the ones they have when they are performing their canonical function (Amblee & Jeffery, 2015). As we can see in Table 2AB, GAPDH and PGK have different experimental molecular weight and pI when they are located at the cytosolic fraction or at the cell surface, and both are not found among the immunogenic surface proteins at 13°C, which could be indicative of moonlighting.

Moonlighting proteins, in addition to the importance of their roles in physiological processes, often have a role as virulence factors in many pathogenic bacteria when they are located on bacterial surface, acting as cell surface receptors for collagen, fibronectin or plasminogen, or modulating different cell signaling processes (Gründel *et al.* 2015; Amblee & Jeffery, 2015; Jeffery 2015). For example, the presence of GAPDH at the surface of different pathogenic streptococci plays an important role in their virulence, promoting bacterial invasion of host cells and modulating host immune system response (Terao *et al.* 2006; Madureira *et al.* 2007; Oliveira *et al.* 2015). In *L. garvieae* this enzyme generally appears as a strongly immunogenic protein and can induce a protective immune-response in fish (Shin *et al.* 2007, 2009; Tsai *et al.* 2012).

Normally, proteins are translocated over the cytoplasmic membrane by defined transport systems as they possess appropriate signal peptides. However, many moonlighting proteins located at the surface lack a signal sequence. Two main theories are proposed to

explain the mechanisms by which these proteins ended at an extracellular location. On one hand, some experiments showed that only a fraction of the cytosolic proteins appears in both surface and secretome, suggesting an unknown transporting mechanism (Boël *et al.* 2004; Pasztor *et al.* 2010; Wang *et al.* 2013; Götz *et al.* 2015). On the other hand, the fact that these moonlighting proteins have no signal peptide suggests that they are released by cell lysis and are further captured by the surface of live bacteria (Oliveira *et al.* 2012; Terrase *et al.* 2015). In *L. garvieae* we observed that at 18°C, the count colony plate assay yielded about 2 log lower colony counts than the same plates incubated at 30 or 37°C (2.0×10^3 versus 1.2×10^5 CFU mL⁻¹) and this difference was statistically significant (P-value <0.05). This lysis phenomenon could be related with the over-expression of the gen25 of contig19, encoding for a phage lysozyme-like enzyme (gi|518123066) at 18°C which appears as an immunogenic protein at the cell surface also at this temperature (Table 2). The involvement of phage lytic enzymes induced by specific temperature on the pathogenicity of carrier bacteria has been demonstrated in different species (Morales *et al.* 2010; Shan *et al.* 2014; Terrasse *et al.* 2015).

The results of the present study indicate that temperature does not seem to be a key factor for the *in vitro* over-expression of any of the currently or putative virulence factors, such as pili or fimbriae described for *L. garvieae* (Gibello *et al.* 2016). However, due to the complexity of the host-pathogen interactions, the possibility that bacterial gene expression during infection has not to be necessarily the same as that observed in *in vitro* experiments should be considered. In this sense, several immunogenic proteins in *L. garvieae* are expressed only when the pathogen grows *in vivo* (Schmidtke & Carson, 2003).

Many factors are hypothesized to contribute to the progression of the lactococcosis from a carrier state, being water temperature the best known predisposing factor (Vendrell *et al.* 2006). Water temperature at which lactococcosis occurs is within the optimal range of temperature for rainbow trout and therefore it is not likely it can assert any effect in the normal function of the immune system of trout that could explain the onset of the lactococcosis outbreaks (Selong *et al.* 2001; Kollner & Kotterba, 2002; Nikoskelainen *et al.* 2004). Nevertheless, the water temperature can also affect the fish metabolism rate and the quality of other parameters in the water, such as oxygen availability (Kollner *et al.* 2002; Wang *et al.* 2016). These situations can lead to a stress condition for the trout affecting to its immune response (Purcell *et al.* 2016) and therefore increasing its susceptibility to infection. Elevated temperatures can increase the growth rate and virulence of microbial pathogens

(Marcogliese, 2008). In this sense, the higher growth rate of *L. garvieae* at 18°C in comparison to 13°C (unpublished data) could represent a competitive advantage for a subsequent faster fish tissue colonization facilitating the overcome of the immune system defenses.

Overall, this work shows that water temperature, although necessary, it should not be a trigger factor on their own for the development of clinical manifestations in infected fish. Moreover, the surface immunogenic protein profile at 18°C suggests that the lysozyme-like enzyme, WxL surface proteins and some putative moonlighting proteins might represent new virulence factors implicated in the onset of *L. garvieae* infection. However, further studies are needed to confirm their specific role in the virulence/pathogenicity of *L. garvieae*.

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Tables

Table 1. Genes differentially regulated at 18°C in comparison with 13°C.

Table 2. A. Immunogenic proteins from the whole protein fraction of *L. garvieae* 8831 grown at 18°C, identified by MALDI-TOF-TOF MS. **B.** Immunogenic proteins from membrane fraction of *L. garvieae* 8831 grown at 18°C, identified by MALDI-TOF-TOF MS.

Table 3. Immunogenic proteins from membrane fraction of *L. garvieae* 8831 grown at 13°C, identified by MALDI-TOF-TOF MS.

Figure legends

Figure 1. Heat map of microarray results at different growth temperatures in Lg8831. Comparison of transcriptional profiles at 13°C, 18°C (this work) and 37°C (Aguado-Urda *et al.* 2013) evidenced that the variation between profiles at 13°C and 18°C is minimal. Blue to orange = low to high signals.

Figure 2. A. 2D-PAGE of surfome proteins of *L. garvieae* grown at 18°C. **B.** 2D-PAGE of surfome proteins of *L. garvieae* grown at 13°C. The proteins excised from the gel following the immunoreactivity observed in the western blot are encircled. The excised proteins were further analyzed by MALDI-TOF/TOF MS.

Supporting Information

Supplementary Table 1. Genes showing significant regulation by microarray hybridization in *Lactococcus garvieae* 8831 at 18°C and 13°C and comparative expression at both temperatures and 37°C. Genes up-regulated and down-regulated in one condition with respect

to the other are highlighted in red and green respectively. We consider a significant regulation when the expression ratio between the analyzed conditions was ≥ 2 .

Supplementary Figure 1. A. Primers used for RT-qPCR experiments. **B.** Correlation between RT-qPCR and microarray analysis data. The $\log_{10}$ values of the RT-qPCR results were plotted against the microarray analysis $\log_{10}$ values. The expression levels for the 4 selected genes are shown.

Supplementary Figure 2. 2D-PAGE of whole protein fraction of *L. garvieae* grown at 18°C.

Table 1. Genes differentially regulated at 18°C in comparison with 13°C.

Fold change			UniqueID/symbol	Gene	Function	Pairwise significant
(18 vs 13)	(13 vs 37)	(18 vs 37)				
9.47	0.11	1.04	TSno3_c001_g013		transcriptional regulator	(1, 2), (1, 3)
8.05	0.14	1.12	TSno3_c001_g020		hypothetical protein	(1, 2), (1, 3)
6.94	0.15	1.02	TSno3_c001_g021		hypothetical protein	(1, 2), (1, 3)
6.74	0.12	0.83	TSno3_c001_g022		hypothetical protein	(1, 2), (1, 3)
3.91	0.23	0.89	TSno3_c001_g023		hypothetical protein	(1, 2), (1, 3)
10.65	0.09	0.96	TSno3_c001_g024		hypothetical protein	(1, 2), (1, 3)
2.08	0.70	1.47	TSno3_c009_g019	LACR_2598	hypothetical protein	(1, 2)
5.86	0.21	1.23	TSno3_c019_g007		predicted phage tail tape measure proteins	(1, 2), (1, 3)
8.36	0.22	1.80	TSno3_c019_g012		predicted protein component of bacteriophage	(1, 2), (1, 3)
9.54	0.22	2.07	TSno3_c019_g014		hypothetical protein	(1, 2), (1, 3)
12.22	0.15	1.86	TSno3_c019_g017		predicted major phage protein	(1, 2), (1, 3)
6.32	0.25	1.59	TSno3_c019_g021		putative terminase large subunit	(1, 2), (1, 3)
2.40	1.19	2.86	TSno3_c019_g023		hypothetical protein	(1, 2), (3, 2)
4.51	1.08	4.85	TSno3_c019_g024		CHAP domain protein	(1, 2), (3, 2)
7.38	0.44	3.26	TSno3_c019_g025		putative phage lysozyme	(1, 2), (1, 3), (3, 2)
7.02	0.22	1.51	TSno3_c019_g027		predicted protein with signal peptide	(1, 2), (1, 3)
3.74	0.39	1.46	TSno3_c019_g028		replication protein	(1, 2), (1, 3)
2.55	0.28	0.72	TSno3_c037_g041	frdC	fumarate reductase flavoprotein subunit	(1, 2), (1, 3)
4.76	0.98	4.65	TSno3_c058_g039		putative lysin	(1, 2), (3, 2)
3.84	0.91	3.51	TSno3_c058_g040	lmrA	multidrug resistance ABC transporter ATP binding and permease protein	(1, 2), (3, 2)
2.54	0.17	0.44	TSno3_c077_g014	coaE	dephospho-CoA kinase	(1, 2), (1, 3), (2, 3)
3.89	0.28	1.08	TSno3_c077_g065		hypothetical protein	(1, 2), (1, 3)

Fold change			UniqueID/symbol	Gene	Function	Pairwise significant
(18 vs 13)	(13 vs 37)	(18 vs 37)				

0.22	3.81	0.83	TSno3_c006_g026		hypothetical protein	(2, 1), (3, 1)
0.36	4.58	1.63	TSno3_c009_g039	phoU	phosphate transport system regulator	(2, 1), (3, 1), (3, 2)
0.24	4.90	1.17	TSno3_c009_g040	pstA	phosphate transporter ATP-binding protein	(2, 1), (3, 1)
0.25	3.76	0.92	TSno3_c009_g041	LACR_1878	ABC-type phosphate transport system, ATPase	(2, 1), (3, 1)
0.31	2.56	0.80	TSno3_c009_g042	pstC	phosphate ABC transporter permease protein	(2, 1), (3, 1)
0.23	4.02	0.92	TSno3_c009_g043	pstD	phosphate transport system permease protein pstD	(2, 1), (3, 1)
0.20	5.52	1.11	TSno3_c009_g044	pstE	phosphate ABC transporter substrate binding protein	(2, 1), (3, 1)
0.04	39.49	1.57	TSno3_c009_g045	pstF	phosphate transport substrate binding protein pstF	(2, 1), (3, 1)
0.46	4.03	1.85	TSno3_c013_g007	llmg_1552	putative ABC type transport system permease	(3, 1)
0.45	1.51	0.67	TSno3_c013_g017	ysjA	amino acid permease	(2, 1), (3, 1), (2, 3)
0.32	37.48	12.15	TSno3_c018_g013	LACR_1033	fusion of IIA, IIB, IIC component of mannitol/fructose-specific phosphotransferase system	(3, 1), (3, 2)
0.27	66.67	17.78	TSno3_c018_g014	fruB	1-phosphofructokinase	(3, 1), (3, 2)
0.30	102.74	30.37	TSno3_c018_g015	fruR	lactose transport regulator	(2, 1), (3, 1), (3, 2)
0.47	1.03	0.48	TSno3_c028_g019	treA	putative trehalose-6-phosphate hydrolase TreA	(2, 1), (2, 3)
0.31	1.42	0.44	TSno3_c028_g020	yedF	beta-glucoside-specific PTS system IIABC component	(2, 1), (2, 3)
0.25	6.58	1.64	TSno3_c032_g009	msmK	multiple sugar-binding transport ATP-binding protein	(2, 1), (3, 1)
0.43	5.61	2.43	TSno3_c036_g022	potD	spermidine/putrescine ABC transporter substrate-binding protein	(2, 1), (3, 1), (3, 2)
0.47	6.89	3.26	TSno3_c036_g025	potA	spermidine/putrescine ABC transporter ATP-binding protein	(3, 1), (3, 2)
0.37	4.50	1.65	TSno3_c042_g040		vanZ family protein	(2, 1), (3, 1)
0.48	3.19	1.53	TSno3_c042_g041	add	adenosine deaminase	(2, 1), (3, 1), (3, 2)
0.47	2.11	0.99	TSno3_c048_g086	ytcC	hypothetical protein	(2, 1), (3, 1)
0.48	1.25	0.60	TSno3_c060_g023	rbsD	D-ribose pyranase	(2, 1), (2, 3)
0.27	5.20	1.39	TSno3_c065_g023		putative transcriptional regulator (PspC)	(2, 1), (3, 1), (3, 2)
0.21	8.16	1.70	TSno3_c065_g024		hypothetical protein	(2, 1), (3, 1), (3, 2)
0.47	3.76	1.76	TSno3_c066_g004		hypothetical protein	(2, 1), (3, 1), (3, 2)
0.37	0.10	0.04	TSno3_c081_g024		gamma-aminobutyric acid:hydrogen symporter activity	(2, 1), (1, 3), (2, 3)
0.48	3.18	1.52	TSno3_c090_g015		sulfate transmembrane-transporting ATPase activity	(2, 1), (3, 1), (3, 2)
0.44	5.49	2.40	TSno3_c096_g016		putative CAAX amino terminal protease self-immunity	(3, 1)

Table 2A. Immunogenic proteins from the whole protein fraction of *L. garvieae* 8831 grown at 18°C, identified by MALDI-TOF-TOF MS.

Spot number	Gi number in NCBI	Identified Protein* Annotation/species	Theoretical MW (Da)/ pI	Experimental ^a MW (Da)/ pI	Mascot score ^b	Coverage (%)
1	gi 347520670	Phosphocarrier protein Hpr/ <i>L. garvieae</i> ATCC49156	9162/ 4.87	15417/ 4.7	316	87
2	gi 347521504	50S ribosomal protein L7/L12/ <i>L. garvieae</i> ATCC49156	12443/ 4.45	16987/ 4.9	103	50
3	gi 518122979	Stress response regulator Gls24/ <i>L. garvieae</i>	18639/ 4.73	23173/ 4.6	89	45
4	gi 518121306	UDP-Glucose 4-epimerase/ <i>L. garvieae</i>	35205/ 4.64	29000/ 5.5	351	39
5	gi 489227749	Glyceraldehyde-3-phosphate dehydrogenase/ <i>L. garvieae</i>	35771/ 5.19	35579/ 4.5	203	34
6	gi 518122123	Hypothetical protein/ <i>L. garvieae</i>	40480/ 4.79	41900/ 4.5	175	43
7	gi 489226842	Ornithine carbamoyltransferase/ <i>L. garvieae</i>	38761/ 4.85	41900/ 4.65	96	44
8	gi 503791466	Phosphoglycerate kinase/ <i>L. garvieae</i>	41851/ 4.86	41900/ 4.8	170	41
9	gi 347521921	Pyruvate kinase/ <i>L. garvieae</i> ATCC 49156	54600/ 4.88	54900/ 4.9	223	54
10	gi 347520695	Arginine deiminase/ <i>L. garvieae</i> ATCC 49156	45983/ 5.29	49300/ 5.5	122	50
11	gi 518121240	Inosine 5'-monophosphate dehydrogenase/ <i>L. garvieae</i>	48228/ 5.69	54900/ 6.0	156	47

Spot	Gi number in	Identified Protein [*]	Theoretical	Experimental ^a	Mascot	Coverage
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Table 2B. Immunogenic proteins from membrane fraction of *L. garvieae* 8831 grown at 18°C, identified by MALDI-TOF-TOF MS.

number	NCBI	Annotation/species	MW (Da)/ pI	MW (Da)/ pI	score ^b	(%)
1	gi 489225219	Phosphocarrier protein HPr/L. garvieae	9162/ 4.87	13803/ 6	190	84
2	gi 489225402	Phosphoglycerate mutase/L. garvieae	26376/ 5.17	22387/ 6.4	126	38
3	gi 489224919	Ribosome recycling factor/L. garvieae	20655/ 5.39	22760/ 6.4	54	11
4	gi 503791454	2-amino-3-ketobutyrate CoA ligase/L. garvieae	42412/ 5.24	55949/ 4.89	106	46
5, 6	gi 518123066	Putative phage lysozyme protein/L. garvieae	41626/ 5.92	42657/ 5.3; 39755/ 7.75	131	39
7	gi 489224754	Elongation factor Ts/L. garvieae	36832/ 4.81	39755/ 5.65	99	43
8	gi 503791466	Phosphoglycerate kinase/L. garvieae	41851/ 4.86	38770/ 5.8	159	50
9	gi 503791539	Glyceraldehyde-3-phosphate dehydrogenase/L. garvieae	35800/ 4.99	37826/ 6	181	49
10	gi 489224851	Hypothetical transcriptional regulator/L. garvieae	8832/ 4.46	13489/ 5.2	89	96
11	gi 490379602	Molecular chaperone DnaK	64670/ 4.57	71531/ 5.34	168	42
12	gi 565621696	Hypothetical protein (WxL domain protein)/L. garvieae	27854/ 5.31	111686/ 5.3	97	68

* For protein identification NCBI nr (40910947 sequences; 14639572021 residues) with no taxonomy restriction was searched using MASCOT 2.3 (www.matrixscience.com) through the Global Protein Server v 3.6 from ABSciex.

a: Observed MW and pI of protein spot in the 2-D SDS-PAGE, and calculated from the equation of the regression line.

b: Protein score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 88 are significant ($p < 0.05$).

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Table 3. Immunogenic proteins from membrane fraction of *L. garvieae* 8831 grown at 13°C, identified by MALDI-TOF-TOF MS.

Spot number	Gi number in NCBI	Identified Protein* Annotation/species	Theoretical MW (Da)/ pI	Experimental ^a MW (Da)/ pI	Mascot score ^b	Coverage (%)
1	gi 814596242	Molecular Chaperone DnaK/ <i>L. garvieae</i>	64670/ 4.57	78973/ 5.4	275	64
2	gi 391856485	Oligopeptide ABC transporter substrate binding subunit	60623/ 5.22	65344/ 5.15	200	11
3	gi 814595206	Trigger factor/ <i>L. garvieae</i>	47021/ 4.39	70276/ 5.15	73	41
4	gi 347521921	Pyruvate kinase/ <i>L. garvieae</i> ATCC 49156	54600/ 4.88	58972/ 5.9	223	54
5, 6	gi 814595312	Inosine 5'-monophosphate dehydrogenase/ <i>L. garvieae</i>	52804/ 5.68	65340/ 6.35	156	47
7, 8	gi 518123382	Fumarate reductase/ <i>L. garvieae</i>	52893/ 6.44	60974/ 6.9	106	21
9	gi 749040956	CHAP domain-containing protein/ <i>L. garvieae</i>	46034/ 5.96	53591/ 6.4	71	34
10	gi 503790140	Arginine deiminase/ <i>L. garvieae</i> ATCC 49156	45983/ 5.29	49715/ 6.5	122	50
11	gi 391855686	Hypothetical protein/ <i>L. garvieae</i>	40602/ 4.97	31769/ 6.6	175	43
12	gi 518122123	Hypothetical protein/ <i>L. garvieae</i>	41851/ 4.86	34303/ 6.2	170	41
13	gi 489226842	Ornithine carbamoyl transferase/ <i>L. garvieae</i>	38761/ 4.85	27832/ 6.2	96	44

* For protein identification NCBI nr (40910947 sequences; 14639572021 residues) with no taxonomy restriction was searched using MASCOT 2.3 (www.matrixscience.com) through the Global Protein Server v 3.6 from ABSciex.

a: Observed MW and pI of protein spot in the 2-D SDS-PAGE, and calculated from the equation of the regression line.

b: Protein score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 88 are significant ($p < 0.05$).

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Title:

How does temperature influences the development of lactococcosis? Transcriptomic and immunoproteomic in vitro approaches

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