Comparative genomic analyses of *Lactococcus garvieae* isolated from bovine mastitis in China

Yushan Lin[1#], Jinge Han[2#], Herman W. Barkema[3], Yue Wang[1], Jian Gao[1] John P. Kastelic[3], Bo Han[1*], Shunyi Qin[2*], Zhaoju Deng[1*]

[1]Department of Clinical Veterinary Medicine, College of Veterinary Medicine, China Agricultural University, Beijing, 100193, P. R. China

[2]College of Animal Science and Veterinary Medicine, Tianjin Agricultural University, Tianjin, 300384, P. R. China

[3]Department of Production Animal Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, AB, Canada, T2N 4N1

[#]Both contributed equally

***Correspondence:** Bo Han: hanbo@cau.edu.cn; Shunyi Qin: qinshunyi@163.com; Zhaoju Deng: zhaoju2020@hotmail.com

Running title: Comprehensive genomic analyses of *Lactococcus garvieae*

No. of words: 4157

No. of Figures: 7

No. of Tables: 3

ABSTRACT *Lactococcus garvieae* is an emerging zoonotic pathogen, but there are few reports regarding bovine mastitis. The recent prevalence of *L. garvieae* poses an increasing disease threat and global public health risk. A total of 39 *L. garvieae* isolates were obtained from 2899 bovine clinical mastitis milk samples in 6 provinces of China from 2017 to 2021. Five clonal complexes were determined from 32 MLST types of *L. garvieae*; ST46 was the predominant sequence type and 13 novel MLST types were identified. All isolates were resistant to chloramphenicol and clindamycin, but susceptible to penicillin, ampicillin, amoxicillin-clavulanic acid, imipenem, ceftiofur, enrofloxacin, and marbofloxacin. Based on genomic analyses, *L. garvieae* had 6310 genes, including 1015, 3641 and 1654 core, accessory and unique genes. All isolates had virulence genes coding for collagenase, fibronectin-binding protein, Glyceraldehyde-3-phosphate dehydrogenase, superoxide dismutase and NADH oxidase. Most isolates had *lsaD* and *mdtA* AMR genes. Based on COG results, the functions of defense, transcription and replication, recombination and repair were enhanced in unique genes, whereas functions of translation, ribosomal structure and biogenesis were enhanced in core genes. The KEGG functional categories enriched in unique genes included human disease and membrane transport, whereas COG functional categories enriched in core genes included energy metabolism, nucleotide metabolism and translation. No gene was significantly associated with host specificity. In addition, core genome SNPs analysis suggested potential host adaptation of some

isolates in several sequence types. Therefore, this study characterized *L. garvieae* isolated from mastitis and assessed host adaptation of *L. garvieae* to various hosts.

IMPORTANCE This study provides important insights on bovine mastitis key topic pathogen *Lactococcus garvieae*, which constitutes mastitis concerns. However, comprehensive genomic analyses of *L. garvieae* from dairy farms have not been performed. This study gives a detailed and comprehensive novel feature in *L. garvieae*, an important but poorly characterized bacterium, recovered in the past 5 years in 6 Chinese provinces. We documented diverse contributory genetic processes, including predominant sequence type ST46 and 13 novel MLST types. *L. garvieae* had 6310 genes, including 1015, 3641 and 1654 core, accessory and unique genes. All isolates had virulence genes coding for collagenase, fibronectin-binding protein, Glyceraldehyde-3-phosphate dehydrogenase, superoxide dismutase and NADH oxidase, and resistant to chloramphenicol and clindamycin. Most isolates had *lsaD* and *mdtA* antimicrobial resistance genes. No gene was significantly associated with host specificity. This is the first absolute quantification of *L. garvieae* isolated from mastitis and identified host adaptation of *L. garvieae* to various hosts.

KEYWORDS bovine mastitis, *Lactococcus garvieae*, population structure, virulence genes, antimicrobial resistance, host adaptation

INTRODUCTION

Bovine mastitis is a prevalent and costly disease on dairy farms worldwide **(1, 2)**. It is a multifactorial disease, often caused by bacteria **(2)**. Bacterial pathogens associated with bovine mastitis are broadly classified as major (*Staphylococcus aureus*, *Escherichia coli*, *Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis*, *Enterococcus* spp. etc.) and minor pathogens (Non-*aureus Staphylococci* spp., *Lactococcus* spp., Corynebacterium spp., etc.) **(3)**. Most studies have focused on major pathogens, with only limited studies of minor pathogens.

Lactococcus garvieae is a zoonotic pathogen reported to cause infections in fish **(4)** and humans **(5–8)**. It is also considered a minor pathogen for bovine mastitis, with transmission attributed to environmental reservoirs **(2)**. There are limited reports on bovine mastitis caused by *L. garvieae* **(9–15),** primarily descriptive studies of phenotypes or genotypes. However, detailed whole genome characterization of *L. garvieae* associated with bovine mastitis is lacking.

Predominant strain types of mastitis pathogens have been described for various pathogens **(16–20)**. Elucidating population structure and diversity of mastitis pathogens informs evidence-based mastitis control programs that target those prevalent strain types.

Bacterial pathogenicity is primarily determined by virulence factors; some facilitate adhesion and invasion, whereas antimicrobial resistance, particularly multi-drug resistance, is an important threat to public health **(21)**. For *L. garvieae,*

several virulence factors and antimicrobial resistance genes were identified using traditional methods (e.g., PCR) targeted at specific virulence genes **(22–24).** However, a comprehensive profiling of its virulence and antimicrobial resistance genes are lacking.

Host adaptation of bovine mastitis associated pathogens have been reported for *Staphylococcus aureus* **(25)** and *Streptococcus agalactiae* **(26)**. Infections caused by *L. garvieae* in humans, fish and cattle have been reported, but potential host adaptation of *L. garvieae* has not been studied. Therefore, our objectives were to: 1) resolve the population structure; 2) identify virulence genes and antimicrobial resistance genes; and 3) determine genes associated with host specificity.

RESULTS

A total of 39 *L. garvieae* isolates from 2899 clinical mastitis composite milk samples collected from 13 large dairy farms in 6 provinces in Northern China from April 2017 to September 2021. Detailed information of these isolates is provided in **Table 1**.

MLST and Minimum Spanning Tree. MLST analysis assigned the 86 *L. garvieae* isolates into 32 STs (**Table 2**), of which 13 were novel STs: ST46 to ST58. The most common sequence type was ST46 (n = 13), followed by ST48 (n = 9). The 32 STs were grouped into 5 CCs and 18 singletons: ST 3, 4, 13 and 38 into CC 1; ST 16 and 17 into CC 2; ST 10, 12, 21 and 35 into CC 3; ST 49 and 52 into CC4; and ST 47 and 50 into CC 5 (**Fig. 1**). Among the 39 isolates, 6 and 9 isolates formed clonal

101 complex CC4 and CC5, respectively, whereas, the remaining 24 isolates were
102 singletons.

103 **Antimicrobial Resistance Profile and Genes.** Antimicrobial resistance patterns
104 of *L. garvieae* are listed in **Table 3**. All isolates were resistant to chloramphenicol and
105 clindamycin. There were also high resistance rates for amikacin (90%), cefpodoxime
106 (82.5%), cefazolin (45%), and gentamicin (37.5%), whereas few isolates (5%) were
107 resistant to erythromycin. Meanwhile, all isolates were susceptible to penicillin,
108 ampicillin, amoxicillin-clavulanic acid, imipenem, ceftiofur, enrofloxacin and
109 marbofloxacin. Regarding multidrug resistance, there were 5, 14, 12, 6, 2 and 1 isolates
110 resistant to 3, 4, 5, 6, 7, and 8 antimicrobials, respectively.

111 Distribution of antimicrobial resistance (AMR) genes with country, host and clonal
112 complex information are presented in **Fig. 2**. The most common genotypic AMR
113 markers were: (i) multidrug protein first reported in *L. garvieae*, represented by the
114 *lsaD* gene (97% of isolates); (ii) resistance-nodulation-cell division antibiotic efflux
115 pump, represented by the mdtA gene (97% isolates); and (iii) tetracycline-resistant
116 ribosomal protection protein, coded by *tetS* gene (14 isolates, including 12 from current
117 bovine samples). Other tetracycline-resistant genes, *tetL and tetM,* were identified in
118 only 3 isolates. Three isolates, namely LG728, LG791 and MGYG-HGUT-00230,
119 harbored the most abundant AMR genes, the gene number of which was 14, 7, 5
120 respectively. Genes *cat, dfrG, ermA* and *lunD* were only present in LG728, and gene
121 *lnuD* was only in MGYG-HGUT-00230. Genes *ermB, fexA* and *optrA* were present in

LG728 and LG791. Genes *acc(6')-aaph(2'')* and *ant(6)-la* were identified in LG728 and MGYG-HGUT-00230. However, no AMR genes were detected in 3 isolates that belonged to ST18: A1, DCC43 and FDAARGOS_893.

Virulence Genes. The occurrence and distribution of putative virulence genes are shown in **Fig. 3**. The putative virulence factors were classified into 5 functional categories: toxin, iron uptake, capsule formation, adherence, and enzyme.

Among the 3 toxin-related genes, *hyl-II* was present in all isolates, whereas *hyl-I* and *hyl-III* were absent in ST18 only (represented by 3 isolates: A1, DCC43 and FDAARGOS_893). Nine iron uptake genes (*fepB, fepC, fepD, fecB, fecC, fecD, fecE, feoA,* and *feoB*) were detected in > 81 isolates. In contrast, *fecE* was absent only in EP01. However, *fecB* was absent in EP01 and FDAARGOS_893. *fecD, feoA feoB, fepB, fepC* and *fepD* were absent in 4 isolates: A1, EP01, DCC43 and FDAARGOS_893. Furthermore, *fecC* was absent in 5 isolates: A1, EP01, DCC43, FDAARGOS_893 plus FDAARGOS_1063. With respect to the 9 adherence-associated virulence factors, *adhPavA* and putative collagenase were identified in all isolates. The gene *adhPsaA* was absent in the same 4 isolates above (A1, EP01, DCC43 and FDAARGOS_893), whereas *Adhesin* was detected in 22 isolates. Genes expressing LPxTG proteins (LPxTG-1, LPxTG-2, LPxTG-3, LPxTG-4, LPxTG-5, and LPxTG-6) were in 4 to 43% of isolates. Six enzyme-related virulence factors were detected in almost all isolates, although *eno* and *srtA* were exclusively absent from 2 (MGBC116427 and UBA11300) and 4 isolates (A1, EP01, DCC43 and FDAARGOS_893). Regarding 16 genes

encoding for the capsule gene cluster, the number of gene presented in isolates varied from 0 to 16. Lg2 and JJJN1 were identified with all 16 genes, whereas 47 isolates had 0 genes.

Co-occurrence of virulence genes was visualized in **Fig. 4**, where each box had a phi coefficient value. A phi value of 1.0 indicates a perfect positive relationship between the 2 variables, whereas values > 0.7 indicates a fair positive relationship. Most associations among virulence genes were very weak. However, there were strongly positive correlations between 8 genes, including *adhPsaA*, *srtA* and 6 iron uptake genes (*fecD*, *feoA*, *feoB*, *fepB*, *fepC* and *fepD*). In addition, toxin-related genes hyl-I and -3 were also strongly positively associated with each other and those 8 genes. Furthermore, fecC was strongly positively associated with hyl-I, hyl-III and the 8 genes. LPxTG-4 was strongly positively associated with *Adhesin*, whereas *fecB* had strong associations with the 8 genes. However, there were no strong negative associations among the presence of virulence genes.

Pan-Genome Analyses. The pan-genome of 86 *L. garvieae* isolates tested in this study had 6310 genes. The core genome (shared by 100% isolates) consisted of 1015 genes. The accessory genome (genes in >2 isolates but not in all) consisted of 3641 genes, and the unique genome was composed of 1654 genes. According to BPGA's calculation, the pan genome was open but approached convergence (**Fig. 4A**). Functional annotation of genes in the pan-genome performed using the COG and KEGG databases revealed a distribution of functional categories among 3 pan-genome

sets (**Fig. 4B, 4C and 4D**). The functions of defense mechanisms, transcription and replication, recombination and repair were enhanced in unique genes, whereas the functions of translation, ribosomal structure and biogenesis were enhanced in core genes in KEGG functional pathways (**Fig. 4B**). The COG functional categories enriched in the unique genome included human disease and membrane transport (**Fig. 4C**). By contrast, COG categories enriched in the core genome included energy metabolism, nucleotide metabolism and translation (**Fig. 4D**).

Phylogenetic Analyses. A phylogenetic tree was constructed based on the core genes of 86 *L. garvieae* genomes (**Fig. 5**). The longer the branch, the more distant the evolutionary relationship. All trees had 3 clades that contained 4, 38 and 44 isolates respectively. All local isolates except Hebei-B-22 were in the same clades. EP01 was phylogenetically distant from the others. There were signs of host adaptation, which consisted of isolates from various hosts.

Phylogenetic tree based on 16s rRNA (**Fig. 5A**) were similar to that using the core genes with minor differences. For example, LG9 was assigned to clade A in core gene based phylogenetic tree but in clade B in 16S rRNA based phylogenetic tree.

Both trees corresponded well with STs and CCs predicted by GrapeTree analysis. For core gene based phylogenetic tree (**Fig. 5B**), all isolates that belonged to the same CC were grouped in the same cluster, except 2 isolates (LG9 and IBB3403) in the 16S rRNA-based phylogenetic tree.

Pan-Genome-Wide Association Analyses. No significant association was detected between genes and either country or host.

Core-genome SNPs Analyses. The core-genome single nucleotide polymorphism (SNPs) based phylogenetic tree with metadata annotation is displayed in **Fig. 6**. The numbers of core-genome SNPs among 86 isolates are provided in Supplementary **Table S1**. Several isolates from various hosts were phylogenetically closely related in core SNPs. For example, the number of SNPs between isolates within the same MLST but a different host were: DM12426 (human) and CT2 (fish) was 0, 1001287H_170206_H11 (human) and UBA5784 (metal) was 5, Lg-ilsanpaik-gs201105 (human) and Hebei-B-22 (cow) was 11, 21881 (human) and M14 (cow) was 105, which indicates potential host adaptation in *L. garvieae*.

Associations between the Co-occurrence of Virulence Genes. Co-occurrence of virulence genes was visualized in **Fig. 7**. SortaseA, LPxTG-6, and adhensin PsaA was in co-occurrence with *fecB*, *fecC*, *fecD*, *feoA*, *feoB*, *fepB*, *fepC*, *fepD*, *hemolysin I*, and *hemolysin III*.

DISCUSSION

Although *L. garvieae* was first isolated as a causative agent of bovine mastitis **(27)**, most reports have focused more on the epidemiology of fish and human cases. In addition, many studies used sequencing to investigate genotypic characteristics of *L. garvieae* isolates **(8, 28–40)**. Therefore, we collected 39 *L. garvieae* isolates from

bovine mastitis in China and conducted comparative genome sequence analysis of *L. garvieae*.

The prevalence of *L. garvieae* from clinical mastitis sample was 1.35% during 2017-2021, which increased from 0% in 2017 to 4.10% in 2020 in China. That *L. garvieae* has been misclassified into *Streptococcus spp.* **(41)** has resulted in underreporting of *L. garvieae*. Similarly, the true incidence of human infective endocarditis is difficult to assess due to misidentification with other gram-positive cocci **(42)**. This is the first report regarding the prevalence of *L. garvieae* in Chinese dairy herds.

The MLST analysis clustered 86 *L. garvieae* isolates into 32 distinct STs, with 5 CCs and 18 singletons, which is consistent with the population structure in isolates from other hosts **(37, 38, 43)**, the environment, or foods **(44)**. All strains, except Hebei-B-22, were new STs and phylogenetically close to each other. However, they were distant from isolates of bovine mastitis in other countries, which might indicate geographic effects on the phylogeny. Meanwhile, new STs profiles are comprised of new alleles in gene loci (e.g., *als*, *gyrB* and *galP*), perhaps due to a different evolution rate of those loci **(38)**.

Understanding phylogenetic relationships between strains is important for characterizing pathogen transmission. In this study, 3 phylogenetic trees were constructed using core genes and core genome SNPs as well as 16S rRNA, respectively. Core-genes tree and 16S rRNA trees produced similar clades but 16S rRNA failed to

resolve relationships toward tree tips. Furthermore, a core-gene tree is in line with MLST and CC. This was not surprising, as 16S rRNA tree is based on only 1 gene, representing only a very small portion of the whole genome. Therefore, many studies recommended core genes for inferring phylogenies **(45, 46)**.

Although pathogenicity of *L. garvieae* is poorly understood, some mechanisms have been determined, including presence of a capsule, hemolytic activity via secreted proteins **(47)** and production of siderophores **(48)**. Capsulated *L. garvieae* Lg2 was more virulent in fish than the non-capsulated isolate ATCC 49156 **(49).** The capsule gene cluster, located in a genomic island, were identified in Lg2 but absent in ATCC49156, which could be crucial for virulence of *L. garvieae* in fish **(40)**. However, existence of the capsule gene cluster has not been detected in all clinical fish isolates from Japan, Spain, Italy, France, Turkey **(22)**, USA **(39),** or India **(50)**, nor in any human isolates **(31, 69)**. In this study, only 2 of 86 isolates had the complete capsule gene cluster, which confirmed that it was not essential for virulence.

Proteases are among the important virulence factors causing rapid and extensive destruction of host tissue. For example, enolase **(50)** can cleave an extracellular proteinaceous matrix and therefore break a host's structural barrier during colonization. Hemolysin genes might act with secreted proteases to promote host tissue destruction. Genes encoding biosynthesis of iron uptake may be involved in iron acquisition during host colonization **(48)**. LPxTG protein (Leu-Pro-any-Thr-Gly), an important virulence factor in *L. garvieae*, binds to the peptidoglycan of cell wall by

transpeptidase enzymes called sortases **(40)**. In a previous study, *L. garvieae* strain isolated from rainbow trout colonized non-phagocytic cells with the help of LPxTG proteins **(51)**. LPxTG proteins and sortases have important roles binding pathogenic bacteria to their host. In this study, genes coding for adhesin, proteases, hemolysin, iron uptake and LPxTG protein were detected in most or all isolates. Strong positive associations within LPxTG-4, *adhPsaA*, *srtA* and iron uptake genes suggest they might act together to promote host tissue destruction and colonization. Gene *pgm,* identified in all isolates, produces protein with an important role in antibody production **(52)**.

The minimum inhibitory concentrations (MIC) results were consistent with reports that *L. garvieae* isolates from dairy farms were susceptible to penicillin, ampicillin and amoxicillin-clavulanic acid, imipenem, ceftiofur, enrofloxacin, vancomycin, and marbofloxacin **(10, 53)**. However, compared to a previous report **(10)**, there were variable degrees of increasing resistant rates for 8 antibiotics, including clindamycin (93.6 to 100%), chloramphenicol (6.4 to 100%), amikacin (2.1 to 90%), cefpodoxime (0 to 82.5%), cephalothin (0 to 10%), cefazolin (40.4 to 45%), gentamicin (0 to 37.5%), and erythromycin (0 to 5%). High resistance of clindamycin has been described as intrinsic for *L. garvieae* and proposed as a selection criterion to distinguish between *L. garvieae* and *L. lactis* **(54)**. The *lsaD* gene, identified in most isolates (83/86), could be responsible for intrinsic resistance. *lsaD* is a novel lsa-type family gene detected in lincomycin-resistant strains isolated from fish **(55)**. The lsa-type genes are responsible for cross-resistance to lincosamides, streptogramins or pleuromutilins (hereinafter

268 referred to as LSA(P)-resistant phenotype), by coding ATP-binding cassette F
269 proteins in Gram-positive pathogens including Staphylococci, **(56)** Streptococci **(57)**,
270 enterococci **(58)**, and lactococci **(55)**. Increasing resistance against cephalosporins
271 might be related to increasing use of these antibiotics for treatment of infectious
272 diseases on Chinese dairy farms **(59)**. The multidrug transporter, *mdtA* is another AMR
273 gene present in most isolates. This gene originally conferred resistance to macrolides,
274 lincosamides, streptogramins and tetracycline in *L. lactis* **(60)**, but mutations present
275 in the C-motifs of *mdtA* from *L. garvieae* confer susceptibility to erythromycin and
276 tetracycline **(53)**. Furthermore, all 39 isolates with *mdtA* had a limited resistance rate
277 to erythromycin (5%). Some isolates (16/86) harbor the *tetS* gene, including 10 local
278 isolates. Three isolates from the human gut in China, LG729, LG729 and
279 MGYG-HGUT-00230, contained the most abundant AMR genes. Notably, 9 AMR
280 gene were only present in the 3 isolates, including *cat, dfrG, ermA*, *ermB, lunD, fexA,*
281 *optrA, acc(6')-aaph(2'')* and *ant(6)-la*. The *optrA* gene, first identified in enterococci,
282 has been reported in *Staphylococci*, and *Streptococci*, *Clostridium perfringens* and
283 *Campylobacter coli*; it confers resistance to oxazolidinones and phenicol and has
284 identified on a plasmid of *L. garvieae* **(61)**. The spread of antibiotic resistance genes in
285 bacterial populations is aided by various mechanisms of horizontal gene transfer, with
286 plasmid-mediated transfer being the main mechanism for transmission of resistance
287 genes **(62).** Horizontal gene transfer between bacteria is largely mediated by
288 specialized mobile genetic elements, including plasmids, bacteriophages, transposon,

289 insert sequences (IS), intergon, etc., and has been reported in *L. garvieae*. Both *tetS*
290 and *tetM* were associated with conjugative transposon-associated gene in isolates
291 from healthy fish intestines **(63)**. Most of the IS in *L. garvieae* had substantial
292 homology to *Lactococcus lactis* elements, implying movement of IS between these 2
293 species that are phylogenetically closely related **(64, 65).** That these 9 AMR genes
294 were only reported in humans does not support the assertion that AMR genes are
295 transferred to humans from fish or dairy products. Regardless, *L. garvieae* could be a
296 reservoir for antibiotic resistance genes for other bacteria.

297 In this study, no genes were associated with host specificity, consistent with the
298 phylogenetic analysis and the core-genome SNP analysis that host adaptation occurs in
299 *L. garvieae* isolates. Previous research **(5)** summarized human *L. garvieae* infections
300 associated with consumption of raw fish, seafood, or unpasteurized milk. The core
301 genome SNP analysis underlies the potential host adaptation of *L. garvieae*. Meanwhile,
302 adhesins, haemolysin, fibronectin-binding proteins, penicillin acylase and WxL
303 domain-containing proteins are considered to actively promote bacterial colonization
304 **(66)**; most had high similarity across host in those coding sequences. Regardless,
305 underlying mechanisms remain unclear. Consequently, further studies are needed to
306 determine host adaptation mechanisms of *L. garvieae*.

307

308 **CONCLUSIONS**

309 This was apparently the first study on comparative genomic analyses of *L. garvieae*
310 isolates from mastitis cows in China. The incidence of *L. garvieae* mastitis was 1.35%
311 in China. Most isolates (38/39) were novel sequence types, 3 antimicrobial resistance
312 genes (*mdtA*, *lsaD* and *tetS)* were identified and there was evidence of host adaptation
313 in these isolates.
314
315 **MATERIALS AND METHODS**
316 **Statement of Ethics.** This study was conducted in accordance with ethical
317 guidelines and standard biosecurity and institutional safety procedures of China
318 Agricultural University (CAU; Beijing, China). Ethical approval was not needed, as no
319 animal study was involved.
320 **Sample Collection and Bacteria Identification.** Milk samples from clinical
321 cases of mastitis were collected aseptically from dairy cows on Chinese dairy farms and
322 sent to the Mastitis Diagnostic Laboratory at the College of Veterinary Medicine, CAU,
323 Beijing, China. Pathogens were identified by bacteriological culture, colony
324 morphology and 16S rRNA sequencing according to NMC guidelines **(67)**. In brief, 50
325 µl milk was spread on tryptone soy agar with 5% defibrinated sheep blood. The plate
326 was incubated aerobically at 37 °C for 24 h. Bacterial colony morphology was recorded;
327 samples with ≥ 3 morphologically distinct colonies were considered contaminated and
328 excluded from subsequent analyses.

Antimicrobial Susceptibility Testing. For all 39 *L. garvieae* isolates, MIC of 15 antimicrobials (Chinese National Institutes for Food and Drug Control, Beijing, China), commonly used to treat clinical mastitis in China, were determined by the microbroth dilution method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines VET01-A4 (CLSI, 2013), with reported breakpoints **(10).** *Staphylococcus aureus* ATCC 29213 was used as the quality control strain.

Genome Assembly and Annotation. Genomic DNA of putative isolates was extracted using a bacterial DNA extraction kit (TransGen Biotech, Beijing, China) according to the manufacturer's instruction. Extracted DNA was quantified with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) prior to 16S rRNA gene sequencing (Beijing Sunbiotech Inc., Beijing, China). Whole genome DNA was paired-end sequenced (2 × 150 bp) using Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA) at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China). For raw reads, quality control was done with FastQC Version 0.11.9 (https://github.com/s-andrews/FastQC). Low quality bases were trimmed using fastp Version 0.20.1 (https://github.com/OpenGene/fastp) with default settings. Quality trimmed reads were assembled into scaffolds using SPades Version 3.13.1 (https://github.com/ablab/spades) with auto coverage cut-off and shovill Version 1.1.0 (https://github.com/tseemann/shovill) with default settings. Thereafter, 2 assembled scaffolds for each isolate were obtained, and a draft genome of each isolate was selected using Quast Version 5.0.2 (https://github.com/ablab/quast) with N50, L50

from the above-mentioned 2 assembled scaffolds. Assembly completeness was assessed using Busco Version 5.2.2 (https://github.com/WenchaoLin/BUSCO-Mod) with reference to lineage lactobacillales_odb10. Only genomes with completion ≥ 95% were considered "high-quality draft genome" and were included in further analyses **(68).** In addition, whole genome sequence assemblies fasta files of 51 *L. garvieae* (accessed on March 24, 2022) were downloaded from NCBI. To ensure high-quality genomes, all genomes were analyzed by BusCom Version 5.2.2 (lineage lactobacillales_odb10) and 3 assembles were excluded from subsequent analysis. There were 3 ATCC 49156 assemblies; we chose the 1 with the highest assembly level. Therefore, a total of 39 isolates from composite (or quarter) milk samples and 47 assemblies from NCBI were obtained in the subsequent genome annotation and pan-genome analysis. Annotation of the genome was performed using Prokka Version 1.14.6 (https://github.com/tseemann/prokka) with default settings. MLST analyses Multilocus sequence typing (MLST) using whole genome sequences was performed to determine sequence types (ST) of the 86 isolates. A *L. garvieae* MLST database was constructed based on reported datasets **(69)** as there was no publicly available MLST scheme for *L. garvieae*. Similarly, the database was integrated into ABRicate local database, and we aligned the 86 *L. garvieae* genomes against the dataset by ABRicate. Sequence types were assigned to new allele patterns and added to the existing MLST scheme for *L. garvieae* constructed by **(69)**. Clonal complex (CC) was defined as a group of STs in which every ST shared at least 5 of 7 identical allele profiles with at

371 least 1 other ST in the group. The minimum spanning tree (MST) was constructed by
372 the goeBURST algorithm and visualized with the PhyloViz web server
373 (https://online.phyloviz.net/index) to infer phylogenetic relationships among STs.

374 **Identification of Antimicrobial Resistance Genes and Virulence Factors.**

375 Antimicrobial resistance genes were identified by blasting *L. garvieae* genomes against
376 ResFinder database via Resfinder Version 4.1.11
377 (https://cge.cbs.dtu.dk/services/ResFinder/) and The Comprehensive Antibiotic
378 Resistance Database (https://card.mcmaster.ca/) via RGI Version 5.2.1
379 (https://github.com/arpcard/rgi). A set of virulence genes of *L. garvieae* was
380 summarized from previous reports **(22, 69, 70)**; they included haemolysin I, II and III
381 (*hlyI*,-*II* and -*III*), iron uptake genes (*fepB*, *fepC*, *fepD*, *fecB*, *fecC*, *fecD*, *fecE*, *feoA*, and
382 *feoB*), capsule gene cluster (CGC), adhesions (*adh*, *adhPavA*, *adhPsaA*), putative
383 collagenase (*colA*), LPxTG surface proteins 1, 2, 3, 4, 5, 6 (LPxTG-1, LPxTG-2,
384 LPxTG-3, LPxTG-4, LPxTG-5 and LPxTG-6) and enzyme-related virulence factors
385 NADH oxidase(*Nox*), glyceraldehyde-3-phosphate dehydrogenase (GAPDH),
386 phosphoglucomutase (*Pgm*), superoxide dismutase (*Sod*), enolase (*Eno*) and SortaseA
387 (*srtA*). The database was integrated into the ABRicate local database. We blasted the 86
388 (39 from our study and 47 from NCBI) *L. garvieae* genomes against the database using
389 ABRicate to determine virulence genes. The presence of antimicrobial resistance or
390 virulence gene was defined using the cut-off value of 80% sequence coverage and 80%
391 nucleotide identity (ABRicate default settings).

Pan-genome Analyses. The pan-genome of 86 *L. garvieae* isolates was computed using BPGA Version 1.3 (https://iicb.res.in/bpga/) with USEARCH algorithm to cluster orthologous gene families using faa files of local isolates produced by Prokka and retrieved from NCBI directly. For BPGA analysis, a core gene was defined as a gene present in all the genomes; an accessory gene was present in > 1 genome but not all genomes; and a unique gene was only present in a single genome. Functional annotations of core, accessory, and unique genes were obtained after comparing sequences to COG and KEGG databases incorporated in BPGA Version 1.3.

Phylogenetic Analyses. A 16S rRNA phylogenetic tree was constructed based on 16s rRNA genes. In addition, we also constructed another phylogenetic tree using alignment of core genes produced by BPGA. For 16S rRNA phylogenetic analysis, Barrnap Version 0.9 (https://github.com/tseemann/barrnap) was used to extract 16S rRNA gene from the whole genome sequence. The 16S rRNA gene sequences were edited and aligned using MAFFT multiple sequence alignment algorithm (stargety "L-IINS-I"; https://github.com/GSLBiotech/mafft). Maximum-likelihood (ML) trees based on this alignment were constructed using FastTree Version 2.1 (https://github.com/PavelTorgashov/FastTree). Visualization of the phylogenetic tree was performed using iTOL (https://itol.embl.de/) with metadata (clonal complex, source of host and country) of the isolates.

Pan-genome Wide Association Analyses. To identify genes potentially associated with traits, such as host, clonal complex, and country, we performed

pan-genome wide association analysis using Scoary. Annotation of whole genome sequences of the 86 *L. garvieae* isolates were performed using Prokka Version 1.14.6 (https://github.com/tseemann/prokka), and the resultant gff files were used for pan-genome analysis with Roary Version 3.13.0 (http://sanger-pathogens.github.io/Roary/) to produce gene presence and absence data. Thereafter, genes associated with host, country, ST or clonal complex were identified with Scoary VVersion 1.6.16 (https://github.com/AdmiralenOla/Scoary). Categorical traits were dichotomized prior to pan-genome wide association analysis with Scoary.

Core-genome SNPs Analyses. In addition to pan-genome wide association analyses, we also performed core-genome SNPs analyses. Core genome alignment and single nucleotide polymorphism (SNP) were detected for all 86 genome sequences using parsnp Version 1.7.2 (https://github.com/marbl/parsnp). Meanwhile, the exact numbers of SNPs among genomes from various hosts in the same MLST group and closely related in core-gene based phylogenetic tree were determined with snp-dists Version 0.8.2 (http://sanger-pathogens.github.io/snp-sites/) using the sequence alignment file produced from parsnp. Phylogenetic tree based on core genome SNPs was annotated with iTOL.

Associations between the Co-occurrence of Virulence Genes. Co-occurrence of virulence genes was determined with phi coefficient using the Phi function in psych package Version 2.2.5 (https://cran.r-project.org/web/packages/psych/) with R Version 4.1.3 (https://www.r-project.org/) and $P < 0.05$ was considered significant in a 2-tailed

test. The pair-wised phi coefficients between the presences of virulence genes were visualized using ggplot2 Version 3.3.6 (https://cran.r-project.org/web/packages/ggplot2/).

Data Availability. All whole genome sequence data used in this study are available without restriction from NCBI under BioProject no. PRJNA848370.

ACKNOWLEDGMENTS

This study was supported financially by the following: National Natural Science Foundation of China (No. 32172928), the High-End Foreign Experts Recruitment Program (No. 110000202720220022), and Beijing Municipal Natural Science Foundation (No. 6222031).

B.H. and Y.L. designed and supervised the study. Y.L., J.H., Y.W., J.G. and S.Q. performed the experiments, and wrote the manuscript. Z.D. and H.W.B. assisted in the analyses and re-edited the manuscript. B.H., J.P. K. and H.W.B. revised the manuscript. All authors read and approved the final manuscript.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only. **Table S1.xlsx** file, 45 KB.

REFERENCES

1. Barkema HW, von Keyserlingk MAG, Kastelic JP, Lam TJGM, Luby C, Roy J-P, LeBlanc SJ, Keefe GP, Kelton DF. 2015. Invited review: Changes in the dairy industry affecting dairy cattle health and welfare. *J Dairy Sci* 98:7426–7445.

2. Ruegg PL. 2017. A 100-year review: Mastitis detection, management, and prevention. *J Dairy Sci* 100:10381–10397.

3. Acharya KR, Brankston G, Slavic D, Greer AL. 2021. Spatio-temporal variation in the prevalence of major mastitis pathogens isolated from bovine milk samples between 2008 and 2017 in Ontario, Canada. *Front Vet Sci* 8:1–12.

4. Meyburgh CM, Bragg RR, Boucher CE. 2017. *Lactococcus garvieae*: an emerging bacterial pathogen of fish. *Dis Aquat Organ* 123:67–79.

5. Gibello A, Galán-Sánchez F, Blanco MM, Rodríguez-Iglesias M, Domínguez L, Fernández-Garayzábal JF. 2016. The zoonotic potential of *Lactococcus garvieae*: An overview on microbiology, epidemiology, virulence factors and relationship with its presence in foods. *Res Vet Sci* 109:59–70.

6. Choksi TT, Dadani F. 2017. Reviewing the Emergence of *Lactococcus garvieae*: A case of catheter associated urinary tract infection caused by *Lactococcus garvieae* and *Escherichia coli* coinfection. *Case Rep Infect Dis* 2017:5921865.

7. González-Bravo DH, Alegre-Boschetti S, Silva-Cantillo R, Mercado-Maldonado J, Ramos-Márquez R, Torres-Rivera G, Cortés C, Mercado-Crespo J. 2021. *Lactococcus garvieae*: An uncommon human pathogen causing infective

endocarditis in a valve-in-valve transcatheter aortic valve replacement. *Case Reports Cardiol* 2021:5569533.

8. Francés-Cuesta C, Ansari I, Fernández-Garayzábal JF, Gibello A, González-Candelas F. 2022. Comparative genomics and evolutionary analysis of *Lactococcus garvieae* isolated from human endocarditis. *Microb Genomics* 8: 000771.

9. Tejedor JL, Vela AI, Gibello A, Casamayor A, Domínguez L, Fernández-Garayzábal JF. 2011. A genetic comparison of pig, cow and trout isolates of *Lactococcus garvieae* by PFGE analysis. *Lett Appl Microbiol* 53:614–619.

10. Plumed-Ferrer C, Barberio A, Franklin-Guild R, Werner B, McDonough P, Bennett J, Gioia G, Rota N, Welcome F, Nydam D V, Moroni P. 2015. Antimicrobial susceptibilities and random amplified polymorphic DNA-PCR fingerprint characterization of *Lactococcus lactis* ssp. lactis and *Lactococcus garvieae* isolated from bovine intramammary infections. *J Dairy Sci* 98:6216–6225.

11. Eraclio G, Ricci G, Moroni P, Santisteban C, Plumed-Ferrer C, Bennett J, Fortina MG. 2019. Sand bedding as a reservoir for *Lactococcus garvieae* dissemination in dairy farms. *Can J Microbiol* 65:84–89.

12. Scillieri Smith JC, Moroni P, Santisteban CG, Rauch BJ, Ospina PA, Nydam D V. 2020. Distribution of Lactococcus spp. in New York State dairy farms and the association of somatic cell count resolution and bacteriological cure in clinical mastitis samples. *J Dairy Sci* 103:1785–1794.

13. Song X, Huang X, Xu H, Zhang C, Chen S, Liu F, Guan S, Zhang S, Zhu K, Wu C. 2020. The prevalence of pathogens causing bovine mastitis and their associated risk factors in 15 large dairy farms in China: An observational study. *Vet Microbiol* 247:108757.

14. Rowe SM, Godden SM, Royster E, Timmerman J, Boyle M. 2021. Postcalving udder health and productivity in cows approaching dry-off with intramammary infections caused by non-aureus Staphylococcus, Aerococcus, Enterococcus, Lactococcus, and Streptococcus species. *J Dairy Sci* 104:6061–6079.

15. de Oliveira RP, Aragão BB, de Melo RPB, da Silva DMS, de Carvalho RG, Juliano MA, Farias MPO, de Lira NSC, Mota RA. 2022. Bovine mastitis in northeastern Brazil: Occurrence of emergent bacteria and their phenotypic and genotypic profile of antimicrobial resistance. *Comp Immunol Microbiol Infect Dis* 85:101802.

16. Zadoks RN, Middleton JR, McDougall S, Katholm J, Schukken YH. 2011. Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. *J Mammary Gland Biol Neoplasia* 16:357–372.

17. Kempf F, Slugocki C, Blum SE, Leitner G, Germon P. 2016. Genomic comparative study of bovine mastitis *Escherichia coli*. *PLoS One* 11:1–22.

18. Li T, Lu H, Wang X, Gao Q, Dai Y, Shang J, Li M. 2017. Molecular characteristics of *Staphylococcus aureus* causing bovine mastitis between 2014 and 2015. *Front Cell Infect Microbiol* 7:1–10.

517 19. Cheng J, Zhou M, Nobrega DB, Barkema HW, Xu S, Li M, Kastelic JP, Shi Y,
518 Han B, Gao J. 2021. Genetic diversity and molecular epidemiology of outbreaks
519 of *Klebsiella pneumoniae* mastitis on two large Chinese dairy farms. *J Dairy Sci*
520 104:762-775.
521 20. Zheng Z, Gorden PJ, Xia X, Zheng Y, Li G. 2022. Whole-genome analysis of
522 *Klebsiella pneumoniae* from bovine mastitis milk in the U.S. *Environ Microbiol*
523 24:1183–1199.
524 21. Holmes AH, Moore LSP, Sundsfjord A, Steinbakk M, Regmi S, Karkey A, Guerin
525 PJ, Piddock LJ V. 2016. Understanding the mechanisms and drivers of
526 antimicrobial resistance. *Lancet* 387:176–187.
527 22. Ture M, Altinok I. 2016. Detection of putative virulence genes of *Lactococcus*
528 *garvieae*. *Dis Aquat Organ* 119:59–66.
529 23. Meyburgh CM, Bragg RR, Boucher CE. 2018. Detection of virulence factors of
530 South African *Lactococcus garvieae* isolated from rainbow trout, Oncorhynchus
531 mykiss (Walbaum). *Onderstepoort J Vet Res* 85:e1–e9.
532 24. Rao S, Chen MY, Sudpraseart C, Lin P, Yoshida T, Wang PC, Chen SC. 2022.
533 Genotyping and phenotyping of *Lactococcus garvieae* isolates from fish by
534 pulse-field gel electrophoresis (PFGE) and electron microscopy indicate
535 geographical and capsular variations. *J Fish Dis* 45:771–781.
536 25. Ballhausen B, Kriegeskorte A, van Alen S, Jung P, Köck R, Peters G, Bischoff M,
537 Becker K. 2016. The pathogenicity and host adaptation of livestock-associated

538 MRSA CC398. *Vet Microbiol* 200:39-45.

539 26. Gori A, Harrison OB, Mlia E, Nishihara Y, Chan JM, Msefula J, Mallewa M, Dube
540 Q, Swarthout TD, Nobbs AH, Maiden MCJ, French N, Heyderman RS. 2020.
541 Pan-GWAS of *Streptococcus agalactiae* highlights lineage- specific genes
542 associated with virulence and niche adaptation. *mBio* 11:e00728-20.

543 27. Garvie EI, Farrow JAE, Phillips BA. 1981. A taxonomic study of some strains of
544 streptococci which grow at 10°C but not at 45°C including *Streptococcus lactis*
545 and *Streptococcus cremoris*. *Zentralblatt fur Bakteriol Angew und Okol Microbiol*
546 *Abt1 OrigC Hyg* 2:151–165.

547 28. Aguado-Urda M, Gibello A, Blanco M del M, Fernández-Garayzábal JF,
548 López-Alonso V, López-Campos GH. 2013. Global transcriptome analysis of
549 *Lactococcus garvieae* strains in response to temperature. *PLoS One* 8:e79692.

550 29. Aguado-Urda M, López-Campos GH, Fernández-Garayzábal JF, Martín-Sánchez F,
551 Gibello A, Domínguez L, Blanco MM. 2010. Analysis of the genome content of
552 *Lactococcus garvieae* by genomic interspecies microarray hybridization. *BMC*
553 *Microbiol* 10:79.

554 30. Hoai TD, Nishiki I, Yoshida T. 2016. Properties and genomic analysis of
555 *Lactococcus garvieae* lysogenic bacteriophage PLgT-1, a new member of
556 Siphoviridae, with homology to *Lactococcus lactis* phages. *Virus Res* 222:13–23.

557 31. Kim JH, Kang D-H, Park SC. 2015. Draft genome sequence of human-pathogenic
558 *Lactococcus garvieae* LG-ilsanpaik-gs201105 that caused acute acalculous
559 cholecystitis. *Genome Announc* 3:e00464-15.
560 32. Shahi N, Mallik SK. 2020. Emerging bacterial fish pathogen *Lactococcus garvieae*
561 RTCLI04, isolated from rainbow trout (Oncorhynchus mykiss): Genomic features
562 and comparative genomics. *Microb Pathog* 147:104368.
563 33. Moumene M, Drissi F, Croce O, Djebbari B, Robert C, Angelakis E, Benouareth
564 DE, Raoult D, Merhej V. 2016. Complete genome sequence and description of
565 *Lactococcus garvieae* M14 isolated from Algerian fermented milk. *New Microbes*
566 *New Infect* 10:122–131.
567 34. Morita H, Toh H, Oshima K, Yoshizaki M, Kawanishi M, Nakaya K, Suzuki T,
568 Miyauchi E, Ishii Y, Tanabe S, Murakami M, Hattori M. 2011. Complete genome
569 sequence and comparative analysis of the fish pathogen *Lactococcus garvieae*.
570 *PLoS One* 6:e23184.
571 35. Nishiki I, Oinaka D, Iwasaki Y, Yasuike M, Nakamura Y, Yoshida T, Fujiwara A,
572 Nagai S, Katoh M, Kobayashi T. 2016. Complete genome sequence of
573 nonagglutinating *lactococcus garvieae* strain 122061 isolated from yellowtail in
574 Japan. *Genome Announc* 4:e00592-16.
575 36. Chang CI, Hung PH, Wu CC, Cheng TC, Tsai JM, Lin KJ, Lin CY. 2012.
576 Simultaneous detection of multiple fish pathogens using a naked-eye readable
577 DNA microarray. *Sensors (Basel)* 12:2710–2728.

578 37. Thiry D, Billen F, Boyen F, Duprez JN, Quenault H, Touzain F, Blanchard Y,
579 Clercx C, Mainil JG. 2021. Genomic relatedness of a canine *Lactococcus garvieae*
580 to human, animal and environmental isolates. *Res Vet Sci* 137:170–173.
581 38. Ferrario C, Ricci G, Milani C, Lugli GA, Ventura M, Eraclio G, Borgo F, Fortina
582 MG. 2013. *Lactococcus garvieae*: Where is it from? A first approach to explore
583 the evolutionary history of this emerging pathogen. *PLoS One* 8:e84796.
584 39. Nelson MC, Varney JS, Welch TJ, Graf J. 2016. Draft genome sequence of
585 *Lactococcus garvieae* strain PAQ102015-99, an outbreak strain isolated from a
586 commercial trout farm in the Northwestern United States. *Genome Announc*
587 4:e00781-16.
588 40. Miyauchi E, Toh H, Nakano A, Tanabe S, Morita H. 2012. Comparative genomic
589 analysis of *Lactococcus garvieae* strains isolated from different sources reveals
590 candidate virulence genes. *Int J Microbiol* 2012:728276.
591 41. Werner B, Moroni P, Gioia G, Lavín-Alconero L, Yousaf A, Charter ME, Carter
592 BM, Bennett J, Nydam D V., Welcome F, Schukken YH. 2014. Short
593 communication: Genotypic and phenotypic identification of environmental
594 streptococci and association of *Lactococcus lactis* ssp. lactis with intramammary
595 infections among different dairy farms. *J Dairy Sci* 97:6964–6969.
596 42. Malek A, De la Hoz A, Gomez-Villegas SI, Nowbakht C, Arias CA. 2019.
597 *Lactococcus garvieae*, an unusual pathogen in infective endocarditis: case report
598 and review of the literature. *BMC Infect Dis* 19:301.

599 43. Kotzamanidis C, Malousi A, Bitchava K, Vafeas G, Chatzidimitriou D, Skoura L,
600 Papadimitriou E, Chatzopoulou F, Zdragas A. 2020. First report of isolation and
601 genome sequence of *L. petauri* strain from a rainbow trout Lactococcosis outbreak.
602 *Curr Microbiol* 77:1089–1096.
603 44. Reguera-Brito M, Galán-Sánchez F, Blanco MM, Rodríguez-Iglesias M,
604 Domínguez L, Fernández-Garayzábal JF, Gibello A. 2016. Genetic analysis of
605 human clinical isolates of *Lactococcus garvieae*: Relatedness with isolates from
606 foods. *Infect Genet Evol* 37:185–191.
607 45. Chun J, Oren A, Ventosa A, Christensen H, Arahal DR, da Costa MS, Rooney AP,
608 Yi H, Xu XW, De Meyer S, Trujillo ME. 2018. Proposed minimal standards for
609 the use of genome data for the taxonomy of prokaryotes. *Int J Syst Evol Microbiol*
610 68:461–466.
611 46. Naushad S, Adeolu M, Goel N, Khadka B, Al-Dahwi A, Gupta RS. 2015.
612 Phylogenomic and molecular demarcation of the core members of the
613 polyphyletic pasteurellaceae genera actinobacillus, haemophilus, and pasteurella.
614 *Int J Genomics* 2015:198560.
615 47. Kimura H, Kusuda R. 1982. Studies on the pathogenesis of streptococcal infection
616 in cultured yellowtails, Seriola spp.: effect of crude exotoxin fractions from cell-
617 free culture on experimental streptococcal infection. *J Fish Dis* 5:471–478.
618 48. Schmidtke LM, Carson J. 2003. Antigen recognition by rainbow trout
619 (Oncorhynchus mykiss) of whole cell proteins expressed by *Lactococcus garvieae*

620 when obtained directly from fish and under iron limited culture conditions. *Vet*
621 *Microbiol* 93:63–71.
622 49. Kawanishi M, Yoshida T, Kijima M, Yagyu K, Nakai T, Okada S, Endo A,
623 Murakami M, Suzuki S, Morita H. 2007. Characterization of *Lactococcus*
624 *garvieae* isolated from radish and broccoli sprouts that exhibited a KG+
625 phenotype, lack of virulence and absence of a capsule. *Lett Appl Microbiol*
626 44:481–487.
627 50. Shahi N, Mallik SK, Sahoo M, Chandra S, Singh AK. 2018. First report on
628 characterization and pathogenicity study of emerging *Lactococcus garvieae*
629 infection in farmed rainbow trout, Oncorhynchus mykiss (Walbaum), from India.
630 *Transbound Emerg Dis* 65:1039–1048.
631 51. Aguado-Urda M, Rodríguez-Bertos A, de las Heras AI, Blanco MM, Acosta F, Cid
632 R, Fernández-Garayzábal JF, Gibello A. 2014. Experimental *Lactococcus*
633 *garvieae* infection in zebrafish and first evidence of its ability to invade
634 non-phagocytic cells. *Vet Microbiol* 171:248–254.
635 52. Tsai MA, Wang PC, Cao TT, Liao PC, Liaw LL, Chen SC. 2013.
636 Immunoprotection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
637 from *Lactococcus garvieae* against Lactococcosis in tilapia. *J Gen Appl Microbiol*
638 59:437–449.
639 53. Walther C, Rossano A, Thomann A, Perreten V. 2008. Antibiotic resistance in
640 Lactococcus species from bovine milk: presence of a mutated multidrug

641 transporter mdt(A) gene in susceptible *Lactococcus garvieae* strains. *Vet*
642 *Microbiol* 131:348–357.
643 54. Elliott JA, Facklam RR. 1996. Antimicrobial susceptibilities of *Lactococcus lactis*
644 and *Lactococcus garvieae* and a proposed method to discriminate between them. *J*
645 *Clin Microbiol* 34:1296–1298.
646 55. Shi YZ, Yoshida T, Fujiwara A, Nishiki I. 2021. Characterization of lsa(D), a novel
647 gene responsible for resistance to lincosamides, streptogramins A, and
648 pleuromutilins in fish pathogenic *Lactococcus garvieae* Serotype II. *Microb Drug*
649 *Resist* 27:301–310.
650 56. Kehrenberg C, Ojo KK, Schwarz S. 2004. Nucleotide sequence and organization of
651 the multiresistance plasmid pSCFS1 from *Staphylococcus sciuri*. *J Antimicrob*
652 *Chemother* 54:936–939.
653 57. Malbruny B, Werno AM, Murdoch DR, Leclercq R, Cattoir V. 2011.
654 Cross-resistance to lincosamides, streptogramins A, and pleuromutilins due to the
655 lsa(C) gene in *Streptococcus agalactiae* UCN70. *Antimicrob Agents Chemother*
656 55:1470–1474.
657 58. Singh K V, Murray BE. 2005. Differences in the Enterococcus faecalis lsa locus
658 that influence susceptibility to quinupristin-dalfopristin and clindamycin.
659 *Antimicrob Agents Chemother* 49:32–39.
660 59. Ali T, Ur Rahman S, Zhang L, Shahid M, Zhang S, Liu G, Gao J, Han B. 2016.
661 ESBL-producing *Escherichia coli* from cows suffering mastitis in China contain

662 clinical class 1 integrons with CTX-M linked to ISCR1. *Front Microbiol* 7:1–11.

663 60. Perreten V, Schwarz F V., Teuber M, Levy SB. 2001. Mdt(A), a new efflux protein

664 conferring multiple antibiotic resistance in *Lactococcus lactis* and *Escherichia*

665 *coli*. *Antimicrob Agents Chemother* 45:1109–1114.

666 61. Cai J, Chen J, Schwarz S, Wang Y, Zhang R. 2021. Detection of the plasmid-borne

667 oxazolidinone/phenicol resistance gene optrA in *Lactococcus garvieae* isolated

668 from faecal samples. *Clin Microbiol Infect* 27:1358-1359.

669 62. Baker S, Thomson N, Holt KE. 2018. Genomic insights into the emergence and

670 spread of antimicrobial-resistant bacterial pathogens *Science* 360: 738:733–738.

671 63. Kim SR, Nonaka L, Suzuki S. 2004. Occurrence of tetracycline resistance genes

672 tet(M) and tet(S) in bacteria from marine aquaculture sites. *FEMS Microbiol Lett*

673 237:147–156.

674 64. Eraclio G, Ricci G, Fortina MG. 2015. Insertion sequence elements in *Lactococcus*

675 *garvieae*. *Gene* 555:291–296.

676 65. Eraclio G, Tremblay DM, Lacelle-Côté A, Labrie SJ, Fortina MG, Moineau S. 2015.

677 A virulent phage infecting *Lactococcus garvieae*, with homology to *Lactococcus*

678 *lactis* phages. *Appl Environ Microbiol* 81:8358–8365.

679 66. Colagrossi L, Costabile V, Scutari R, Agosta M, Onori M, Mancinelli L, Lucignano

680 B, Muda AO, Baldo G Del, Mastronuzzi A, Locatelli F, Trua G, Montanari M,

681 Alteri C, Bernaschi P, Perno CF. 2022. Evidence of pediatric sepsis caused by a

682 drug resistant *Lactococcus garvieae* contaminated platelet concentrate. *Emerg*

683 *Microbes Infect* 11:1325-1334.

684 67. NMC. 2017. Laboratory Handbook on Bovine Mastitis, 3rd ed. National Mastitis

685 Council, Minnesota, USA.

686 68. Bowers RM, Kyrpides NC, Stepanauskas R, Harmon-Smith M, Doud D, Reddy

687 TBK, Schulz F, Jarett J, Rivers AR, Eloe-Fadrosh EA, Tringe SG, Ivanova NN,

688 Copeland A, Clum A, Becraft ED, Malmstrom RR, Birren B, Podar M, Bork P,

689 Weinstock GM, Garrity GM, Dodsworth JA, Yooseph S, Sutton G, Glöckner FO,

690 Gilbert JA, Nelson WC, Hallam SJ, Jungbluth SP, Ettema TJG, Tighe S,

691 Konstantinidis KT, Liu WT, Baker BJ, Rattei T, Eisen JA, Hedlund B, McMahon

692 KD, Fierer N, Knight R, Finn R, Cochrane G, Karsch-Mizrachi I, Tyson GW,

693 Rinke C, Lapidus A, Meyer F, Yilmaz P, Parks DH, Eren AM, Schriml L, Banfield

694 JF, Hugenholtz P, Woyke T. 2017. Minimum information about a single amplified

695 genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria

696 and archaea. *Nat Biotechnol* 35:725–731.

697 69. Lin YS, Kweh KH, Koh TH, Lau QC, Abdul Rahman NB. 2020. Genomic analysis

698 of *Lactococcus garvieae* isolates. *Pathology* 52:700–707.

699 70. Aguado-Urda M, López-Campos GH, Blanco MM, Fernández-Garayzábal JF,

700 Cutuli MT, Aspiroz C, López-Alonso V, Gibello A. 2011. Genome sequence of

701 *Lactococcus garvieae* 21881, isolated in a case of human septicemia. *J Bacteriol*

702 193:4033–4034.

703

FIGURE CAPTIONS

FIG 1 Minimum spanning tree based on Multi-Locus Sequence Typing for 86 *Lactococcus garvieae* isolates involving 32 sequence types performed by geoBURST algorithm and visualized by PhyloViz. Five clonal complexes (CC1-CC5) were clustered with similar STs (5 - 7 shared alleles); the number between nodes indicates the number of distinct alleles within them.

FIG 2 Distribution of antimicrobial resistance genes against each category of antimicrobial resistance, together with source of host, country, and clonal complex (CC) of 86 *Lactococcus garvieae* isolates.

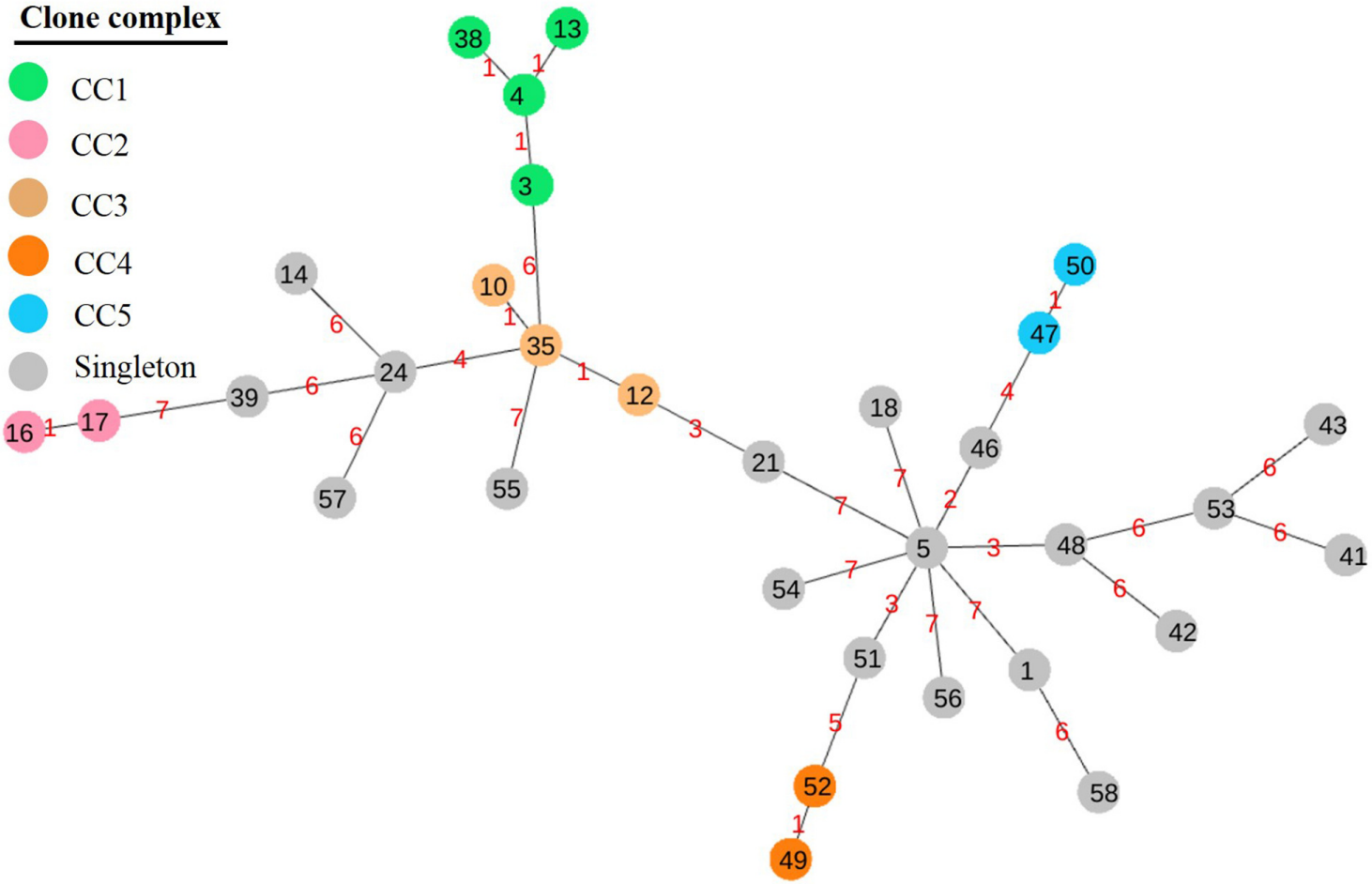
FIG 3 Distribution of virulence factor genes, source of host, country, and clonal complex (CC) of 86 *Lactococcus garvieae* isolates.

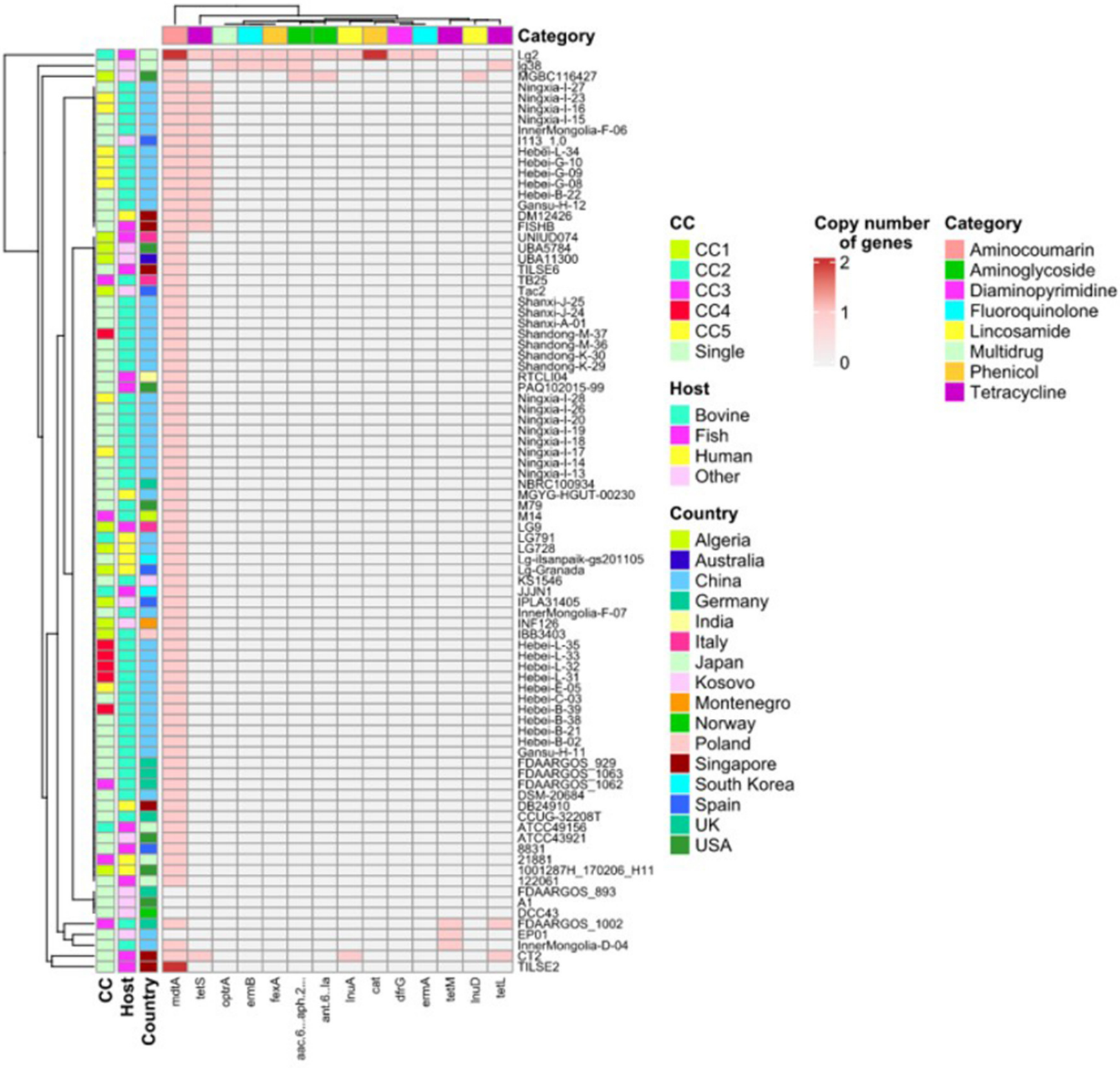
FIG 4 Pan-genome of 86 *Lactococcus garvieae* isolates in this study. The pan-genome consisted of 6310 genes, of which, 1015 core genes, 3641 accessory genes and 1654 unique genes; the size of the genome in the pan-genome increased as the number of isolates increased, but pan-genome size approached convergence. The number of core genes (shared by all isolates) was fairly constant at 1015 genes **(A)**. Distribution of KEGG **(B)** and COG **(C, D)** functional categories in core, accessory and unique genes of 86 *Lactococcus garvieae*.

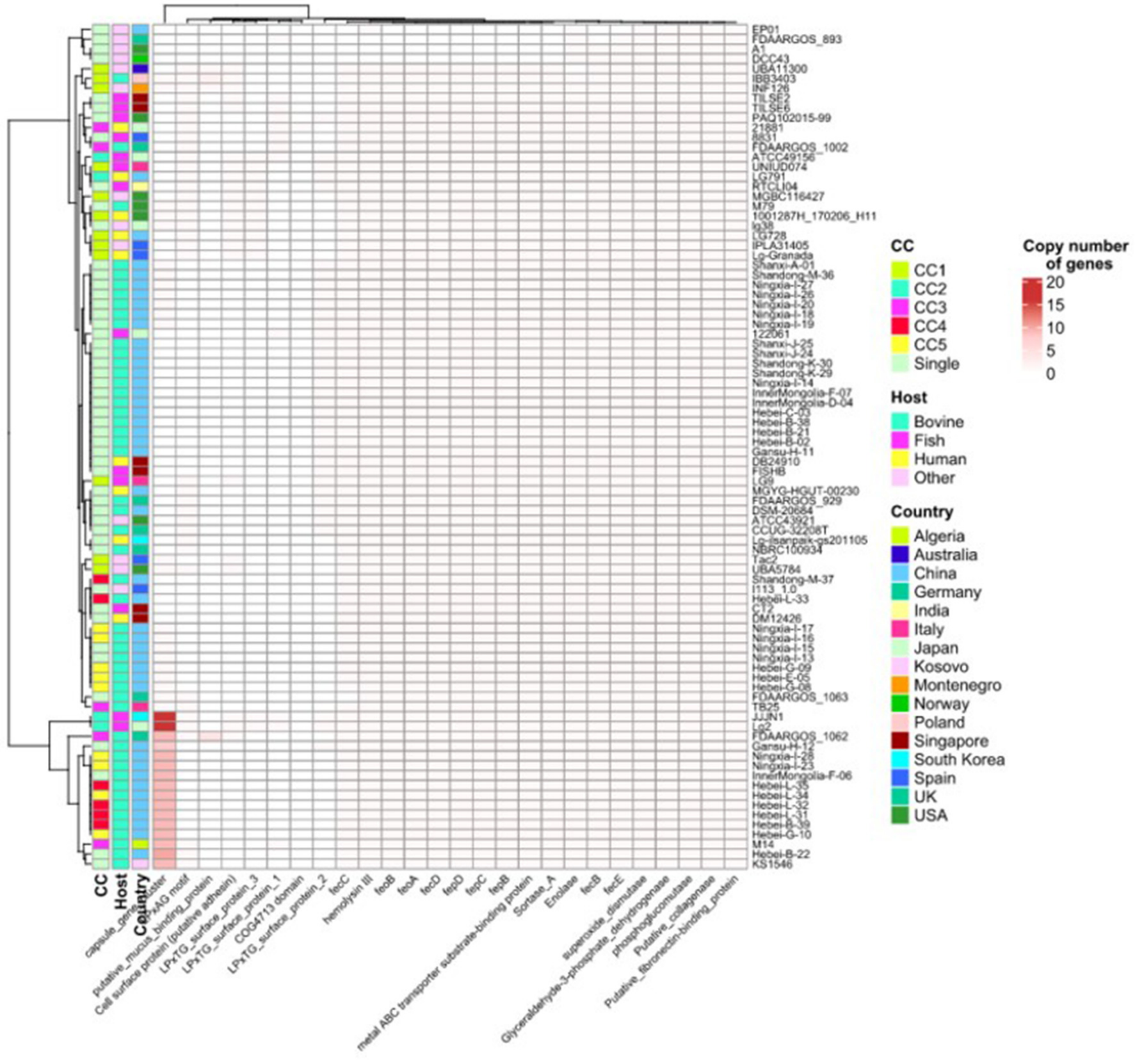
FIG 5 Phylogenetic tree. (A). based on 16S rRNA with source of host (4 hosts, the first ring indicated by a rectangle) and country (16 countries, the second ring indicated by a circle) as well as sequence types (STs, 32 STs, the outermost ring indicated by a triangle) of 86 *Lactococcus garvieae* isolates. **(B).** Phylogenetic tree based on core genes with source of host (4 hosts, the innermost ring indicated by a rectangle) and country (16 countries, the second ring indicated by a circle) as well as sequence types (STs, 32 STs, the outermost ring indicated by a triangle) of 86 *Lactococcus garvieae* isolates.

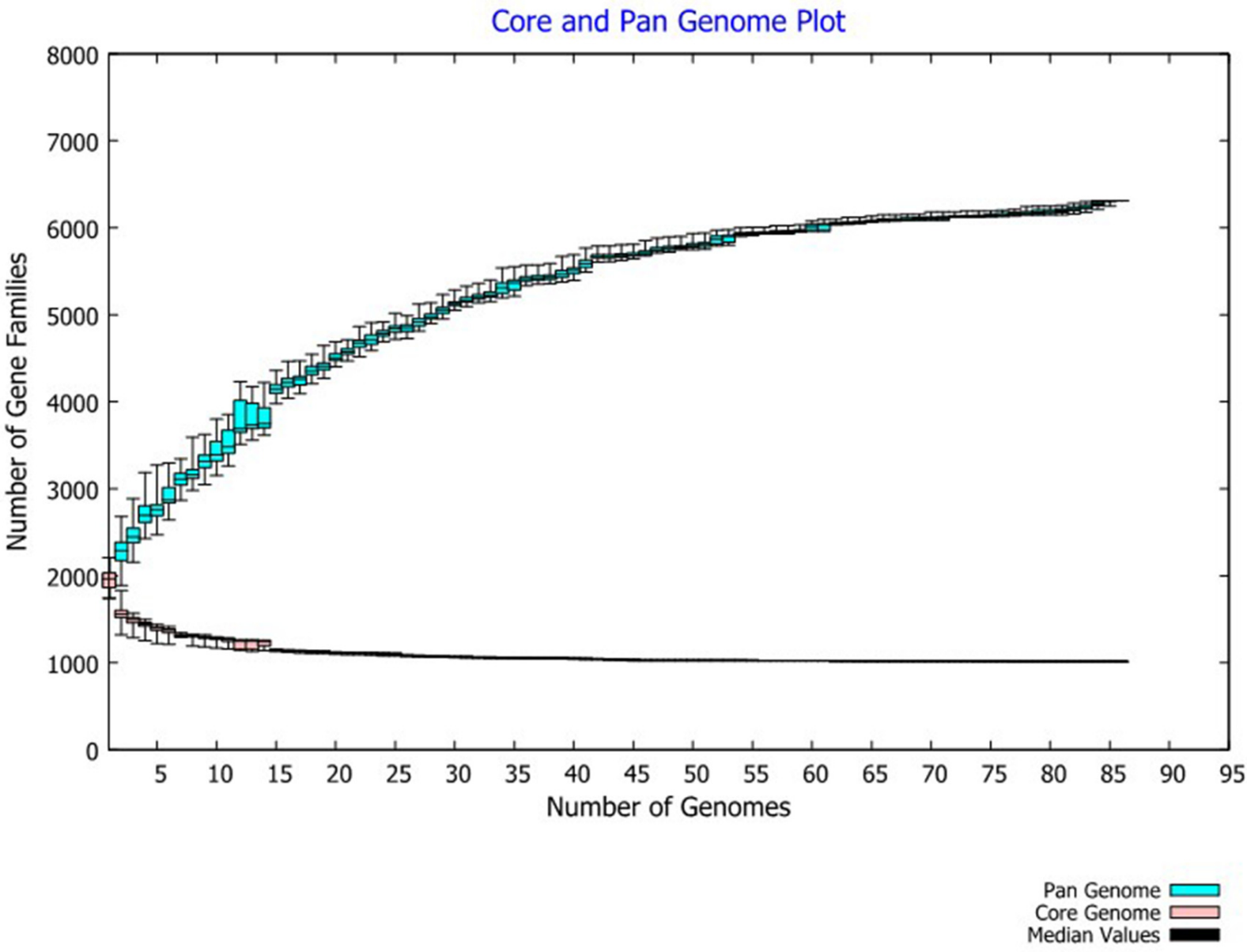
FIG 6 Phylogenetic tree based on core genome single nucleotide polymorphism of 86 *Lactococcus garvieae* isolates.

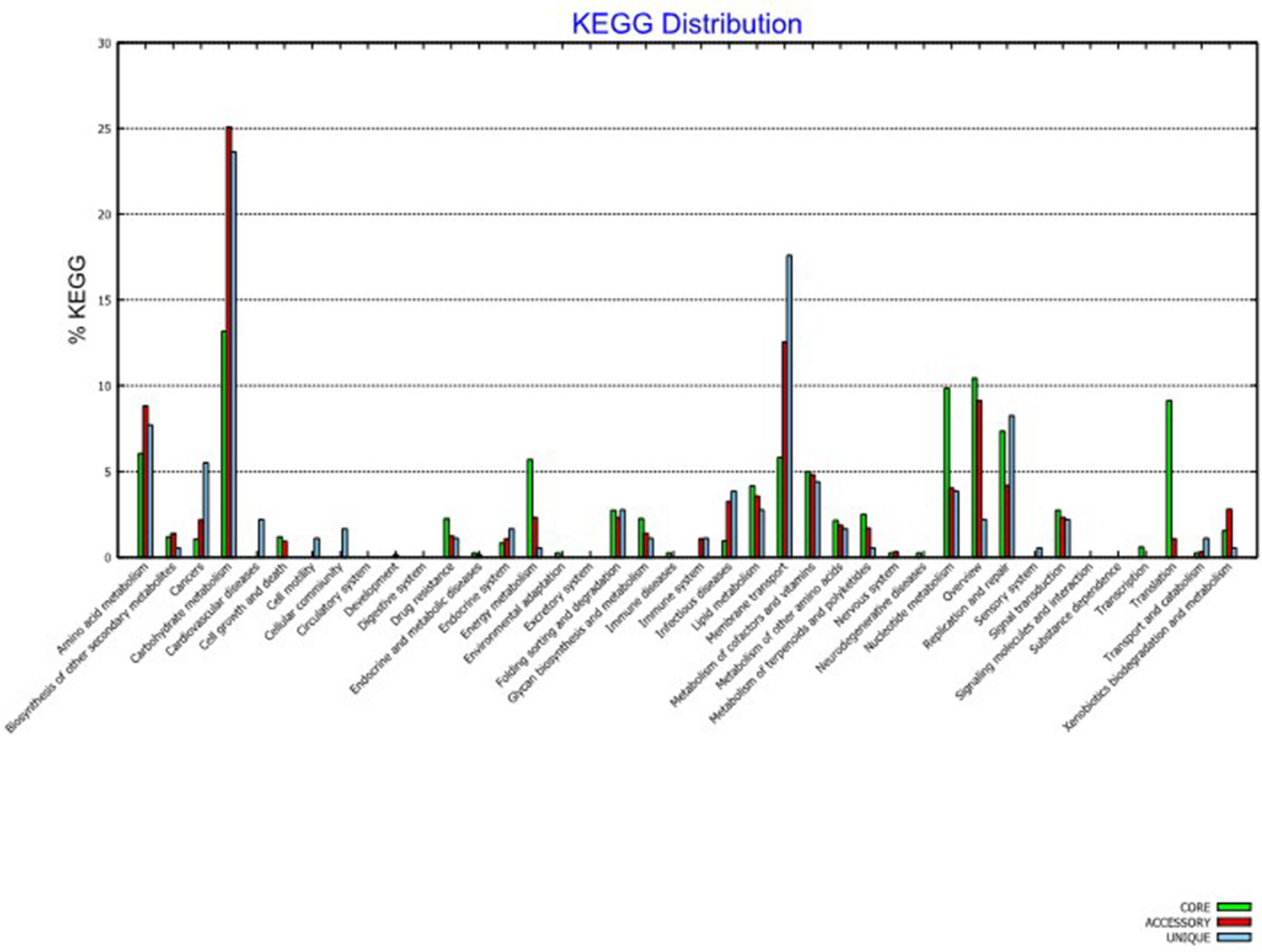
FIG 7 Co-occurrence of virulence genes in 86 *Lactococcus garvieae* isolates.

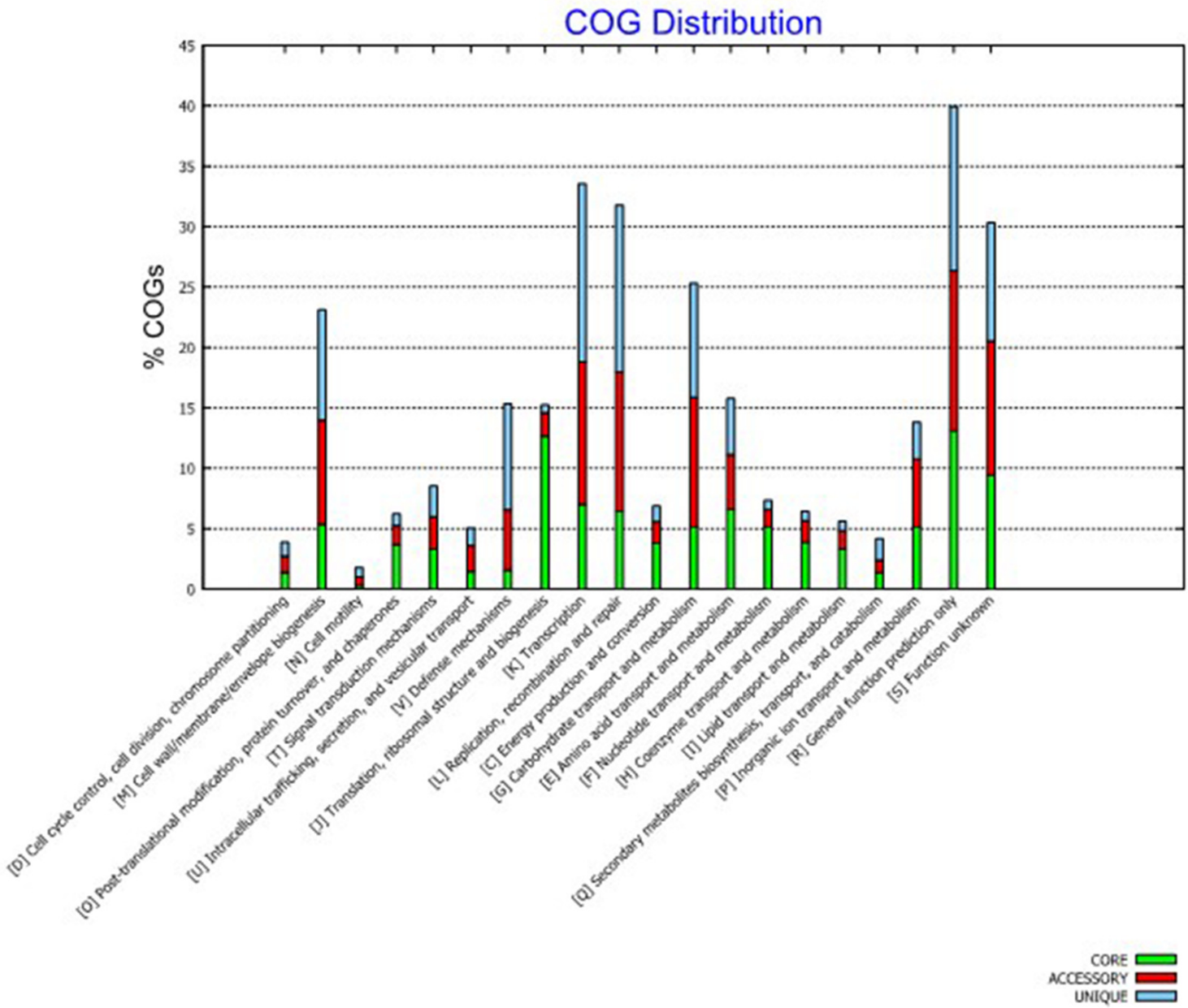


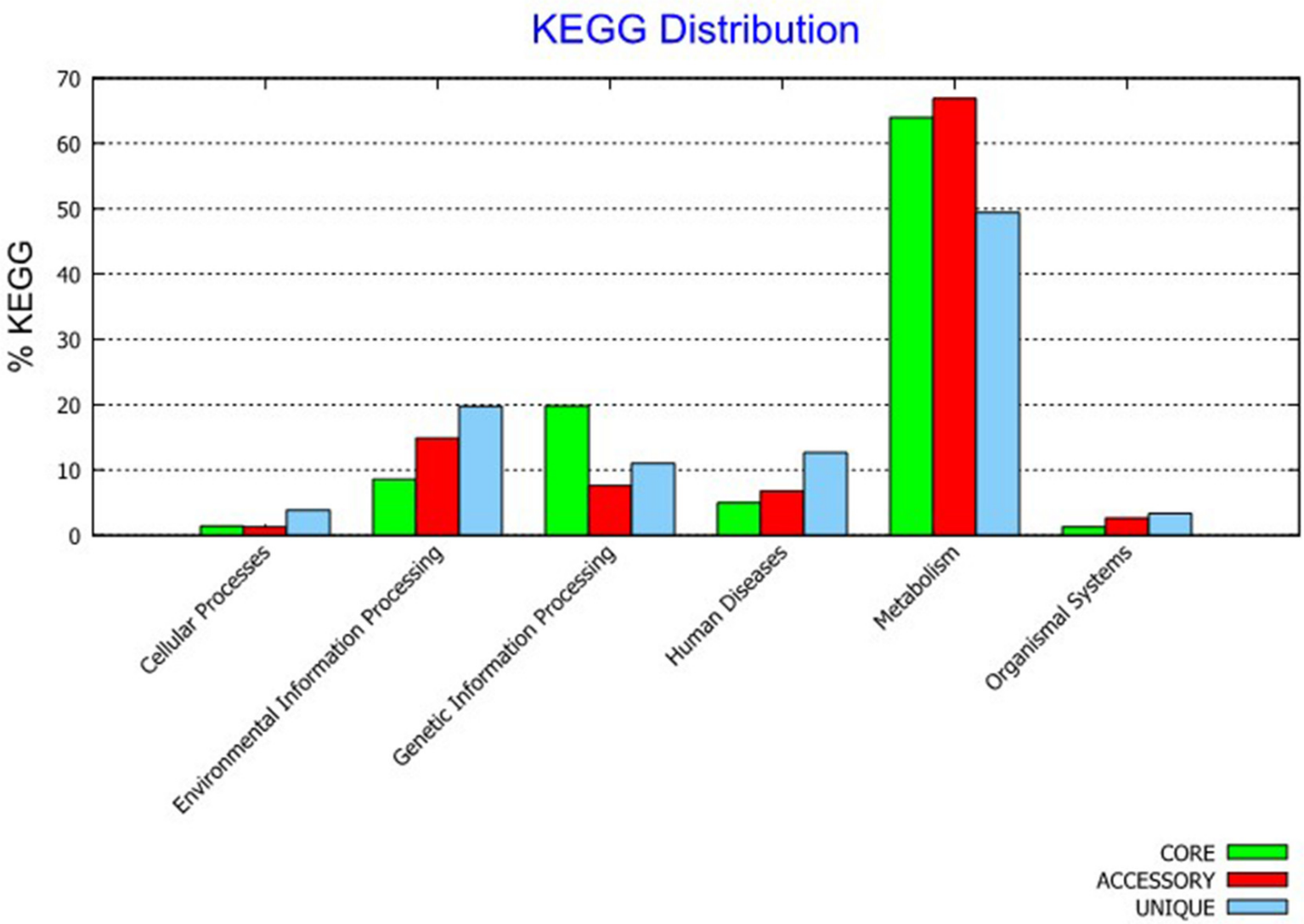


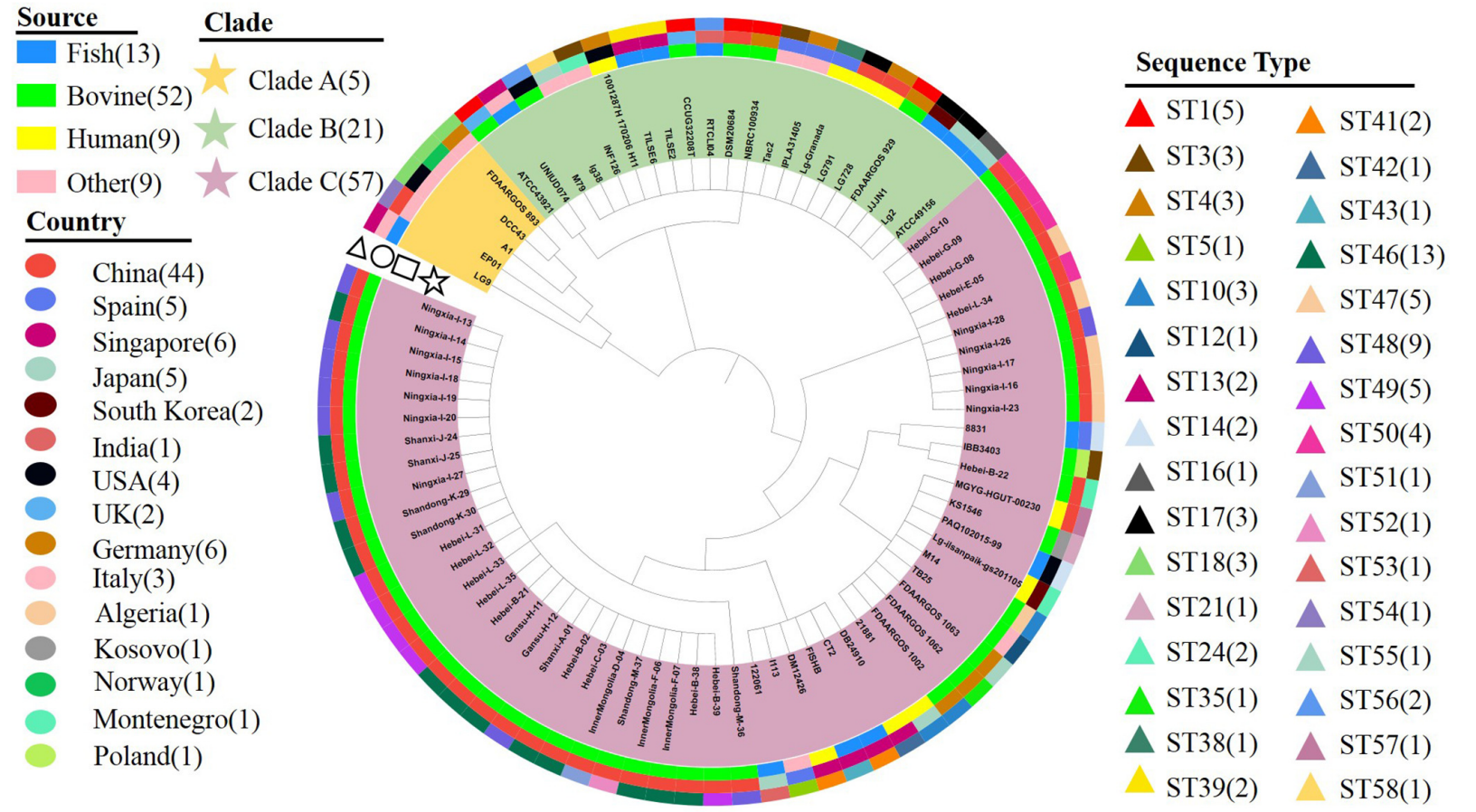


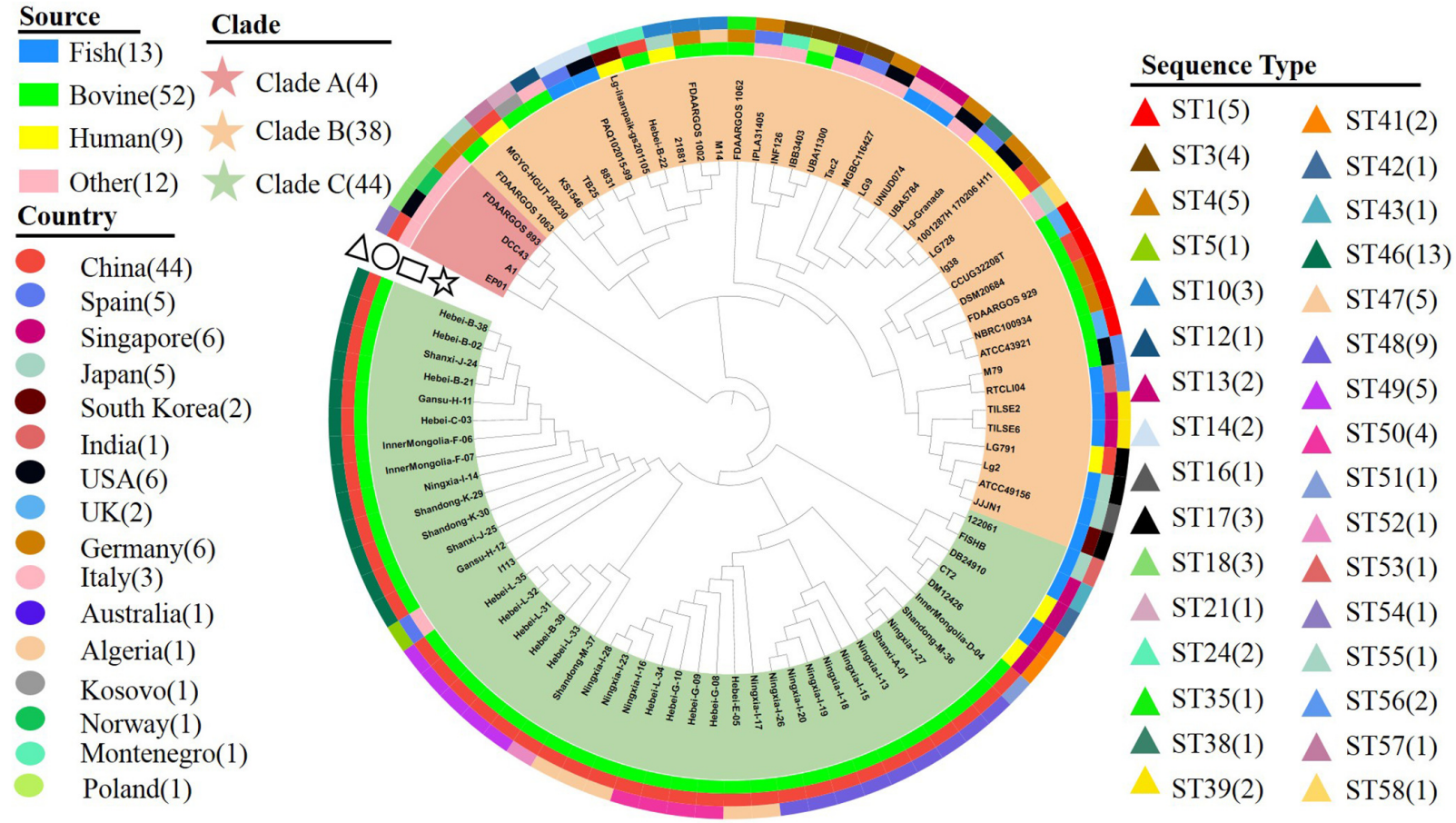


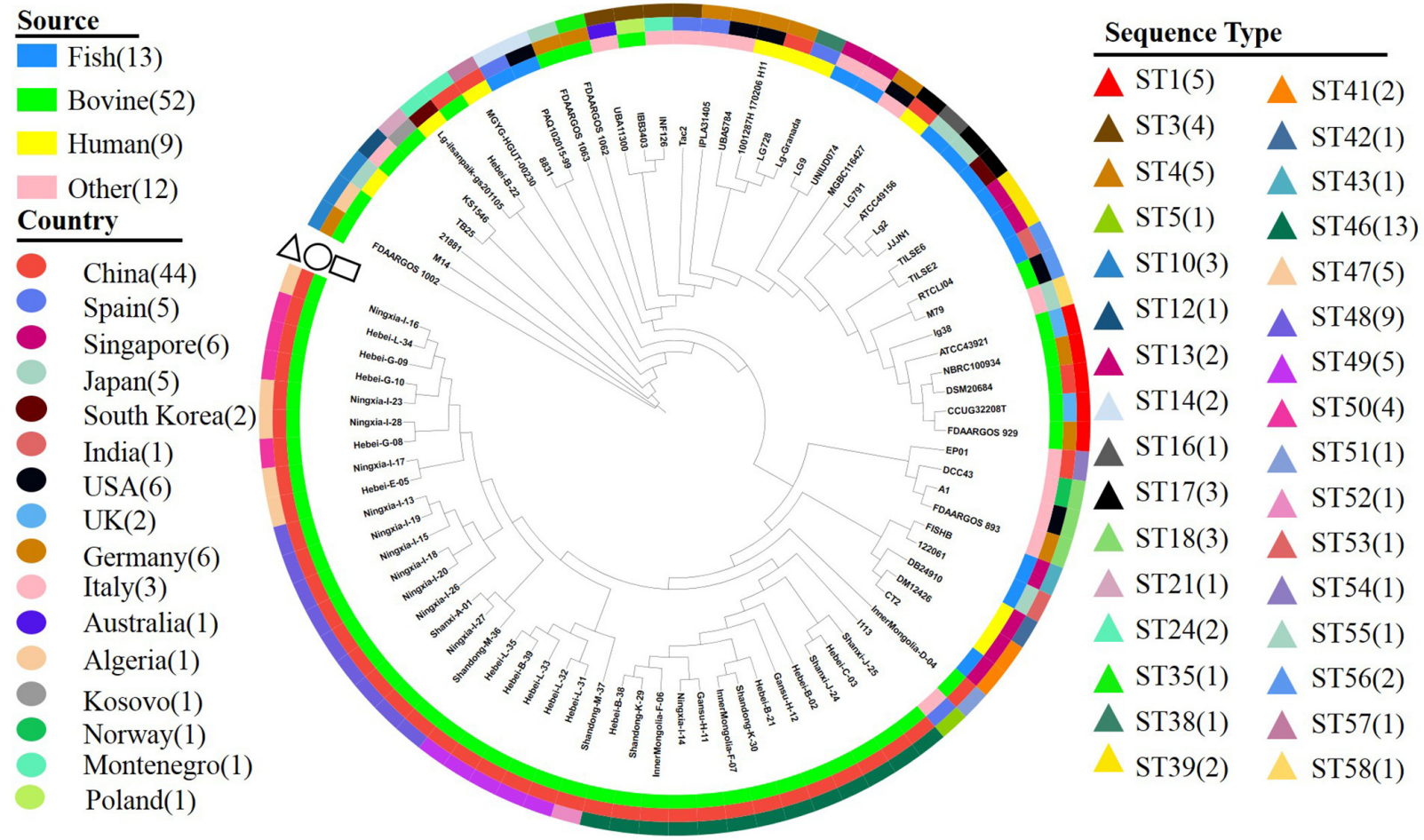












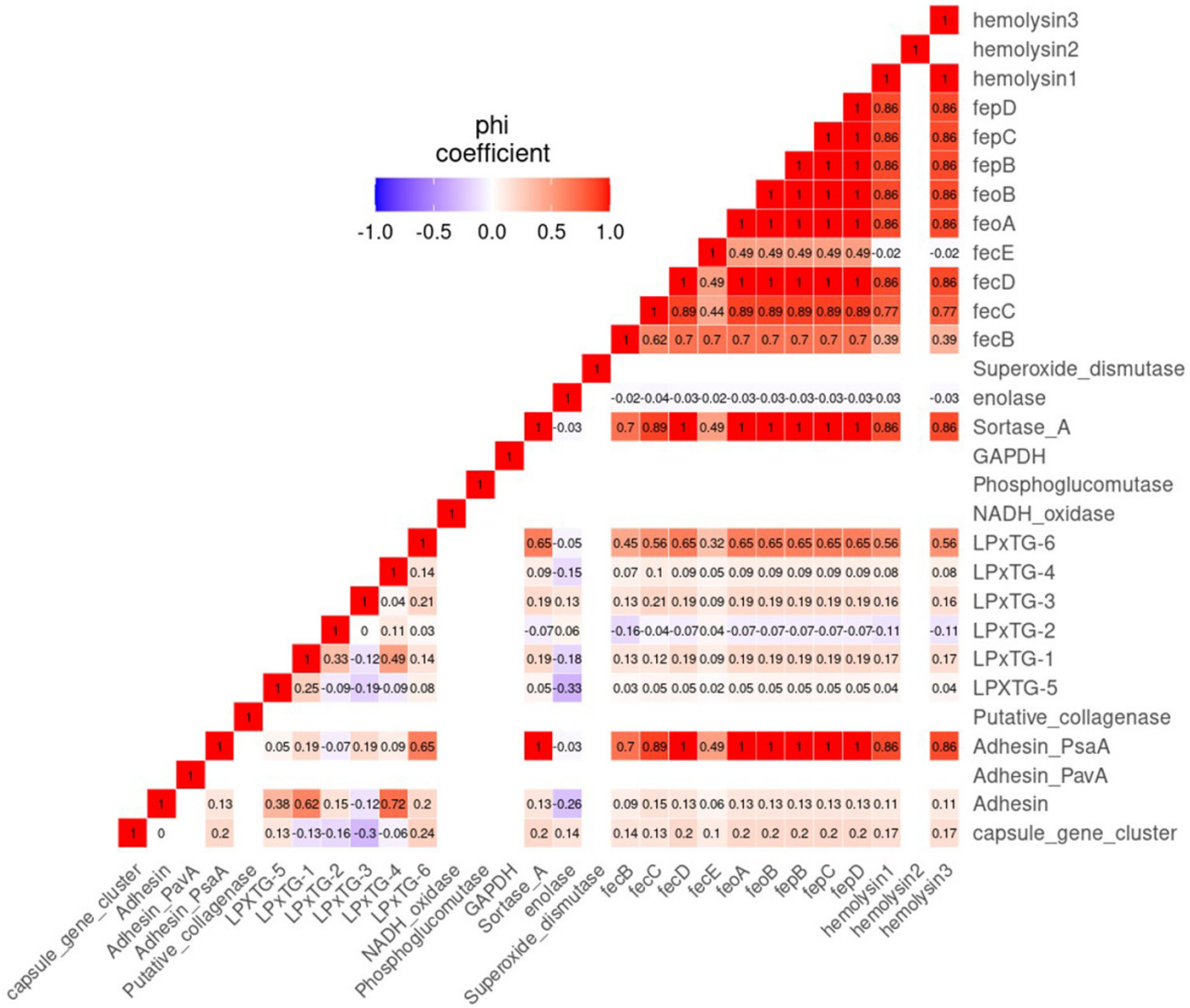


Table 1 *Lactococcus garvieae* isolates (n=39) recovered from bovine mastitis from 2899 bovine mastitis (CM) milk samples collected from farms in China.

Isolate	Province	Farm	Date
Shanxi-A-01	Shanxi	A	2018.1.29
Hebei-B-02	Hebei	B	2018.4.9
Hebei-C-03	Hebei	C	2018.5.11
InnerMongolia-D-04	Inner Mongolia	D	2018.6.26
Hebei-E-05	Hebei	E	2018.7.16
InnerMongolia-F-06	Inner Mongolia	F	2019.6.25
InnerMongolia-F-07	Inner Mongolia	F	2019.6.25
Hebei-G-08	Hebei	G	2019.6.29
Hebei-G-09	Hebei	G	2019.6.29
Hebei-G-10	Hebei	G	2019.6.29
Gansu-H-11	Gansu	H	2020.8.12
Gansu-H-12	Gansu	H	2020.8.12
Ningxia-I-13	Ningxia	I	2020.8.12
Ningxia-I-14	Ningxia	I	2020.8.12
Ningxia-I-15	Ningxia	I	2020.8.12
Ningxia-I-16	Ningxia	I	2020.8.12
Ningxia-I-17	Ningxia	I	2020.8.24
Ningxia-I-18	Ningxia	I	2020.8.24
Ningxia-I-19	Ningxia	I	2020.8.24
Ningxia-I-20	Ningxia	I	2020.8.24
Hebei-B-21	Hebei	B	2020.8.27
Hebei-B-22	Hebei	B	2020.8.27
Ningxia-I-23	Ningxia	I	2020.10.2
Shanxi-J-24	Shanxi	J	2020.11.1
Shanxi-J-25	Shanxi	J	2020.11.1
Ningxia-I-26	Ningxia	I	2020.11.6
Ningxia-I-27	Ningxia	I	2020.11.6
Ningxia-I-28	Ningxia	I	2021.1.29
Shandong-K-29	Shandong	K	2021.6.9
Shandong-K-30	Shandong	K	2021.6.9
Hebei-L-31	Hebei	L	2021.6.10
Hebei-L-32	Hebei	L	2021.6.10
Hebei-L-33	Hebei	L	2021.6.10
Hebei-L-34	Hebei	L	2021.6.10
Hebei-L-35	Hebei	L	2021.6.10
Shandong-M-36	Shandong	M	2021.6.28
Shandong-M-37	Shandong	M	2021.6.28

Hebei-B-38	Hebei	B	2021.7.12

Table 2 Allelic profiles, sequence types and clonal complexes of 86 *Lactococcus garvieae* isolates.

Isolate id	ST	CC	Allelic profile						
			als	galP	gapC	gyrB	atpA	rpoC	tuf
ATCC43921	ST1	Sing	1	1	1	1	1	1	1
CCUG32208T	ST1	Sing	1	1	1	1	1	1	1
DSM20684	ST1	Sing	1	1	1	1	1	1	1
FDAARGOS_929	ST1	Sing	1	1	1	1	1	1	1
NBRC100934	ST1	Sing	1	1	1	1	1	1	1
IBB3403	ST3	CC1	3	3	2	3	3	3	3
INF126	ST3	CC1	3	3	2	3	3	3	3
Tac2	ST3	CC1	3	3	2	3	3	3	3
UBA11300	ST3	CC1	3	3	2	3	3	3	3
1001287H_170206_H11	ST4	CC1	3	3	2	3	3	3	4
IPLA31405	ST4	CC1	3	3	2	3	3	3	4
LG728	ST4	CC1	3	3	2	3	3	3	4
MGBC116427	ST4	CC1	3	3	2	3	3	3	4
UBA5784	ST4	CC1	3	3	2	3	3	3	4
I113	ST5	Sing	4	4	3	4	4	4	5
21881	ST10	CC3	9	9	4	7	7	9	3
FDAARGOS_1002	ST10	CC3	9	9	4	7	7	9	3
M14	ST10	CC3	9	9	4	7	7	9	3
TB25	ST12	CC3	9	9	2	7	7	10	3
LG9	ST13	CC1	10	3	2	3	3	3	4
UNIUD074	ST13	CC1	10	3	2	3	3	3	4
8831	ST14	Sing	11	8	2	9	6	11	10
PAQ102015-99	ST14	Sing	11	8	2	9	6	11	10
ATCC49156	ST16	CC2	12	12	6	10	8	13	6
JJJN1	ST17	CC2	12	12	7	10	8	13	6
Lg2	ST17	CC2	12	12	7	10	8	13	6
LG791	ST17	CC2	12	12	7	10	8	13	6
A1	ST18	Sing	13	13	8	11	9	14	12
DCC43	ST18	Sing	13	13	8	11	9	14	12
FDAARGOS_893	ST18	Sing	13	13	8	11	9	14	12
KS1546	ST21	Sing	9	9	10	7	11	10	3
Hebei-B-22	ST24	Sing	17	16	2	7	12	9	3
Lg-ilsanpaik-gs201105	ST24	Sing	17	16	2	7	12	9	3
FDAARGOS_1062	ST35	CC3	9	9	2	7	7	9	3
Lg-Granada	ST38	CC1	3	23	2	3	3	3	4
TILSE2	ST39	Sing	5	24	2	23	17	19	6
TILSE6	ST39	Sing	5	24	2	23	17	19	6
CT2	ST41	Sing	25	25	9	25	10	21	21
DM12426	ST41	Sing	25	25	9	25	10	21	21
DB24910	ST42	Sing	26	25	13	20	18	22	22

FISHB	ST43	Sing	27	26	9	26	19	23	23
Gansu-H-11	ST46	Sing	29	4	3	27	4	4	5
Gansu-H-12	ST46	Sing	29	4	3	27	4	4	5
Hebei-B-02	ST46	Sing	29	4	3	27	4	4	5
Hebei-B-21	ST46	Sing	29	4	3	27	4	4	5
Hebei-B-38	ST46	Sing	29	4	3	27	4	4	5
Hebei-C-03	ST46	Sing	29	4	3	27	4	4	5
InnerMongolia-F-06	ST46	Sing	29	4	3	27	4	4	5
InnerMongolia-F-07	ST46	Sing	29	4	3	27	4	4	5
Ningxia-I-14	ST46	Sing	29	4	3	27	4	4	5
Shandong-K-29	ST46	Sing	29	4	3	27	4	4	5
Shandong-K-30	ST46	Sing	29	4	3	27	4	4	5
Shanxi-J-24	ST46	Sing	29	4	3	27	4	4	5
Shanxi-J-25	ST46	Sing	29	4	3	27	4	4	5
Hebei-E-05	ST47	CC5	29	27	3	27	4	24	14
Ningxia-I-16	ST47	CC5	29	27	3	27	4	24	14
Ningxia-I-17	ST47	CC5	29	27	3	27	4	24	14
Ningxia-I-23	ST47	CC5	29	27	3	27	4	24	14
Ningxia-I-28	ST47	CC5	29	27	3	27	4	24	14
Ningxia-I-13	ST48	Sing	4	4	3	20	4	24	14
Ningxia-I-15	ST48	Sing	4	4	3	20	4	24	14
Ningxia-I-18	ST48	Sing	4	4	3	20	4	24	14
Ningxia-I-19	ST48	Sing	4	4	3	20	4	24	14
Ningxia-I-20	ST48	Sing	4	4	3	20	4	24	14
Ningxia-I-26	ST48	Sing	4	4	3	20	4	24	14
Ningxia-I-27	ST48	Sing	4	4	3	20	4	24	14
Shandong-M-36	ST48	Sing	4	4	3	20	4	24	14
Shanxi-A-01	ST48	Sing	4	4	3	20	4	24	14
Hebei-B-39	ST49	CC4	30	28	3	28	21	4	18
Hebei-L-31	ST49	CC4	30	28	3	28	21	4	18
Hebei-L-32	ST49	CC4	30	28	3	28	21	4	18
Hebei-L-33	ST49	CC4	30	28	3	28	21	4	18
Hebei-L-35	ST49	CC4	30	28	3	28	21	4	18
Hebei-G-08	ST50	CC5	29	29	3	27	4	24	14
Hebei-G-09	ST50	CC5	29	29	3	27	4	24	14
Hebei-G-10	ST50	CC5	29	29	3	27	4	24	14
Hebei-L-34	ST50	CC5	29	27	3	27	4	24	14
InnerMongolia-D-04	ST51	Sing	31	4	3	4	14	4	14
Shandong-M-37	ST52	CC4	31	28	3	28	21	4	18
122061	ST53	Sing	32	26	14	28	10	25	14
EP01	ST54	Sing	33	Ab	15	29	22	26	24
FDAARGOS_1063	ST55	Sing	34	21	16	30	7	27	25
M79	ST56	Sing	35	30	17	31	23	28	26
RTCLI04	ST56	Sing	35	30	17	31	23	28	26

MGYG-HGUT-00230	ST57	Sing	36	6	2	32	24	8	27
lg38	ST58	Sing	37	31	1	33	25	29	28

CC, clonal complexes; Sing, singleton; ST, sequence type; Ab, absent.

Table 3 Minimum inhibitory concentration (MIC) of the 15 antimicrobials tested for 39 *Lactococcus garvieae* isolates isolated from clinical mastitis composite milk samples in China and control strain ATCC 43921 [1].

Antimicrobial	MIC (μg/mL)										Resistance rate (%)	MIC50 (μg/mL)	MIC90 (μg/mL)
	0.12	0.25	0.5	1	2	4	8	16	32	64			
Penicillin		9	7	19	5						0	1	2
Ampicillin		3	9	24	4						0	1	1
Amoxicillin/clavulanic acid				1	4	26	9				0	4	8
Imipenem	11	17	11	1							0	0.25	0.5
Cephalothin						10	26	4			10	8	8
Cefazolin				2	20	11	6	1			45	2	8
Cefpodoxime				2	5	21	12				82.5	4	8
Ceftiofur	16	17	7								0	0.25	64
Amikacin								4	5	31	90	64	64
Gentamicin				1	12	12	15				37.5	4	8
Erythromycin	19	6	13	2							5	0.25	0.5
Clindamycin							1	23	11	5	100	16	32
Enrofloxacin	1	3	17	15	3	1					0	0.5	1
Marbofloxacin			2	20	15	3					0	1	2
Chloramphenicol							18	14	2	6	100	16	64
Vancomycin			15	22	3						0	1	1

1. Resistance breakpoints are highlighted by dark gray shading; intermediate breakpoints are highlighted by light gray shading; cells without shading indicate that no breakpoints were available in the literature.