Detection of virulence-related genes in *Lactococcus garvieae* and their expression in response to different conditions

Giovanni Eraclio [1], Giovanni Ricci [1], Mattia Quattrini [1], Paolo Moroni [2,3], Maria Grazia Fortina [1]*

[1] Università degli Studi di Milano, Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente, Via Celoria 2, 20133 Milan, Italy

[2] Università degli Studi di Milano, Dipartimento di Medicina Veterinaria, Via Celoria 10 20133 Milan, Italy

[3] Cornell University, Animal Health Diagnostic Center, Quality Milk Production Services, 240 Farrier Road, Ithaca, NY, 14853, USA

*Corresponding author: Maria Grazia Fortina

Phone: +39 0250319131

Fax: +39 0250319238

E-mail: grazia.fortina@unimi.it

Aknowledgments

We thank DR Milda Stuknite for providing language help and writing assistance. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Abstract

L. garvieae has emerged as an important zoonotic pathogen. However, information regarding mechanisms and factors related to its pathogenicity is lacking. In the present study we investigated the distribution and functionality of genes related to virulence factors in *L. garvieae* strains isolated from different niches, using both post-genomic and genotypic analysis. Putative genes encoding hemolysin, fibronectin binding protein and penicillin acylase were detected in all analyzed genomes/strains. Their expression was significantly induced by bile salts stress. Putative genes encoding bile salt hydrolase were found in a few strains from dairy and human sources, as well as the mobilizable *tet* genes. Finally, all genomes possessed a folate gene cluster, in which mutations in the dihydropteroate synthase gene (*folP*) could be related to sulfonamides resistance.

To the best of our knowledge, this is the first study aimed to explore the pathogenic potential of *L. garvieae* through the analysis of numerous *L. garvieae* genomes/strains, coming from different sources. This approach allowed the detection of virulence-related genes not yet investigated in the species and the study of their expression after exposure to different environmental stresses. The results obtained suggest a virulence potential in some *L. garvieae* strains that can be exploited for survival in the human gastrointestinal tract.

Keywords: *Lactococcus garvieae*; Emerging zoonotic pathogen; Virulence genes; Gene expression studies

Introduction

Lactococcus garvieae is regarded as an important fish pathogen, affecting different farmed and wild fish species in many countries (Vendrell et al. 2006). Recent developments in discriminating molecular analyses have enabled linking this species also with human infections. Human infections most commonly manifest as infective endocarditis, liver abscess, diverticulitis, peritonitis,

endophthalmitis and spondylodiscitis (Chan et al. 2011, Kim et al. 2013, Ramussen et al. 2014, Sachdeva et al. 2016). Even though the source of infection is still uncertain in many cases, the consumption of contaminated fish, in conjunction with immune-deficient subjects, is suspected to be the most probable cause of the infection. The frequent recovery of *L. garvieae* isolates from milk, dairy products, meat products and vegetables (Aquilanti et al. 2007, Kawanishi et al. 2007, Ferrario et al. 2012, 2013) and their genetic relatedness with human *L. garvieae* isolates (Reguera-Brito et al. 2016) suggest that also these foods might represent important sources of human *L. garvieae* infection.

Little information, in some cases contradictory, is available on the precise mechanism and factors related to pathogenicity of *L. garvieae*. Several studies (Barnes et al. 2002, Kawanishi et al. 2006, 2007) have reported that capsulated fish isolates showed strong virulence in fish (with a few exceptions), whereas none of the isolates (capsulated or not), obtained from terrestrial animals, were clearly lethal to fish. The authors did not show any relationship between the strains' pathogenicity, capsule and niche of isolation, but assumed that a difference in polysaccharide composition of the capsule could explain the non-virulence of some strains, regardless of the presence of capsule. Furthermore, studies on the host specificity of the bacterium have also revealed a behavior depending more on the strain than on the environment of isolation: isolates pathogenic to fish did not show pathogenicity in mice. (Kawanishi et al. 2007, Reimundo et al. 2011). Moreover, a strain isolated from a human patient affected by endocarditis provoked by the bacterium was virulent neither for fish nor for mice (Reimundo et al. 2011).

Recently, the availability of genome sequences of several *L. garvieae* strains has provided specific information on genes related to virulence. For example, analysis of the genome of a fish-isolated virulent strain (Morita et al. 2011) has revealed the presence of a capsule gene cluster composed of 15 genes that was not found in the non-virulent strain used for comparison. Strains isolated from a human with endocarditis, seemed to be pathogenic in human. However, they did not possess genes encoding extracellular envelope (Kim et al. 2015). Comparative genome analysis of

these strains indicated that the presence of a capsule gene cluster is not the only factor leading to pathogenicity. Indeed, other potential virulence factors could be found in the *L. garvieae* genome, such as adhesion surface proteins, hemolysins, NADH oxidase, resistance to antibiotics, which all could have an important role in pathogenicity both in humans and in animals. The real involvement of these genes in virulence remains to be studied, as well as their association with strains isolated in specific environments and their specificity at the strain-level.

Studies on biodiversity in *L. garvieae* using different genetic approaches (Ferrario et al. 2012, 2013), have revealed that *L. garvieae* population could be separated into independent genomic lineages. However, this discrimination was not coherent with the ecological niche of origin. Another study on comparative genome analysis (Miyauchi et al. 2012) did not allow grouping of strains associated with infection both in animals and in humans.

In the present study we investigated the distribution and functionality of genes related to virulence factors in *L. garvieae* strains isolated from different niches, using both post-genomic and genotypic analysis. The study also aimed to evaluate, through RT-qPCR experiments the expression profile of the detected genes in different conditions.

Materials and methods

Data source for comparative genome analysis

Information of publicly available *L. garvieae* genomes was retrieved from the National Center for Biotechnology Information (NCBI, available at http://www.ncbi.nlm.nih.gov/). Genome analysis was carried out using the Rapid Annotation using Subsystem Technology (RAST) Server (Aziz et al. 2008). The NCBI BLAST software was used for sequence-similarity search (Altschul et al. 1997).

Bacterial strains and culture conditions

The source and origin of the tested strains are listed in Table 1. Strains were maintained in M17 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 1 g/L glucose (M17-G) at 30 °C for 24 h; serial transfer was minimized to prevent the occurrence of mutations as a result of adaptation to laboratory medium and conditions. Stock cultures were maintained at -80 °C in M17-G with 15% glycerol.

To study gene expression in different conditions, selected strains were grown in M 17-G, as reference medium for all experiments, M 17-G supplemented with sub-lethal antibiotic concentrations (M17-GAR), M17-G with addition of 0.2% bile salts salts (Sigma-Aldrich, St. Louis, MO, USA), (M17-GBS), Brain Heart Infusion (BHI) broth (Sigma-Aldrich) and Meat Simulation medium (MSM) broth (Leroy and De Vuyst 2005). The exponentially growing cell cultures in M17-G (OD_{600} =0.9) were collected through centrifugation (9600 x *g* at 4 °C for 10 min), washed three times with phosphate buffered saline (PBS: 130 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4), resuspended in sterile water and distributed into the selected prewarmed media. These conditions were applied for 1 h at 30 °C for MSM and at 37 °C for BHI and M17-GBS. For M17-GAR cells were incubated at 30 °C for 8 h before RNA was extracted for gene expression analysis. Samples were taken subsequently, centrifuged and the pellets obtained used for RNA extraction.

DNA extraction and quantification

For strains grown in pure culture, DNA was extracted as previously described by Fortina et al. (2003).The concentration and purity of the DNAs were determined using a UV-Vis spectrophotometer (SmartSpec™ Plus, Bio-Rad, Hercules, CA, USA).

Gene targets and DNA amplification

Internal fragments of loci *hly* III, *fbp, pva, bsh1, bsh2, and folP* identified through BLAST and codifying for hemolysin, fibronectin binding protein, penicillin acylase, bile salt hydrolases, and dihydropteroate synthase respectively, were amplified using primers developed in this study. The specific primers and conditions used and their amplification products are reported in Table 2. PCRs were performed in a 25 μL reaction mixture containing 100 ng of bacterial DNA, 2.5 μL of 10× reaction buffer (Thermo Fisher Scientific, Vilnius, Lithuania), 200 μM of each dNTP, 2.5 mM $MgCl_2$, 0.5 μM of each primer, and 0.5 U of *Taq* polymerase (Thermo Fisher Scientific). After incubation for 2 min at 94 °C, samples were subjected to 35 cycles of 60 s at the annealing temperature (Table 2), followed by 1 min at 72 C; the reaction was completed by 7 min at 72 C and kept at 4 °C using a PCR instrument Mastercycler® 96 (Eppendorf, Hamburg, Germany). Amplification products were separated on a 1.5% agarose gel stained with ethidium bromide in 1× TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) buffer and photographed.

RNA extraction and RT-qPCR experiments

L. garvieae cells, obtained as above reported, were washed three times with PBS buffer and resuspended in 1.5 mL RNase-free water (Macherey-Nagel, Düren, Germany). Total RNA was isolated using NucleoSpin RNA II (Macherey-Nagel), according to manufacturer's protocol.RNA concentration and purity were optically determined at 260 and 280 nm using a UV-Vis spectrophotometer (SmartSpecTM Plus, Bio-Rad). RNA integrity was assessed by electrophoresis. Residual contaminating DNA was hydrolyzed with DNaseI (RNase-free) (Thermo Fisher Scientific) at 37 °C for 1h. Subsequently, 1 μg of RNA was converted into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) in accordance with manufacturer's instructions.

153 The level of gene expression was determined fluorimetrically using SYBR Green I (Bio-Rad). A
154 master mix containing, per sample, 7.5 µL of qPCR Master mix (EURx, Gdansk, Poland), 1.78 µL
155 RNase-free water, 0.36 µL of each primer (final concentration 300 nM), was combined with 5 µL
156 of each cDNA sample. This mixture was placed into a LineGene 9620 (BIOER Technol.,
157 Hangzhou, China) for real time analysis of PCR amplification. The qPCR temperature procedure
158 was performed according to the manufacturer's recommendations and corresponded to an initial
159 cycle of denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation 95 °C for 30 sec,
160 annealing and extension 60 °C for 30 sec. Fluorescence acquisition was done at the end of each
161 amplification. A melting curve analysis was performed to verify the identity of the PCR products.
162 The optimization of the qPCR protocols (temperature of annealing, concentrations of primers and
163 reaction efficiency) was carried out before gene expression quantification. The housekeeping gene
164 *gapC* (encoding a glyceraldehyde-3-phosphate dehydrogenase) was used as internal control to
165 which all other gene expressions were normalized. The primers used are reported in Table 2.
166 Samples were examined for differences in gene expression using relative quantification in which
167 relevant gene expression is normalized to a housekeeping gene, using the $2^{-\Delta\Delta CT}$ method, as reported
168 elsewhere (Livak and Schmittgen, 2001). All gene expression results are based upon independent
169 experiments per environmental condition. Statistical comparison of means was performed using
170 Student's test.
171
172 **Results and discussion**
173
174 *L. garvieae* has gained recognition as an opportunistic emerging human pathogen and a transient or
175 commensal intestinal bacterium (Kubota et al. 2010). However, its pathogenic mechanism is yet
176 poorly understood. A comparative genomic analysis of four *L. garvieae* strains (Miyauchi et al.
177 2012) confirmed that a capsule gene cluster was found only in one fish-pathogenic strain and
178 reported other possible virulence genes, such as genes encoding hemolysins and adhesins. The

179 availability of other sequenced strains, isolated from various environments, could allow refining the
180 pan-genome structure and predicting new candidate genes responsible for host specificity of *L.*
181 *garvieae.*

182 In this study, we carried out a comparative genomic analysis on 15 sequenced *L. garvieae*
183 genomes; the detected virulence-related genes, also searched in the genome of 22 *L. garvieae*
184 strains of our collection, coming from different sources, were subjected to gene expression studies
185 to improve our understanding on adaptive biology of this emerging pathogen. There is an almost
186 complete lack of knowledge on gene expression in *L. garvieae*. Aguado-Urda et al. (2013) carried
187 out a whole genome DNA microarray of two strains, evidencing an efficient adaptive response of
188 the strains to low-temperature conditions. No other data regarding the expression of virulence genes
189 in *L. garvieae* are available.

190 Among the 15 genomes of *L. garvieae* available in Genebank (Table 3), five were related to
191 strains coming from diseased fish, four to strains of the dairy sector (cheeses or bovine mastitis),
192 two to strains isolated from meat, two from human and two from animals. The search of
193 hypothetical virulence factors carried out by RAST server showed the presence of genes that could
194 be involved in pathogenicity. In particular, we found an ORF exhibiting significant similarity to the
195 known sequences of hemolysin genes (*hly*). This ORF was present in all genomes tested and its
196 nucleotide sequence seemed highly conserved among the genomes (92-100%). *L. garvieae*
197 hypothetical hemolysin exhibited the highest levels of similarity to a hemolysin III of *Lactococcus*
198 *lactis* (69% identity) (accession number WP_012897263). It also exhibited significant similarity to
199 hemolysin III of *Lactococcus raffinolactis* (CCK20048) and *Enterococcus faecalis*
200 (WP_033624268) (64 and 56% identity, respectively). We referred to this ORF as the *L. garvieae*
201 *hly* gene. *L. garvieae* genomes also contained two ORFs, previously annotated in LG2 genome
202 (Morita et al. 2011) as encoding for putative hemolysins. However, the proteins contained
203 conserved domains not related to the domain of the hemolysin III superfamily. The functions of
204 these proteins remain still unknown.

Among the candidate genes encoding cell surface adhesins, we found an ORF codifying a protein that exhibited significant similarity to the known sequences of fibronectin binding proteins (FnBPs) belonging to the family of the FnBP A N-terminus (FBPA) of *L. lactis* (81%) (KRO21949), *Streptococcus mitis* (64%) (EFM31516.1) and *Streptococcus pyogenes* (60%) (ESA50946). Also in this case this ORF was present and highly conserved in all genomes tested (nucleotide similarity ranging from 94 to 100%): we referred to this ORF as the *L garvieae fbp* gene. Most of the understanding of bacterial FnBPs has emerged from the study of the proteins of *Staphylococcus aureus* and *S. pyogenes* (Terao et al. 2002, Speziale et al. 2009). The binding of FnBPs to fibronectin mediates not only the adherence of these pathogens to extracellular matrices but also to the surface of a number of host cell types, including endothelial and epithelial cells. It has been demonstrated that *S. aureus*–endothelium interaction mediated by the FnBPs can cause inflammation, procoagulant activity and endocarditis formation (Heying et al. 2009). In the last decade, several studies have revealed that a wide range of bacteria possess adhesin-like proteins with very different characteristics, but able to bind to fibronectin, such as proteins containing the FbpA motif. This motif, occurring widely in *Streptococcus* spp., could play a direct role in bacterial colonization and bacteria–host interactions (Henderson et al. 2010).

Concerning the genes involved in bile salts hydrolysis, the analysis carried out by RAST server showed the presence of ORFs that exhibited significant similarity to the known sequences of genes encoding different enzymes belonging to the superfamily of linear amide CN hydrolases. This superfamily comprises bile salt hydrolases (BSHs, also designated as conjugated bile acid hydrolases, CBH or conjugated bile acid hydrolases, CBAH) that catalyze the hydrolysis of bile salts; it also contains penicillin V acylases (PVAs), capable of hydrolyzing the same type of chemical bond, but using penicillin as substrate (Lambert et al. 2008). Even if it has been suggested that they may hydrolyze bile acids, the role of PVAs has not yet been elucidated (Begley et al. 2005). In our strains, the amino acid sequence encoded by one of these ORFs, present in all

genomes tested, showed the highest similarity to penicillin acylases of *L. lactis* (60%) (NP_267291). We referred to this ORF as the *L. garvieae pva* gene.

Two other ORFs were present only in the genome of specific strains. In particular, in the genome of strains TB25 and M14 (from dairy products), and 21881 (from human source) we found a candidate gene showing the highest similarity with a gene codifying a bile salt hydrolase that exhibits greatest homology to the BSHs of *Enterococcus mundtii* (WP_019723927) (68% amino acid identity), and *Enterococcus durans* (WP_053109026) (66% identity). These ORFs were named *L. garvieae bsh1* genes. In strain TB25 we found another putative BSH-encoding gene, sharing 64% identity with *bsh1*; this ORF, named *bsh2*, exhibited significant similarity to *bsh* genes present in *Enterococcus faecium* (WP_010724277) (85% identity) and *Enterococcus faecalis* (WP_016622461) (81% identity).

PCR experiments carried out with primers designated on *L. garvieae* conserved sequences, testing a collection of strains coming from different sources (Table 1), confirm that *hly, fbp* and *pva* genes could be considered as part of the core genome of the *L. garvieae* species. On the contrary, putative genes encoding BSH, which contributes to survival of strains in the intestinal tract, can be considered as part of the dispensable genome of *L. garvieae*. Indeed, only one strain of the collection (G01), in addition to the three sequenced genomes, showed a positive PCR signal for *bsh1*.

Regarding genes encoding antibiotic-resistance determinants, the mobilizable *tet* genes could be considered as part of the dispensable genome of *L. garvieae;* indeed *tet*M determinants were found only along the genome of two *L. garvieae* strains, IPLA 31405 and I113, this last also harboring the *tet*S determinant. The multidrug transporter *mdt*(A) gene, previously detected in some *L. garvieae* strains (Walther et al. 2008), was present on all available genomes: sequence analysis of all mdt(A) revealed the presence of mutations generating the two substitutions Val154Phe and Ile296Val in each antiporter motif C, that have been related to the susceptibility of *L. garvieae* to erythromycin. In agreement with reported data, all strains of the collection were susceptible to erythromycin (data

not shown). On the contrary, the 22 strains of the collection resulted resistant to sulfonamides (SUL) (data not shown). SUL acts as competitive inhibitor of the dihydropteroate synthase (DHPS), enzyme involved in bacterial pathway leading to formation of tetrahydrofolate (Huovinen et al. 1995). SUL resistance can be mediated by the acquisition of alternative DPHPS genes (*sul1, sul 2, sul3*) (Byrne-Bailey et al. 2009, Wang et al. 2014), or by mutations in the DHPS gene (*folP*) (Yun et al. 2012). Along the 15 *L. garvieae* genomes, no *sul* genes were detected; on the contrary, the sequenced strains harbored a folate biosynthesis cluster that includes *folA*, encoding dihydrofolate reductase, *folB*, predicted to encode neopterine aldolase, *folE*, predicted to encode GTP cyclohydrolase I, *folP*, predicted to encode dihydropteroate synthase and *folC*, predicted to encode folate synthetase. Sequence comparison showed an identical genetic organization with folate cluster present in *L. lactis*, with an amino acid sequence identity ranging from 63 to 85%. Amino acid sequences of DHPS from the 15 *L. garvieae* genomes, were all identical and contained, within the conserved loop 1 and 2 regions (two catalytic sites of the enzyme), mutations that have been associated to sulfa drug-resistance in other bacteria (Baca et al. 2000, Babaoglu et al. 2004).

To verify the expression of the putative virulence-associated genes and to determine the extent to which this expression could be influenced by infection-relevant environmental cues, we quantified virulence factor-encoding mRNA following growth in different conditions, and compared it to mRNA abundance in laboratory medium-grown cultures, using quantitative real-time PCR. For these experiments, we selected strains TB25 and LG9, as representatives of the two different lineages within that *L. garvieae* population can be distributed (Ferrario et al. 2013) and designed environmental conditions simulating those associated with both infections and foods. Brain Heart Infusion (BHI) broth was used due to association of *L. garvieae* with different cases of endocarditis; M17 with addition of 0.2% bile salts and meat simulation medium (MSM) were prepared to represent the digestive tract of the host and the conditions in meat products respectively.

Even if the *hly*, *pva*, and *fbp* expression was different under the selected growth conditions and in relation to *L. garvieae* strains, the three genes were up-regulated in response to bile salts stress

(Table 4). In particular, *fbp* and *hly* genes in strain TB25 were significantly over-expressed after exposure to bile salts (12- and 9-fold). Transcription of *bsh1* and *bsh2* genes was also significantly induced by TB25 strain in response to bile salts (3.9- and 7.5-fold respectively). Lower values of up-regulation were observed in MSM medium. While statistically significant, the expression of all genes tested in BHI cultures was modest.

It is known that enteric bacteria respond to bile by altering protein production and by increasing mechanisms of resistance (Begley et al. 2005). Gram negative bacteria can also utilize bile as a signal for the temporal production of virulence factors (Gunn 2000). This is the case, for example, of *Vibrio* spp., the growth of which in presence of bile salts increases the production of hemolysin (Osawa and Yamai 1996); other studies indicate that bile salts can stimulate bacterial co-aggregation, bacterial adhesion to human cells and biofilm formation (Pumbwe et al. 2007, Ruiz et al. 2013). On the contrary, the effect of bile on Gram positive bacteria is not well understood, even if the presence of the same mechanisms found in Gram negative bacteria has been hypothesized (Gunn 2000).

Finally, when the strains were grown in presence of sub-lethal concentration of sulfamethoxazole (1 mg/mL), no change of expression in *folP* was observed, compared to levels in reference medium without antibiotic. The constitutive levels of expression of *folP* in presence of SUL, the absence of *sul* drug resistance genes (in all sequenced genomes) and the high sulfamide resistance in all *L. garvieae* strains tested, suggest that the observed mutations in *folP* gene (and consequently in DHPS catalytic site) could be responsible of sulfa-drug-resistance in *L. garvieae* The correlation between these mutations and SUL resistance has been already described in other species (Baca et al. 2000, Babaoglu et al. 2004).

Conclusions

This is the first study aimed to explore the pathogenic potential of *L. garvieae* through the analysis of numerous *L. garvieae* genomes/strains, coming from different sources. This approach allowed the detection of virulence-related genes not yet investigated in the species. Additionally, to the best of our knowledge, this is the first study on the expression of virulence-related genes in *L. garvieae* exposed to different environmental stresses. The results obtained suggest a virulence potential in some *L. garvieae* strains that can be exploited for survival in the human gastrointestinal tract. This evidence agrees with the genetic relatedness previously found between human and food isolates and the hypothesis that contaminated foods can represent important sources of human *L. garvieae* infection (Reguera-Brito et al. 2016). Further studies are currently in progress to verify this hypothesis.

Conflit of interests

The authors declare that they have no conflict of interest.

References

Aguado-Urda M, Gibello A, Blanco MM, Fernández-Garayzábal JF, López-Alonso V, López-Campos GH (2013) Global transcriptome analysis of *Lactococcus garvieae* strains in response to temperature. PLoS ONE 8(11): e79692. doi:10.1371/journal.pone.0079692

Aguado-Urda M, López-Campos GH, Blanco MM, Fernández-Garayzábal JF, Cutuli MT, Aspiroz C, López-Alonso V, Gibello A (2011a) Genome sequence of *Lactococcus garvieae* 21881, isolated in a case of human septicemia. J Bacteriol 193: 4033–4034

330 Aguado-Urda M, López-Campos GH, Gibello A, Cutuli MT, López-Alonso V, Fernández-
331 Garayzábal JF, Blanco MM (2011b) Genome sequence of *Lactococcus garvieae* 8831, isolated
332 from rainbow trout lactococcosis outbreaks in Spain. J Bacteriol 193: 4263–4264

333 Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped
334 BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids
335 Res 25: 3389–3402

336 Aquilanti L, Garofalo C, Osimani A, Silvestri G, Vignaroli C, Clementi F (2007) Isolation and
337 molecular characterization of antibiotic-resistant lactic acid bacteria from poultry and swine
338 meat products. J Food Prot 70: 557–565

339 Aziz RK, Bartels D, Bes AA, DeJongh M, Disz T, Edwards RA et al. (2008) The RAST Server:
340 rapid annotation using subsystems technology. BMC genomics 9-75.
341 http://dx.doi.org/10.1186/1471-2164-9-75

342 Babaoglu KJQ, Lee RE, White SW (2004) Crystal structure of 7,8-dihydropteroate synthase from
343 *Bacillus anthracis*: mechanism and novel inhibitor. Design Structure 12: 1705–1717

344 Baca AM, Sirawaraporn R, Turle S, Sirawaraporn W, Hol WGJ (2000) Crystal structure of
345 *Mycobacterium tuberculosis* 6-hydroxymethyl-7,8-dihydropteroate synthase in complex with
346 pterin monophosphate: new insight into the enzymatic mechanism and sulfa-drug action. J Mol
347 Biol 302: 1193–1212

348 Barnes AC, Guyot C, Hansen BG, Mackenzie K, Horne MT, Ellis AE (2002) Resistance to serum
349 killing may contribute to differences in the abilities of capsulate and non-capsulated isolates of
350 *Lactococcus garvieae* to cause disease in rainbow trout (*Oncorhynchus mykiss L.*) Fish and
351 Shellfish Immun 12: 155–168

352 Begley M, Sleator RD, Gahan CGM, Hill C (2005) Contribution of three bile-associated loci, *bsh*,
353 *pva, and btlB*, to gastrointestinal persistence and bile tolerance of *Listeria moncytogenes.* Infect
354 Immunity 73: 894-904

355 Byrne-Bailey KG, Gaze WH, Kay P, Boxall AB, Hawkey PM, Wellington EMH (2009) Prevalence
356 of sulfonamide resistance genes in bacterial isolates from manured agricultural soils and pig
357 slurry in the United Kingdom. Antimicrob Agents Chemother 53: 696–702

358 Chan JFW, Woo PCY, Teng JLL, Lau SKP, Leung SSM, Tam FCC, Yuen KY (2011) Primary
359 infective spondylodiscitis caused by *Lactococcus garvieae* and a review of human *L. garvieae*
360 infections. Infection 39: 259-264

361 Ferrario C, Ricci G, Borgo F, Rollando A, Fortina MG (2012) Genetic investigation within
362 *Lactococcus garvieae* revealed two genomic lineages. FEMS Microbiol Lett 332: 153-161

363 Ferrario C, Ricci G, Milani C, Lugli GA, Ventura M, Eraclio G, Borgo F, Fortina MG (2013)
364 *Lactococcus garvieae*: where is it from? A first approach to explore the evolutionary history of
365 this emerging pathogen. PLoS One 8, e84796.

366 Florez AB, Reimundo P, Delgado S, Fernandez E, Alegria A, Guijarro JA, Mayo B (2012) Genome
367 sequence of *Lactococcus garvieae* IPLA 31405, a bacteriocin-producing, tetracycline-resistant
368 strain isolated from a raw-milk cheese. J Bacteriol 194: 5118-5119

369 Fortina MG, Ricci G, Acquati A, Zeppa G, Gandini A, Manachini PL (2003) Genetic
370 characterization of some lactic acid bacteria occurring in an artisanal protected denomination
371 origin (PDO) Italian cheese, the Toma Piemontese. Food Microbiol 20: 397-404

372 Gabrielsen C, Brede DA, Hernandez PE, Nes IF, Diep DB (2012) Genome sequence of the
373 bacteriocin-producing strain *Lactococcus garvieae* DCC43. J Bacteriol 194: 6976-6977

374 Gunn JS (2000) Mechanisms of bacterial resistance and response to bile. Microbes Infect 2: 907-
375 913

376 Henderson B, Nair S, Pallas J, Williams MA (2010) Fibronectin: a multidomain host adhesin
377 targeted by bacterial fibronectin-binding proteins. FEMS Microbiol Rev 35: 147–200

378 Heying R, van de Gevel J, Que YA, Piroth L, Moreillon P, Beekhuizen H (2009) Contribution of
379 (sub)domains of *Staphylococcus aureus* fibronectin-binding protein to the proinflammatory and
380 procoagulant response of human vascular endothelial cells. Thromb Haemost 101: 495–504

381 Huovinen P, Sundstrom L, Swedberg G, Skold O (1995) Trimethoprim and sulfonamide resistance.
382 Antimicrob Agents Chemother 39: 279-289

383 Kawanishi M, Yoshida T, Kijima M, Yagyu K, Nakai T, Okada S, Endo A, Murakami M, Suzuki S,
384 Morita H (2007) Characterization of *Lactococcus garvieae* isolated from radish and broccoli
385 sprouts that exhibited a KG+ phenotype, lack of virulence and absence of a capsule. Lett Appl
386 Microbiol 44: 481-487

387 Kawanishi M, Yoshida T, Yagashiro S, Kijima M, Yagyu K, Nakai T. Murakami M, Morita H,
388 Suzuki S (2006) Differences between *Lactococcus garvieae* isolated from the genus *Seriola* in
389 Japan and those isolated from other animals (trout, terrestrial animals from Europe) with regard
390 to pathogenicity, phage susceptibility and genetic characterization. J Appl Microbiol 101: 496-
391 504

392 Kim JH, Go J, Cho CR, Kim JI, Lee MS, Park SC (2013) First report of human acute acalculous
393 cholecystitis caused by the fish pathogen *Lactococcus garvieae*. J Clin Microbiol 51: 712–714

394 Kim JH, Kang D-H, Park SC (2015) Draft genome sequence of human-pathogenic *Lactococcus*
395 *garvieae* LG-ilsanpaik-gs201105 that caused acute acalculous cholecystitis. Genome A, 3,
396 e00464-15

Kubota H, Tsuji H, Matsuda K, Kurawa T, Asahara T, Nomoto K (2010) Detection of human intestinal catalase-negative, Gram-positive cocci by rRNA targeted reverse transcription-PCR. Appl Environ Microbiol 76: 5440–5451

Lambert JM, Siezen RJ, de Vos WM, Kleerebezem M (2008) Improved annotation of conjugated bile acid hydrolase superfamily members in Gram-positive bacteria. Microbiology 154: 2492-2500

Leroy F, De Vuyst L (2005) Simulation of the effect of sausage ingredients and technology on the functionality of the bacteriocin-producing *Lactobacillus sakei* CTC494 strain. Intern J Food Microbiol 100: 141-152

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using Real- Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402–408

Miyauchi E, Toh H, Nakano A, Tanabe S, Morita H (2012) Comparative genomic analysis of *Lactococcus garvieae* strains isolated from different sources reveals candidate virulence genes. Intern J Microbiol doi:10.1155/2012/728276

Morita H, Toh H, Oshima K, Yoshizaki M, Kawanishi M, Nakaya K, Suzuki T, Miyauchi E, Ishii Y, Tanabe S, Murakami M, Hattori M (2011) Complete genome sequence and comparative analysis of the fish pathogen *Lactococcus garvieae*. PLoS One, 6(8) e23184

Moumene M, Drissi F, Croce O, Djebbari B, Robert C, Angelakis E, Benouareth DE, Raoult D, Merhej V (2016) Complete genome sequence and description of *Lactococcus garvieae* M14 isolated from Algerian fermented milk. New Microbe New Infect 10: 122–131

Osawa R, Yamai S (1996) Production of thermostable direct hemolysis by *Vibrio parahaemolyticus* enhanced by conjugated bile acids. Appl Environ Microbiol 62: 3023-3025

Pumbwe L, Skilbeck CA, Nakano V, Avila-Campos MJ, Piazza RMF, Wexler HM (2007) Bile salts enhance bacterial co-aggregation, bacterial-intestinal epithelial cell adhesion, biofilm formation and antimicrobial resistance of *Bacteroides fragilis*. Microbial Pathog 43: 78-87

Rasmussen M, Bjork Werner J, Dolk M, Christensson B (2014) *Lactococcus garvieae* endocarditis presenting with subdural haematoma. BMC Cardiovasc Disord 14, 13 doi: 10.1186/1471-2261-14-13

Reguera-Brito M, Galán-Sánchez F, Blanco MM, Rodríguez-Iglesias M, Domínguez L, Fernández-Garayzábal JF, Gibello A (2016) Genetic analysis of human clinical isolates of *Lactococcus garvieae*: relatedness with isolates from foods. Infection Gen Evol 37: 185–191

Reimundo P, Pignatelli M, Alcaraz LD, D'Auria G, Moya A, Guijarro JA (2011) Genome sequence of *Lactococcus garvieae* UNIUD074, isolated in Italy from a lactococcosis outbreak. J Bacteriol 193: 3684–3685

Ricci G, Ferrario C, Borgo F, Eraclio G, Fortina MG (2013) Genome sequences of two *Lactococcus garvieae* strains isolated from meat. Genome Ann 1 (1) e00018-12

Ricci G, Ferrario C, Borgo F, Rollando A, Fortina MG (2012) Genome sequences of *Lactococcus garvieae* TB2.5, isolated from Italian cheese, and *Lactococcus garvieae* LG9, isolated from Italian rainbow-trout. J Bacteriol 194: 1249-1250

Ruiz L, Margolles A, Sànchez B (2013) Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium.* Frontiers Microbiol doi: 10.3389/fmicb.2013.00396

Sachdeva MM, Moshiri A, Leder HA, Scott AW (2016) Endophthalmitis following intravitreal injection of anti-VEGF agents: long-term outcomes and the identification of unusual micro-organisms. J Ophthalmic Inflam Infect 6: 2-7

441 Speziale P, Pietrocola G, Rindi S, Provenzano M, Provenza G, Di Poto A, Visai L, Arciola RC
442 (2009) Structural and functional role of *Staphylococcus aureus* surface components recognizing
443 adhesive matrix molecules of the host. Future Microbiol 4: 1337–1352

444 Terao Y, Kawabata S, Nakata M, Nakagawa I, Hamada S (2002) Molecular characterization of a
445 novel fibronectin-binding protein of *Streptococcus pyogenes* strains isolated from toxic shock-
446 like syndrome patients. J Biol Chem 277: 47428–47435

447 Vendrell D, Balcázar JL, Ruiz-Zarzuela I, de Blas I, Gironés O, Múzquiz JL (2006) *Lactococcus*
448 *garvieae* in fish: a review. Comp Immunol Microbiol Infect Dis 29: 177–198

449 Walther C, Rossano A, Thomann A, Perreten V (2008) Antibiotic resistance in *Lactococcus* species
450 from bovine milk: presence of a mutated multidrug transporter *mdt(A)*gene in susceptible
451 *Lactococcus garvieae* strains. Vet Microbiol 131: 348–357

452 Wang N, Yang X, Jiao S, Zhang J, Ye B, Gao S (2014) Sulfonamide-resistant bacteria and their
453 resistance genes in soils fertilized with manures from Jiangsu province, southeastern China.
454 PLoS One 9(11): e112626. doi:10.1371/journal.pone.0112626

455 Yoshida T, Endo M, Sakai M, Inglis V (1997) A cell capsule with possible involvement in
456 resistance to opsonophagocytosis in *Enterococcus seriolicida* isolated from yellowtail *Seriola*
457 *quinqueradiata*. Dis Aquat Org 29: 233–235

458 Yun M-K, Wu Y, Li Z, Zhao Y, Waddell MB, Ferreira A M, Lee RE, Bashford D, White SW
459 (2012) Catalysis and sulfa drug resistance in dihydropteroate synthase: crystal structures reveal
460 the catalytic mechanism of DHPS and the structural basis of sulfa drug action and resistance.
461 Science 2: 335(6072): 1110–1114. doi:10.1126/science.1214641

462

463

464

Table 1 *L. garvieae* strains used in this study, and their origin

L. garvieae strains	Source of isolation
[a]DSMZ 20684[T]	Cow with mastitis
G27	Cow milk
TB25[b]; G01; G07	Italian cheeses
BL 7; BL 15; BL 23	Bedding litter from American dairy farms
Far 1	Wheat flour
Br 3; Br4; Sed 2; Ins 1	Vegetables
V63; V79; LG9; Lg19	Diseased fish
Po1; Tac 2; Bov 3	Raw meat
I113; Smp3	Meat products

[a] DSMZ= Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; [T]= type strain of the species

[b] TB25 = synonymous of G9 strain reported in Ferrario et al. (2012)

472 **Table 2** PCR primers and conditions used in this study

473

Gene/product	Primer-pair (5'-3')	Annealing temperature (°C)	Amplicon (bp)
hly III (hemolysin)	F: TAGCACTTGTTTGGCTTTGTGC R: CCATAGATGGAGAACCACATCA	60	301
fbp (fibronectin binding protein)	F: CGGTTCGTTCAGGAAGAACATC R: CGGTCATTGCCTACTTGCTCAA	60	181
pva (penicillin V acylase)	F: AGCTCGCGCCTTTTGAATTTG R: ACGACCTGTTGGATCGACAG	60	164
bsh1 (bile salt hydrolase)	F: AATACGGGTTAAGTATGGCTGG R: AGCTAACAGTTCTCTAGCTTC	60	152
bsh2 (bile salt hydrolase)	F: GGCTTATCCATGGCTGGATTAA R: GGGATAAAGGAAGCTCTTCACT	60	196
folP (dihydropteroate synthase*)*	F: GTAAGTCTTCTCGCCCAGG R: TGCCCGCTCGACCATTATTC	58	240
gapC (glyceraldehyde-3-phosphate dehydrogenase)	F: TATCGGTCGTCTTGCTTTCC R: TCAGCCCAGTTGATGTTAGC	58	225

474

475 **Table 3** *L. garvieae* strains used for genome sequence analysis

476

Strain	Lenght (bp)	Number of contigs	Genes	tRNA	% GC	Isolation	Accession number	Reference
LG9	2,087,705	139	2,069	43	38.5	Diseased Rainbow trout	AGQY00000000	Ricci et al. 2012
ATCC 49156	1,950,135	Complete	1,983	79	38.8	Diseased yellowtail	AP009332	Morita et al. 2011
Lg2	1,963,964	Complete	1,998	62	38.8	Diseased yellowtail	AP009333	Morita et al. 2011
UNIUD 074	2,171,966	25	2,178	63	38.7	Diseased Rainbow trout	AFHF00000000	Reimundo et al. 2011
IPLA 31405	2,052,310	23	2,030	46	38.5	Spanish cheese	AKFO00000000	Florez et al. 2012
Tac2	2,242,860	97	2,194	43	38.2	Turkey meat	AMFE00000000	Ricci et al. 2013
NBRC 100934^{T}	2,028,350	56	1,934	47	38.5	Bovine mastitis	BBJW00000000	Unpublished
TRF1	2,204,910	112	2,394	60	38.5	Fecal material of a timber rattlesnake	AVFE00000000	Unpublished
TB 2.5	2,014,328	92	1,995	38	38.1	Italian cheese	AGQX00000000	Ricci et al. 2012
21881	2,164,557	91	2,209	42	37.9	Human blood	AFCF00000000	Aguado-Urda et al. 2011a
8831	2,087,226	87	2,030	48	38.0	Diseased Rainbow trout	AFCD00000000	Aguado-Urda et al. 2011b
M14	2,253,704	13	2,273	45	37.7	Fermented milk	CCXC00000000	Moumene et al. 2016
Lg-ilsanpaik-gs201105	1,958,880	53	1,934	47	38.1	Human gall bladder	JPUJ00000000	Kim et al. 2015
DCC43	2,239,000	67	2,228	49	37.8	Mallard duck intestines	AMQS00000000	Gabrielsen et al. 2012
I113	2,178,730	49	2,151	49	37.9	Pork sausage	AMFD00000000	Ricci et al. 2013

477

478

479

480

Table 4 Influence of different growth conditions on the expression levels of virulence genes in *L. garvieae*[a]

Strain	Gene	Fold change when cultured in /in presence of: 0.2% bile salts	BHI	MSM	Sulfamethoxazole (1 mg/ml)
L. garvieae TB25	*hly*	**9.0**	1.2	**5.5**	
	fbp	**12.0**	**1.5**	**5.2**	
	pva	**4.0**	**0.5**	1.2	
	bsh1	**3.9**	**2.0**	**4.2**	
	bsh2	**7.5**	**4.3**	**6.7**	
	folP				**0.5**
L. garvieae LG9	*hly*	**3.5**	1.5	**3.0**	
	fbp	**4.5**	**1.0**	4.0	
	pva	**5.0**	0.5	**1.2**	
	folP				**0.3**

[a] Values greater than 1 reflect a relative increase in gene expression compared to the reference medium. Values in boldface type represent changes with a statistical significance (*P*) of < 0.05. Each data point is derived from the mean and standard deviation of three independent experiments