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Phylogenetic Analysis of Lactococcosis-Causing Bacteria Isolated from Different Fish Species in Brazil Using Multilocus Sequence Typing

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Abstract

Lactococcosis has emerged as an economically and ecologically significant disease in aquatic animals worldwide. This study employed multilocus sequence typing (MLST) to investigate the genetic diversity of *Lactococcus* spp. strains from Brazilian fish species and evaluate their phylogenetic relationships with global isolates to elucidate potential epidemiological connections involving multiple host species and distinct geographic regions. A total of 55 isolates had their DNA extracted, followed by the amplification and sequencing of the internal fragments of seven housekeeping genes (*als*, *atpA*, *tuf*, *gapC*, *gyrB*, *rpoC* and *galP*). Sequence types (STs) and clonal complexes (CCs) were defined. An unrooted neighbor-joining phylogenetic tree was generated using allele profiles from this study and those previously reported from other aquatic animal species. The isolates comprised 29 STs (11 previously reported, 18 novel ones), which were grouped into species-specific CCs: CC5 (*L. formosensis*); CC4, CC17, CC62 (*L. garvieae*); CC24, CC29, CC97 (*L. petauri*). Considerable genetic divergence was observed, with *L. formosensis* and *L. garvieae* forming heterogeneous populations, while *L. petauri* was more homogeneous. Phylogenetics confirmed groupings within the CCs and revealed significant genetic arrangements. In conclusion, the Brazilian *Lactococcus* spp. strains analyzed in this study constitute a genetically diverse population based on their STs. MLST and phylogenetic analysis demonstrated genetic relatedness between the *L. garvieae* and *L. formosensis* isolates from this study and those from other aquatic animal species. In contrast, all the STs identified for *L. petauri* in this study were unrelated to the MLST lineages responsible for outbreaks in Brazilian Nile tilapia (*Oreochromis niloticus*) and North American rainbow trout (*Oncorhynchus mykiss*). This suggests that piscine *L. petauri* populations in the Americas evolved from distinct ancestral origins.

Keywords: *Lactococcus*; native fish species; ornamental fish; sequence type; clonal complex; phylogenetic relationships

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Introduction

Lactococcosis has emerged as an economically and ecologically significant disease in aquatic animals worldwide [1]. Disease outbreaks and associated mortality have been linked to infections caused by *Lactococcus formosensis*, *L. garvieae* and *L. petauri* in various fishes and prawn species, particularly in aquaculture systems [2–8]. Among the lactococcosis-causing bacteria (LCBs), *L. petauri* has been responsible for the most significant economic losses in commercial rainbow trout (*Oncorhynchus mykiss*) [9] and Nile tilapia (*Oreochromis niloticus*) [3] production in the Americas. Nevertheless, LCBs have been detected in a wide range of fish species, some of which are susceptible to natural infection or experimental challenge [10–21]. In addition to aquatic animals, these three pathogens have also been identified in, terrestrial animals including humans, products destined for human consumption, and in the environment [22–26].

Given the broad range of hosts and wide geographic distribution of these pathogens, genetic characterization studies have become a critical tool in epidemiological investigations. Such studies help elucidate the pathogen's genetic structure and assess genetic relatedness or the divergence among isolates [23]. Different molecular typing methods have been employed for the genotyping of strains of LCBs. These include PCR-based DNA fingerprinting techniques such as repetitive sequence-based PCR (REP-PCR), random amplification of polymorphic DNA (RAPD-PCR), restriction fragment length polymorphism (RFLP), and pulsed-field gel electrophoresis (PFGE) [19,27–29], as well as sequencing-based approaches like multilocus sequence analysis (MLSA) and multilocus sequence typing (MLST) [3,9,22].

Among the sequencing-based methods, MLST is the most widely adopted for assessing genetic diversity in bacterial pathogens, including those that affect aquatic host species [30,31]. MLST is a molecular typing technique that relies on sequencing internal fragments of housekeeping genes and has been extensively used to determine phylogenetic relationships among bacterial isolates [23], infer ancestral genotypes and trace evolutionary lineages [22]. For LCBs, the seven housekeeping genes analyzed—along with their corresponding proteins—are *als* (α-acetolactate synthase), *atpA* (ATP synthase α subunit), *tuf* (elongation factor EF-Tu), *gapC* (glyceraldehyde-3-phosphate dehydrogenase), *gyrB* (DNA gyrase β subunit), *rpoC* (RNA polymerase β subunit) and *galP* (galactose permease) [22]. The combination of alleles from these genes defines an allelic profile, which corresponds to a sequence type (ST). Genetic relatedness among isolates can be inferred by comparing these allelic profiles. Allele and ST designations can be used to classify strains into clonal complexes (CCs) or lineages, thus providing insights into population structure and evolutionary dynamics [32]. Furthermore, curated MLST databases, particularly those hosted by PubMLST [33], offer standardized nomenclature and facilitate phylogenetic analysis to infer evolutionary relationships [32]. This approach enables the differentiation of LCB strains isolated from a variety of hosts and from different geographic regions [3,22–26,34,35].

In Brazil, a recent study evaluated the genetic diversity of isolates from LCBs derived from native fish species using PCR-based DNA fingerprinting techniques (REP-, RAPD-, and BOX-PCR) [19]. The results demonstrated significant genetic heterogeneity among *L. garvieae* strains, whereas *L. petauri* isolates exhibited a more homogeneous population [19]. To date, MLST analysis in Brazil has been restricted to *L. garvieae* and *L. petauri* isolates obtained from Nile tilapia from different commercial farms, revealing that there are only three STs (ST24, ST46 and ST47) in circulation [3]. However, no MLST data are available for LCBs strains from other fish species in the country. This represents a critical knowledge gap since it remains unclear whether the genetic structure of *Lactococcus* spp. infecting non-Nile tilapia hosts mirrors that reported in tilapia-associated strains, or whether the same genotypes are shared between fish farms and wild fish populations. Furthermore, MLST-based surveillance is ideal for assessing potential cross-species transmission and supports evidence-based biosecurity measures in Brazilian aquaculture.

Therefore, this study aimed to evaluate the genetic diversity and population structure of Brazilian LCB strains isolated from multiple fish species using the MLST approach. Additionally, we sought to investigate the phylogenetic relationships between these isolates and globally deposited

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strains by comparing them with the sequences available in the PubMLST database, in order to elucidate potential epidemiological connections among diverse different host species and across geographic regions.

Methods

Bacterial Strains and Identification

This study used a total of 55 *Lactococcus* spp. strains, comprising *L. formosensis* (*n* = 7), *L. garvieae* (*n* = 20) and *L. petauri* (*n* = 28) isolates. The strains were obtained from 16 fish species from wild populations and commercial farms in six Brazilian states (Amazonas, Bahia, Mato Grosso do Sul, Minas Gerais, Pará and São Paulo) between 2012 and 2024 (Table 1). The isolates were obtained during routine diagnostic investigations of bacterial infections in fish, and were identified to the species level via *gyrB* sequencing, following previously described methods [19].

Table 1. Characteristics and allelic profiles of the Brazilian *Lactococcus* spp. isolates analyzed in this study.

Isolate	Species	Origin	Year	Host	MLST							
					Allele						ST	CC
					als	*atpA*	*tuf*	*gapC*	*gyrB*	*rpoC*	*galP*	
167/23-02	*L. formosensis*	BA	2023	*Arapaima gigas*	22	62	18	3	20	4	78	n168	Singleton
167/23-06	*L. formosensis*	BA	2023	*Arapaima gigas*	15	10	14	9	13	15	15	20	Singleton
167/23-09	*L. formosensis*	BA	2023	*Arapaima gigas*	22	62	18	3	20	4	78	n168	Singleton
49/21-29	*L. formosensis*	SP	2021	*Pangasianodon hypophthalmus*	100	4	18	3	20	4	79	n174	Singleton
52MS	*L. formosensis*	MS	2012	*Pseudoplatystoma fasciatum*	91	60	14	9	20	33	81	n179	Singleton
AM-LG05	*L. formosensis*	AM	2022	*Colossoma macropomum*	90	35	14	9	20	38	81	n178	Singleton
LG91-23	*L. formosensis*	MG	2023	*Pseudoplatystoma* sp.	92	4	50	3	20	4	73	n166	Singleton
177	*L. garvieae*	MS	2012	*Pseudoplatystoma fasciatum*	3	3	4	2	59	3	3	122	CC4
31MS	*L. garvieae*	MS	2012	*Pseudoplatystoma fasciatum*	12	8	54	7	27	13	12	n180	CC17
49/21-11	*L. garvieae*	SP	2021	*Pangasianodon hypophthalmus*	5	5	6	2	5	5	5	6	Singleton
CRBP53	*L. garvieae*	AM	2023	*Arapaima gigas*	93	61	51	15	72	61	74	n167	Singleton

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CRBP 54	*L. garvieae*	AM	2023	*Arapaima gigas*	93	61	51	15	72	61	74	n167	Singleton
CRBP 138	*L. garvieae*	AM	2023	*Arapaima gigas*	34	59	27	15	28	19	29	n173	-
CRBP 144	*L. garvieae*	AM	2023	*Arapaima gigas*	34	59	27	15	28	19	29	n173	-
LG09-14	*L. garvieae*	SP	2014	*Pseudoplatystoma corruscans*	3	3	4	2	3	3	3	4	CC4
LG10-14	*L. garvieae*	MG	2014	*Lophiosilurus alexandri*	60	8	6	7	10	45	48	105	Singleton
LG23-16	*L. garvieae*	SP	2016	*Pseudoplatystoma corruscans*	88	22	46	25	71	20	71	n164	Singleton
LG63-21	*L. garvieae*	MG	2021	*Hoplias macrophtalmus*	3	3	4	2	3	3	3	4	CC4
LG64-21	*L. garvieae*	MG	2021	*Xiphophorus maculatus*	87	24	27	15	28	27	29	n176	nCC62
LG66-22	*L. garvieae*	MG	2022	*Phractocephalus hemioliopterus*	12	8	6	7	27	13	12	46	CC17
LG88-23	*L. garvieae*	MG	2023	*Brycon amazonicus*	34	59	27	15	28	19	72	n165	-
LG89-23	*L. garvieae*	MG	2023	*Brycon amazonicus*	34	59	27	15	28	19	72	n165	-
LG114-23	*L. garvieae*	AM	2023	*Hoplias malabaricus*	34	59	27	15	28	19	72	n165	-
LG115-23	*L. garvieae*	MG	2023	*Trichogaster lalius*	21	13	1	2	73	16	75	n169	Singleton
LG116-23	*L. garvieae*	MG	2023	*Cichla sp.*	94	3	52	2	73	62	76	n170	Singleton
LG119-24	*L. garvieae*	MG	2024	*Pseudoplatystoma* sp.	95	69	6	2	5	63	77	n171	Singleton
PA-LG01	*L. garvieae*	PA	2018	*Arapaima gigas*	34	59	27	15	28	19	72	n165	-
86	*L. petauri*	MS	2012	*Pseudoplatystoma* sp.	9	7	3	2	37	9	9	152	nCC29
93	*L. petauri*	MS	2012	*Pseudoplatystoma* sp.	9	7	3	2	37	9	9	152	nCC29
176	*L. petauri*	MS	2012	*Pseudoplatystoma fasciatum*	9	7	3	4	16	9	17	25	Singleton
14MS	*L. petauri*	MS	2012	*Pseudoplatystoma fasciatum*	32	21	7	2	7	11	6	n181	-
167/23-03	*L. petauri*	BA	2023	*Arapaima gigas*	94	6	7	2	7	11	82	n172	Singleton
167/23-04	*L. petauri*	BA	2023	*Arapaima gigas*	9	7	3	4	18	9	9	29	nCC29
167/23-05	*L. petauri*	BA	2023	*Arapaima gigas*	9	7	3	4	18	9	9	29	nCC29
167/23-07	*L. petauri*	BA	2023	*Arapaima gigas*	9	7	3	4	18	9	9	29	nCC29
167/23-08	*L. petauri*	BA	2023	*Arapaima gigas*	9	7	3	4	18	9	9	29	nCC29
167/23-10	*L. petauri*	BA	2023	*Arapaima gigas*	9	7	3	4	18	9	9	29	nCC29

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49/21-21	*L. petauri*	SP	2021	*Pangasianodon hypophthalmus*	9	7	3	4	18	9	9	29	nCC29
89/2	*L. petauri*	MS	2012	*Pseudoplatystoma* sp.	9	7	3	2	37	9	9	152	nCC29
AM-LG02	*L. petauri*	AM	2020	*Colossoma macropomum*	61	6	7	35	7	11	8	n175	Singleton
AM-LG03	*L. petauri*	AM	2022	*Colossoma macropomum*	89	20	26	2	24	25	6	n177	-
AM-LG06	*L. petauri*	AM	2022	*Pterophyllum scalare*	9	7	3	2	7	9	9	35	nCC29
AM-LG07	*L. petauri*	AM	2022	*Brycon amazonicus*	9	7	3	2	7	9	9	35	nCC29
AM-LG08	*L. petauri*	AM	2022	*Brycon amazonicus*	9	7	3	2	7	9	9	35	nCC29
CRBP89	*L. petauri*	AM	2023	*Arapaima gigas*	9	7	3	2	7	9	9	35	nCC29
CRBP98	*L. petauri*	AM	2023	*Arapaima gigas*	9	7	3	2	7	9	9	35	nCC29
CRBP146	*L. petauri*	AM	2023	*Arapaima gigas*	9	7	3	2	7	9	9	35	nCC29
LG03-18	*L. petauri*	MG	2018	*Pseudoplatystoma corruscans*	33	6	10	2	7	11	8	61	-
LG86-23	*L. petauri*	MG	2023	*Pseudoplatystoma* sp.	9	7	3	4	18	9	9	29	nCC29
LG94-23	*L. petauri*	MG	2023	*Pseudoplatystoma* sp.	9	7	3	4	18	9	9	29	nCC29
LG104-23	*L. petauri*	MG	2023	*Pseudoplatystoma* sp.	9	7	3	4	18	9	9	29	nCC29
LG106-23	*L. petauri*	MG	2023	*Pseudoplatystoma* sp.	9	7	3	4	18	9	9	29	nCC29
LG117-23	*L. petauri*	MG	2023	*Pseudoplatystoma* sp.	9	7	3	4	18	9	9	29	nCC29
LG120-24	*L. petauri*	MG	2024	*Carassius auratus*	9	7	3	4	18	9	9	29	nCC29
LG121-24	*L. petauri*	MG	2024	*Carassius auratus*	9	7	3	4	18	9	9	29	nCC29

DNA Extraction

The selected *Lactococcus* spp. strains were cultured from cryopreserved stocks on de Man, Rogosa and Sharp (MRS) agar (Merck, Darmstadt, Germany) and incubated at 28 ºC for 72 h under aerobic conditions. Colonies were harvested and resuspended in 180 µL of lysis buffer (20 mg/mL lysozyme, 20 mM Tris-HCl [pH 8.0], 2mM EDTA, and 1.2% Triton X-100), followed by incubation at 37 ºC overnight. Bacterial DNA was extracted using the Maxwell® 16 Tissue DNA Purification kit (Promega, Madison, USA) in accordance with the manufacturer's protocol. DNA concentration and purity were assessed spectrophotometrically (Nanodrop® 2000, Thermo Fisher Scientific, Wilmington, USA) at 260/280 nm absorbance ratios. DNA samples were stored at -20 ºC until further analysis.

Multilocus Sequence Typing

For the MLST analysis, the isolates were characterized by sequencing internal fragments of seven housekeeping genes, following a modified version of the previously described protocol [22]. In summary, PCR amplification was performed using the Gotaq® PCR Core System kit (Promega) in 25

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µL reaction volumes containing 100 ng of DNA template (2 µL) and 23 µL of PCR mix (1× PCR buffer, 0.2 µM of each primer [Table 2], 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.625 U of DNA polymerase, and nuclease-free water). The primers were synthesized and purified by Invitrogen (Thermo Fisher Scientific).

Table 2. Oligonucleotide primers used in the MLST assay for *Lactococcus* spp. strains and polymorphism observed for each gene.

Gene	Primer pairs (5′-3′)	Annealing temperature (ºC)	Size (bp)	Nº of alleles	Nº of polymorphic sites
als	F: ATTCGGCTCAGACTTAGTTG R: TTCAGCTGCTTCAACATCAA	58	811	22	85
atpA	F: TAYRTYGGKGAYGGDATYGC R: CCRCGRTTHARYTTHGCYTG	56	803	18	51
tuf	F: ATATGCGGCCGCCATYGGHCACGTBGACCA R: AAAATATGCGGCCGCTCNCCNGGCATNACCAT	56	809	15	30
gapC	F: AAGTTGGTATTAACGGTTTCG R: AAGTGTACGAACGAGGTTAG	56	821	8	9
gyrB	F: CATGCTGGTGGTAAATTTGG R: GTCATCCATTTCTCCTAAACC	58	827	16	111
rpoC	F: TTGGTCCACAAAAGGACTGG R: TCACGTCCTTTTGCTTCCAT	58	830	18	58
galP	F: TGGGGAAAATTTAAACCTTGG R: ATCATCAGAACGGCTGGAAG	58	812	21	107

Amplification of *als*, *tuf*, *gapC*, *gyrB*, *rpoC* and *galP* was conducted in a 96-well thermal cycler (Veriti®, Applied Biosystems, Foster City, USA) under the following conditions: initial denaturation at 95 ºC for 5 min; 35 cycles of 94 ºC for 45 s, 56-58 ºC (primer-specific, see Table 2) for 45 s, and 72 ºC for 70 s; and a final extension at 72 ºC for 5 min. The *atpA* gene was amplified using a touchdown protocol: initial denaturation at 95 ºC for 5 min; 3 cycles of 95 ºC for 60 s, 56 ºC for 135 s, and 72 ºC for 75 s; followed by 30 cycles of 95 ºC for 35 s, 56 ºC for 75 s, and 72 ºC for 75 s; with a final extension at 72 ºC for 7 min.

Amplicons were size verified by capillary electrophoresis (QIAxcel Advanced System, Qiagen, Hilden, Germany) using the QX DNA Screening kit (Qiagen) according to the manufacturer's protocol. PCR products were then purified using the Agencourt AMPure® XP kit (Beckman Coulter, Brea, USA). Sanger sequencing was performed using the BigDye® Terminator v3.1. Cycle Sequencing kit (Applied Biosystems) in a genetic analyzer (ABI 3500, Applied Biosystems). Sequencing contigs were assembled and manually curated using Geneious Prime v. 2022.2.2 (Dotmatics, Boston, USA).

Data Analysis

To determine the allelic profiles and STs for each isolate, the assembled contigs were analyzed against the *L. garvieae* typing scheme in the PubMLST database (https://pubmlst.org/organisms/lactococcus-garvieae) [33]. The number of alleles and polymorphic sites were calculated using the BIGSdb Polymorphic Site Analysis plugin. Additional *Lactococcus* spp. strains isolated from aquatic animals with publicly available genome sequences in GenBank databases [36,37], but without prior ST designations in the literature, were selected for analysis (Table S1). The corresponding FASTA sequences were retrieved and subsequently uploaded to the

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PubMLST database via the BIGSdb platform for automated in silico analysis. Novel allelic profiles and STs were assigned to both the newly sequenced strains in this study and the previously deposited genomes.

The genetic relationships among the LCB isolates were inferred using the goeBURST algorithm [38,39], performed in PHYLOViZ software v. 2.0 [40]. Clonal complexes were defined based on single-locus variants (SLVs) using the software's default parameters. The novel STs and CCs identified in this study were designated with the prefix 'n' preceding the ST, or the CC number.

The discriminatory power of the MLST scheme was evaluated using Simpson's diversity index [41], calculated with the Comparing Partitions online tool (http://www.comparingpartitions.info/) [42].

To examine the phylogenetic relationships among the LCB isolates, we constructed an unrooted phylogenetic tree incorporating both novel allele profiles from this study and previously reported alleles from diverse animal aquatic species worldwide (Table S2). The seven housekeeping gene sequences were concatenated and the isolate sequences composed by all the loci were aligned using ClustalW implemented in MEGA12 [43]. A neighbor-joining phylogenetic tree was generated using the Tamura-Nei model, with branch support assessed using 1,000 bootstrap replicates to evaluate topological robustness [44]. The resulting phylogenetic trees were visualized using iTOL v. 6 online tool [45].

Results

MLST Analysis

The MLST analysis of the 55 LCB strains evaluated in this study grouped the isolates into 29 distinct STs (Table 1). The map of the distribution of *L. formosensis*, *L. garvieae*, and *L. petauri* STs are shown in Figure 1. Sequence analysis revealed that all the loci were polymorphic, with the number of variable nucleotide sites ranging from 9 (*gapC*) to 111 (*gyrB*), resulting in 8 (*gapC*) to 22 (*als*) distinct alleles (Table 2).

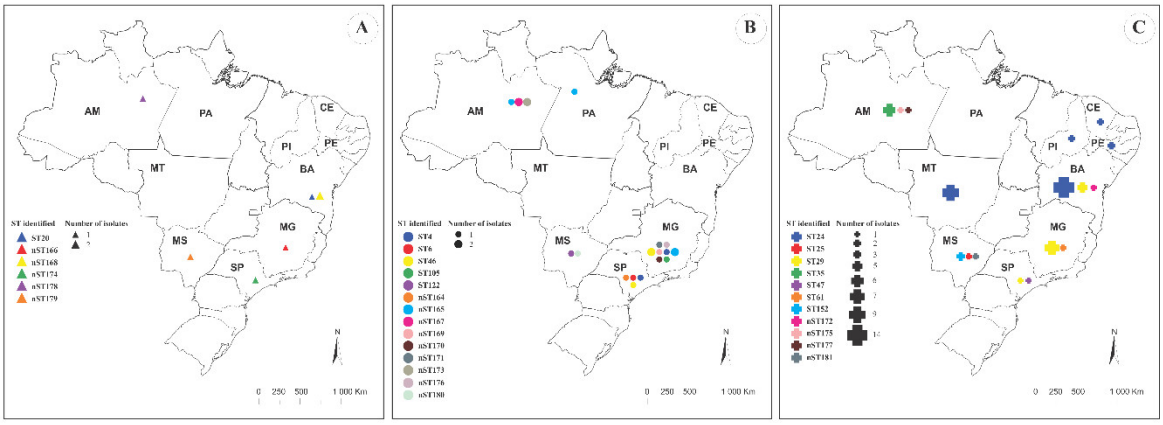

Figure 1. Map of the distribution of *L. formosensis* (A), *L. garvieae* (B) and *L. petauri* (C) sequence types (ST) identified in this study according to Brazilian state. Different colors represent the different STs and symbol sizes are proportional to the number of isolates per ST. *Lactococcus garvieae* ST46, *L. petauri* ST24 and ST47 Nile tilapia-derived isolates were added to demonstrate Brazilian genetic diversity.

Analysis of the 67 LCB genome sequences isolated from aquatic animals and subjected to MLST analysis in PubMLST identified 20 STs, including 10 novel STs. Only the ERR5094895 strain (from rainbow trout in Poland) lacked an assigned ST due to the absence of the *als* gene in its genome sequence (Table S1).

The *L. formosensis* strains used in this study were grouped into 6 different STs, including one previously reported (ST20) and five novel STs (nST166, nST168, nST174, nST178 and nST179). All of

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these STs were characterized as singletons (Table 1, Figure 2). The Simpson's diversity index (SDI) value was 0.933.

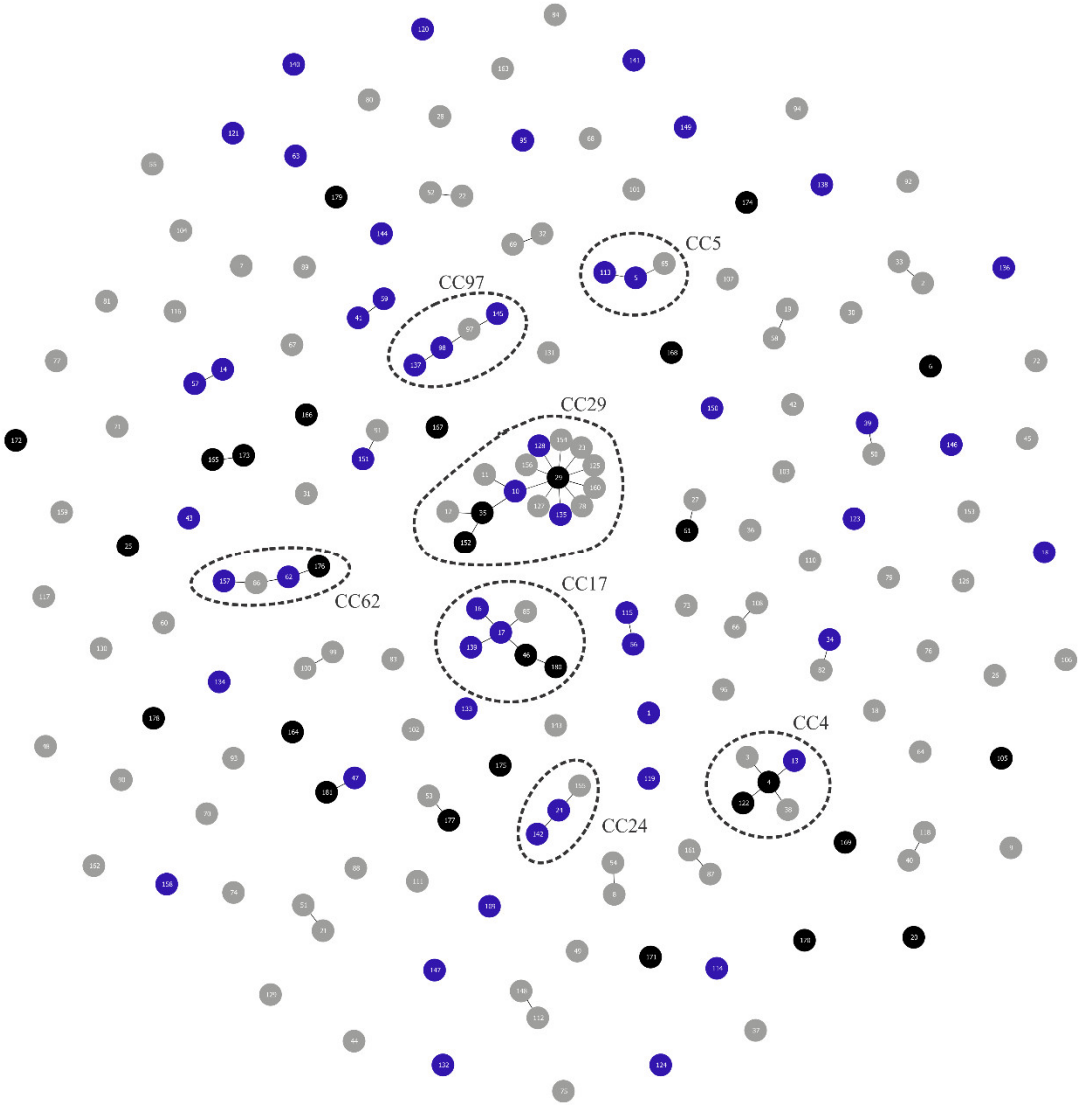

Figure 2. Global optimal eBURST analysis of all sequence types (ST) available to date in the *Lactococcus garvieae* typing scheme in the PubMLST database. Each circle represents an ST. Blue circles represent ST isolated from fish or prawns, black circles represent ST observed in this study, and black lines represent single-locus variants. STs highlighted in dashed lines form a clonal complex.

The *L. garvieae* strains grouped into 14 different STs, including five previously reported STs (ST4, *n* = 2; ST6, *n* = 1; ST46, *n* = 1; ST105, *n* = 1; ST122, *n* = 1) and nine novel STs (nST164, *n* = 1; nST165, *n* = 4; nST167, *n* = 2; nST169, *n* = 1; nST170, *n* = 1; nST171, *n* = 1; nST173, *n* = 2; nST176, *n* = 1; and nST180, *n* = 1). ST4 and ST122 were grouped into CC4. ST46 and nST180 clustered into CC17, and the nST176 belongs to CC62. The nST165 and nST173 clustered together, but differed only in the *galP* gene allele (a 4-nucleotide divergence), without forming a distinct clonal complex. All the isolates belonging to these STs were obtained from fish in northern Brazil, demonstrating genetic similarity associated with geographical origin. Finally, ST6, ST105, nST164, nST167, nST169, nST170 and nST171 were characterized as singletons (Table 1, Figure 2). The SDI value was 0.953.

The *L. petauri* strains grouped into nine different STs, including five previously reported STs (ST25, *n* = 1; ST29, *n* = 13; ST35, *n* = 6; ST61, *n* = 1; ST152, *n* = 3) and four novel STs (nST172, *n* = 1; nST175, *n* = 1; nST177, *n* = 1; nST181, *n* = 1). ST29, ST35 and ST152 were grouped into CC29. ST35 was

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exclusively identified in isolates from fish from the state of Amazonas, and ST152 was found only in *Pseudoplatystoma* ssp. from Mato Grosso do Sul. ST29, however, was detected in different hosts and across various geographical regions. ST61, nST177 and nST181 clustered with ST27, ST53 and ST47, respectively, but did not form distinct CCs. Finally, ST25, nST172 and nST175 were characterized as singletons (Table 1, Figure 2). The SDI value was 0.726.

Phylogenetic Relatedness Between Fish Isolates

The phylogenetic tree, constructed from concatenated MLST allele sequences of piscine *L. formosensis*, *L. garvieae*, and *L. petauri*, are presented in Figures 3, 4 and 5, respectively.

The *L. formosensis* strains clustered into five major groups, with the strains reported in this study forming three distinct clusters. These exhibited phylogenetic divergence from isolates obtained from marine fish of the Carangidae family (ST56 and ST115) from Japan and China (Figure 3).

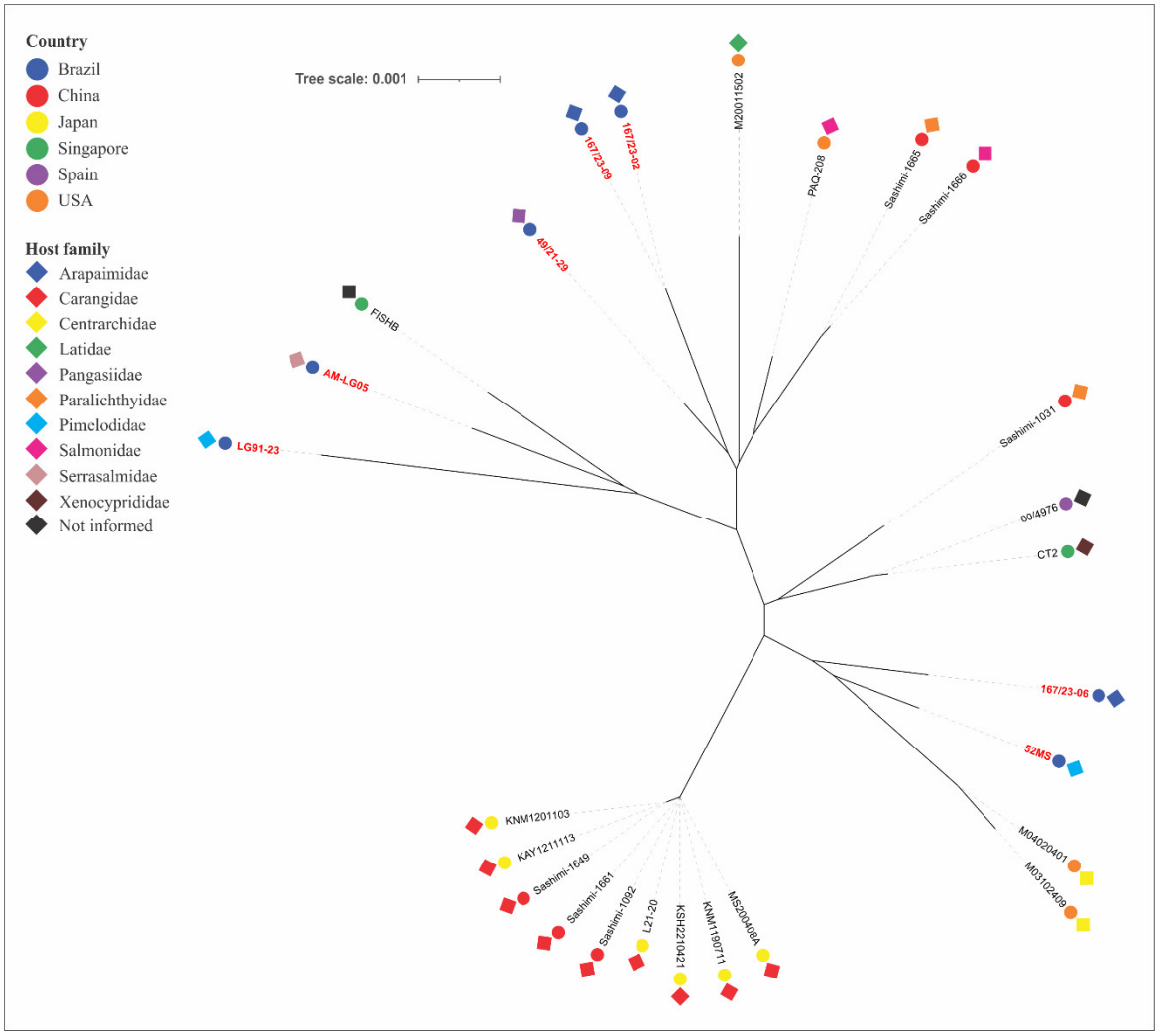

Figure 3. Phylogenetic tree of *Lactococcus formosensis* strains. The unrooted phylogenetic tree was constructed using the neighbor-joining method with the Tamura-Nei model from the 5,713 bp concatenated DNA sequences of the seven loci (*als*, *atpA*, *tuf*, *gapC*, *gyrB*, *rpoC* and *galP*) and a bootstrap analysis of 1,000 replicates to determine the evolutionary relationships among *L. formosensis* strains obtained from aquatic animals. The isolate's name in red denotes strains from this study. The colors of the circles and diamonds indicate the isolate's country of origin and host origin, respectively.

The *L. garvieae* strains clustered into fourteen groups, demonstrating the high genetic heterogeneity of this species. The Brazilian isolates that were not from tilapia grouped independently or alongside other aquatic animal isolates worldwide within eight of these groups. The LG10-14

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(ST105), LG66-22 (ST46) and 31MS (nST180) strains clustered with isolates obtained from disease outbreaks in Nile tilapia in Brazil (Figure 4).

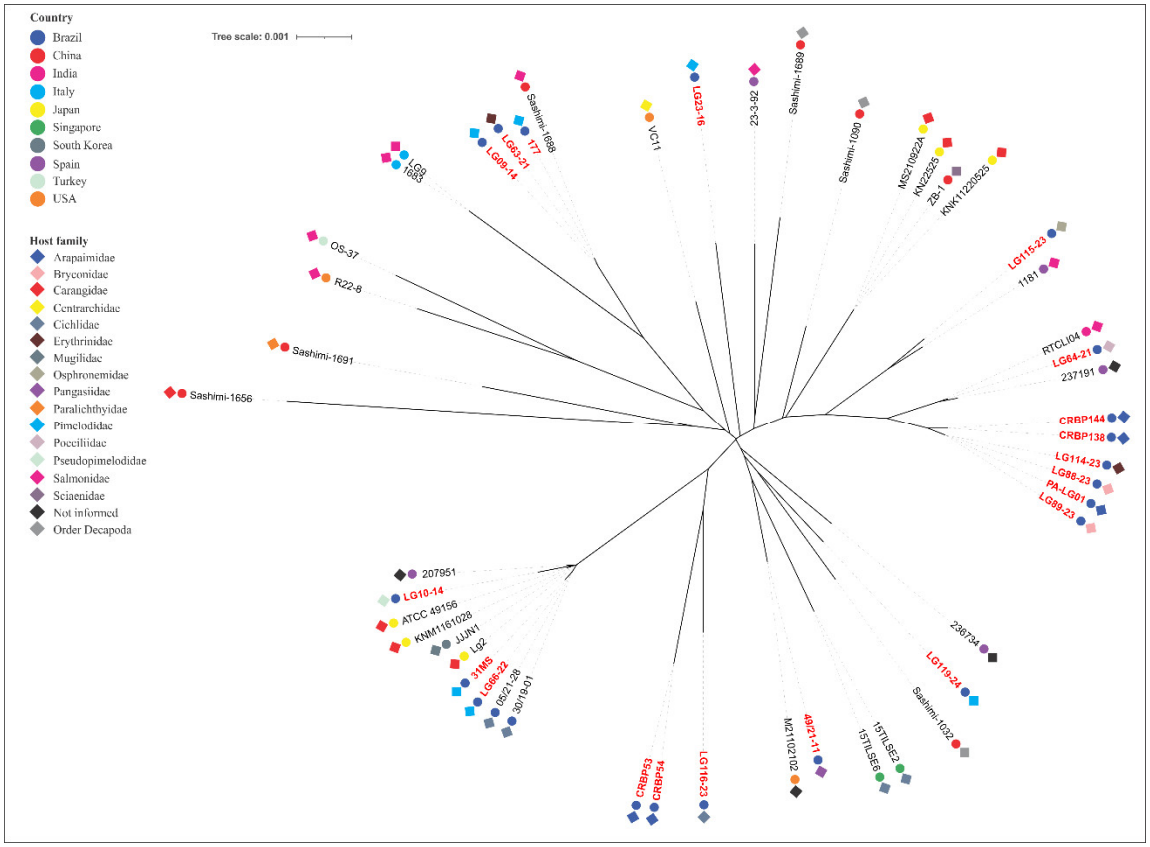

Figure 4. Phylogenetic tree of *Lactococcus garvieae* strains. The unrooted phylogenetic tree was constructed using the neighbor-joining method with the Tamura-Nei model from the 5,713 bp concatenated DNA sequences of the seven loci (*als*, *atpA*, *tuf*, *gapC*, *gyrB*, *rpoC* and *galP*) and a bootstrap analysis of 1,000 replicates to determine the evolutionary relationships among *L. garvieae* strains obtained from aquatic animals. The isolate's name in red denotes strains from this study. The colors of the circles and diamonds indicate the isolate's country of origin and host origin, respectively.

The *L. petauri* strains clustered into nine distinct phylogenetic groups. The Brazilian isolates that were not from tilapia were distributed among five of these clusters, with the majority (82.1%) forming a single predominant cluster. The analysis revealed genetic divergence between these isolates and those obtained from disease outbreaks in rainbow trout in Europe (ST14), the United States (nST145) and Mexico (nST145). Notably, AM-LG02 and AM-LG03 strains clustered with ST47 and ST24 isolates, respectively, which originated from disease outbreaks in Brazilian tilapia farms (Figure 5).

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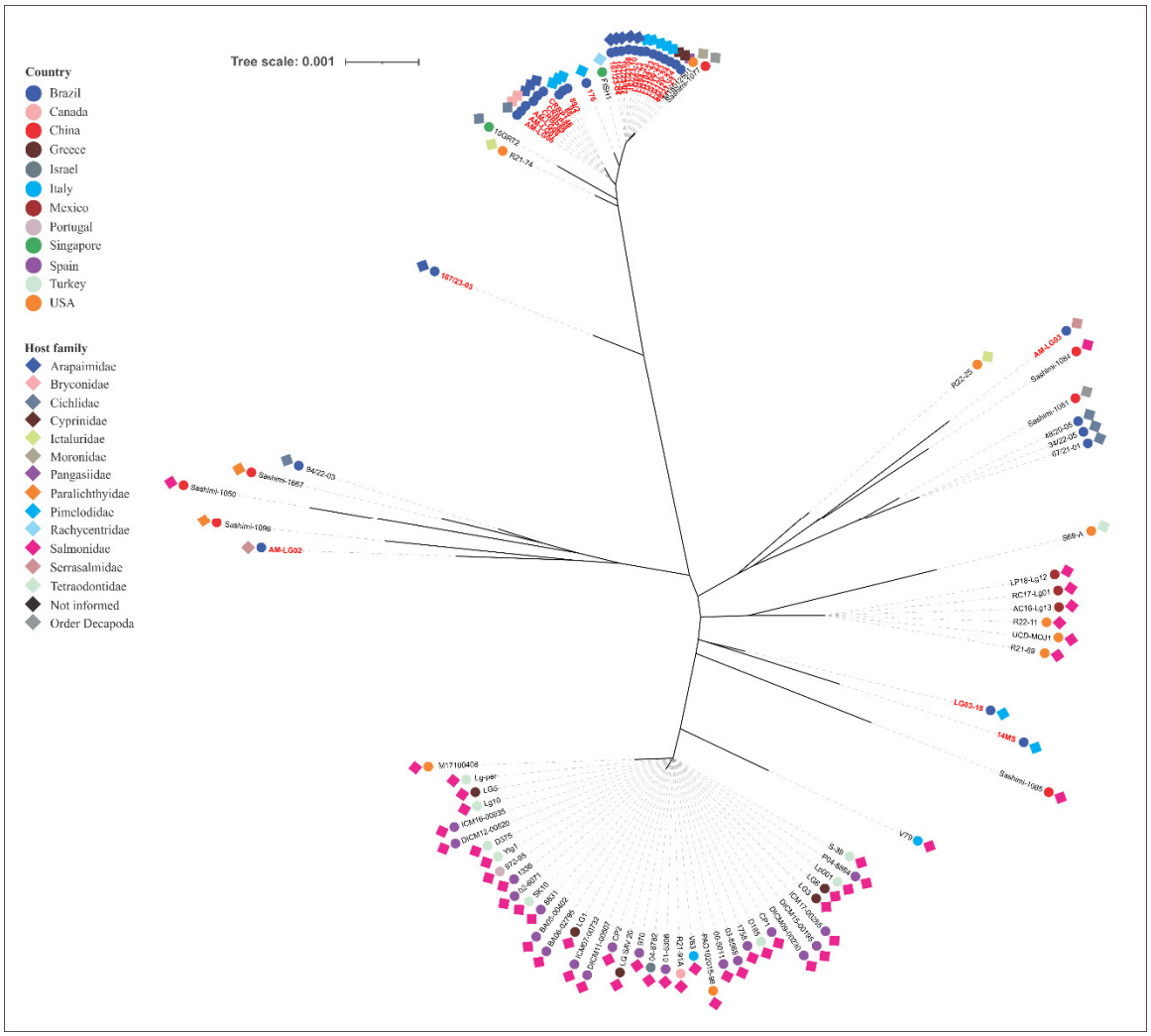

Figure 5. Phylogenetic tree of *Lactococcus petauri* strains. The unrooted phylogenetic tree was constructed using the neighbor-joining method with the Tamura-Nei model from the 5,713 bp concatenated DNA sequences of the seven loci (*als*, *atpA*, *tuf*, *gapC*, *gyrB*, *rpoC*, and *galP*) and a bootstrap analysis of 1,000 replicates to determine the evolutionary relationships among *L. petauri* strains obtained from aquatic animals. The isolate's name in red denotes strains from this study. The colors of the circles and diamonds indicate the isolate's country of origin and host origin, respectively.

Discussion

The present study investigated the population structure and genetic profile of a set of LCB strains obtained from different fish species in Brazil, using MLST as the genotyping method. Based on sequences deposited in the PubMLST database for *L. garvieae*—including the isolates reported in this study—80 STs were assigned to isolates derived from aquatic animals (Figure 2), which include isolates from both clinical disease cases, stool samples and fish meat products. Among these, 18 STs belong to *L. formosensis*, 29 to *L. petauri* and 33 to *L. garvieae* (Table 3), highlighting the genetic heterogeneity among these bacterial species.

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Table 3. Number of aquatic animal-derived sequence types identified in this and previous studies, categorized by bacterial species.

Bacterial species	ST in aquatic animals/ST total[a]	STs identified in this study	STs identified in other studies
L. formosensis	18/39	ST20, nST166, nST168, nST174. nST178, nST179	ST5, ST41, ST43, ST56, ST59, ST113, ST114, ST115, nST140, nST141, nST150, nST151
L. garvieae	33/55	ST4, ST6, ST46, ST105, ST122, nST164, nST165, nST167, nST169, nST170, nST171, nST173, nST176, nST180	ST1, ST13, ST16, ST17, ST39, ST62, ST63, ST95, ST109, ST119, ST120, ST121, ST123, ST124, ST139, nST144, nST147, nST157, nST158
L. petauri	29/85	ST25, ST29, ST35, ST61, ST152, nST172, nST175, nST177, nST181	ST10, ST14, ST15, ST24, ST34, ST47, ST57, ST98, ST128, ST132, ST133, ST134, ST135, ST136, ST137, ST138, nST142, nST145, nST146, nST149
Lactococcus ssp.[b]	0/2	-	-
Total	80/181	29/181	51/181

[a] Proportion of sequence types identified from aquatic animal isolates relative to the total number of STs deposited in PubMLST. [b] Strains currently classified as *Lactococcus garvieae* in PubMLST database but shown by genomic analysis to represent a distinct, yet taxonomically uncharacterized *Lactococcus* species [37].

When the MLST scheme was first developed by Ferrario et al. [22], all the isolates were believed to belong to *L. garvieae*, revealing two distinct genetic populations within the analyzed collection of strains. Subsequent studies, using strains from diverse sources (human, animal, food and environmental), identified a wide range of STs, which indicates the genetic heterogeneity of *L. garvieae* [23,24,34,35]. However, a study conducted in 2017 redefined the *L. garvieae* subgroup A as a new species, named *L. petauri*, and suggested the reassignment of previously characterized isolates [46]. Consequently, various studies have been conducted to improve the speciation within the genus *Lactococcus* [19,37,47]. It was only after 2023 that the first studies using the MLST approach to differentiate genetic profiles among LCB species were published [3,6,25,26], demonstrating high and comparable genetic diversity within each species, based on isolates from both human and animal sources [26]. During this same period, our research team constructed the *L. garvieae* MLST scheme in the PubMLST database. Since then, we have curated all the newly deposited sequences—including alleles, isolates and genomes—to ensure standardized nomenclature for major STs and CCs, integrating and consolidating data from LCB strains, and providing a comprehensive analysis of their genetic and epidemiological characteristics. Thus, by sequencing the seven housekeeping genes of our isolates and utilizing the PubMLST database (accessed 5 August 2025), it was possible to compare the population structure and phylogenetic relationships of *Lactococcus* spp. strains obtained from fish in Brazil, with those of other countries.

Our results demonstrate that the LCB isolates from the fish belong to 11 previously established STs (*L. formosensis*: ST20; *L. garvieae*: ST4, ST6, ST46, ST105 and ST122; *L. petauri*: ST25, ST29, ST35, ST61 and ST152). ST4 and ST122 were previously identified in animal-derived products, including fish meat, from China, Italy and Spain [22,26]. Notably, ST4, ST20, ST29 and ST105 have been associated with human diseases in China, Singapore, Spain and the United States [22,23,26,35]. Additional epidemiological findings include: ST6 reported in vegetable isolates from Italy; ST61 detected in water samples from Spain; ST25, ST35 and ST152 identified in human and swine fecal samples from China and Spain [22,23]. Among the previously reported STs, only ST6 and ST46 have

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been found in diseased fish, in the United States and Brazil, respectively [3,37]. The high values of the SDI show a considerable genetic divergence among the isolates evaluated, with *L. formosensis* and *L. garvieae* being a heterogeneous population and *L. petauri* a more homogeneous population.

It is important to mention that when evaluating the ancestry of the isolates through CC analysis, no cluster comprising three or more STs formed exclusively by isolates from aquatic animals was observed (Figure 2). Our results suggest that, regardless of the bacterial species evaluated, LCBs tend to be adapted to multiple hosts.

L. garvieae CC4, which groups ST4 (LG09-14 and LG63-21 strains) and ST122 (177 strain) identified in this study, appears to be associated with isolates from animal-derived products, particularly samples originating from the European continent [26]. Nonetheless, ST13, which also belongs to this CC, includes isolates from rainbow trout in Italy [22,37]. On the other hand, *L. garvieae* CC17 appears to harbor more STs (ST16, ST17, ST46, and ST139) associated with clinical manifestations of disease in fish [3,22,23,25]. This corroborates results from our study, as 31MS (nST180) and LG66-22 (ST46) strains, isolated from diseased *Pseudoplatystoma fasciatum* and *Phractocephalus hemioliopterus*, respectively, grouped within this CC. A previous study assessed the pathogenicity of the 31MS strain through experimental infection (10^7 CFU/fish) in *Pseudoplatystoma* spp. [48]. During the 21-day monitoring period, 10.6% of the animals exhibited clinical signs of diseases; however, no mortality was observed. Conversely, the LG66-22 strain belongs to the same ST identified in disease outbreaks affecting Nile tilapia in Brazil in 2019 and 2021 [3]. Since the pathogenicity of this specific ST has not been evaluated, future laboratory controlled challenges comparing the susceptibility of Nile tilapia and *Phractocephalus hemioliopterus* is warranted to better understand the pathogenicity of this ST. Finally, *L. garvieae* CC62 includes isolates from fish in India and Spain (ST62 and nST157) [37], and is grouped with a strain from the current study, LG64-21 (nST176), which was obtained from an ornamental fish species. Although a few LCB isolates from ornamental fish were included in this study, the two *L. garvieae* strains possess different STs, demonstrating no clear association between STs and host origin or geographical source.

L. formosensis CC5 is also predominantly associated with isolates from animal-derived products, including fish meat from China (ST5 and ST113) [26]. In the current study, we did not identify any isolates belonging to this CC.

L. petauri CC24 comprises isolates associated with diseases in fish (Nile tilapia – ST24; catfish – nST142) and humans (ST24), as well from human (ST24) and swine (ST155) feces [3,23,35,37]. ST24 has been the predominant genetic profile among *L. petauri* isolates obtained from Nile tilapia in different types of commercial production and different geographic regions in Brazil between 2020 and 2022, and its pathogenicity and high virulence for this aquatic host were confirmed [3]. Interestingly, none of the isolates evaluated in this study shared this ST or belonged to CC24, suggesting that these isolates may have emerged from a distinct ancestor. On the other hand, *L. petauri* CC29 clustered isolates from diverse sources and was the largest CC identified in this study. CC29 clustered isolates from human feces [22,23], fish (cobia and European seabass) and prawn sashimi, such as ST10, ST128 and ST135 [26,35,37]. A total of 22 out of 28 *L. petauri* strains from our study belong to this CC, indicating that isolates of this bacterial species tend to have a more homogeneous genetic profile compared to the other two bacterial species investigated. Other studies utilizing different genotyping methods (DNA fingerprinting approaches) also revealed a more homogeneous population for *L. petauri* strains [19]. Finally, *L. petauri* CC97 contains isolates linked to diseases in fish (ST98 and nST145) and fish meat (ST137) [26,37]. Among these, nST145 has been associated with major mortality outbreaks in rainbow trout in the United States and Mexico between 2016 and 2020 [9,49]. No isolate from this study grouped within this CC.

Other genetic relationships were also identified via goeBURST analysis. For *L. petauri*: ST27 (human feces, Spain) and ST61 (LG03-18 strain); ST47 (Nile tilapia, Brazil) and nST181 (14MS strain); ST53 (bovine mastitis, Spain) and nST177 (AM-LG03 strain); ST34 (red tilapia, Singapore) and ST82 (human feces, China). For *L. garvieae*: nST165 (LG88-23, LG89-23 and PA-LG01 strains) and nST173 (CRBP138 and CRBP144) from Amazonian fish species; ST39 (tilapia, Singapore) and ST50 (bovine

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mastitis, Spain). And for *L. formosensis*: ST41 (carp, Singapore) and ST59 (fish, Spain); ST91 (bovine mastitis, China) and ST151 (barramundi, USA) [25,35,37]. Furthermore, our study observed that many isolates were singletons (lacking a common ancestor with other isolates), underscoring the significant genetic heterogeneity of these bacteria. Despite this, there are currently 181 STs deposited in the PubMLST database. In the future, with the addition of more isolates and allelic profiles, new population structure relationships among LCBs may be revealed.

Phylogenetic analysis of the concatenated housekeeping genes was used to reconstruct the evolutionary relationships among the strains of the tested bacterial species. As expected, the analysis enabled the grouping of strains within the same CC. However, it also revealed arrangements that represented double- or triple-locus variants. The analysis revealed that our *L. formosensis* strains are closely related to others obtained from largemouth bass (nST140 and nST141), barramundi (nST151), and rainbow trout (nST150) in the USA; from a fish with no designated species in Singapore (ST43); and from salmon (ST113) and flounder sashimi (ST5) in China [26,35,37]. The *L. garvieae* strains are related to those obtained from salmon (ST122) and prawn (ST119) sashimi (China), rainbow trout (ST62, India; ST63, Spain), unspecified fish species (ST157 and ST158, Spain; ST6, USA), tilapia (ST39, Singapore; ST46, Brazil), yellowtail (ST16 and ST17, Japan), and so-iuy mullet (ST17, South Korea). This broad host range demonstrates a lack of host specificity and no clear phylogenetic distinction based on geographic origin. Conversely, the *L. petauri* strains demonstrated a more intriguing genetic relationship. Most of our isolates (those related to CC29) are genetically linked to other isolates obtained from tilapia (ST34, Singapore), cobia (ST10, Singapore), hybrid catfish (nST142, USA), European seabass (ST128, USA) and prawn sashimi (ST135, China) [26,35,37]. Isolates from clinical cases of piscine lactococcosis in trout were divided into two distinct phylogenetic clades: one associated with ST14, ST57 and nST146, identified primarily in European countries (with a single representative from the USA and Canada), and another clade associated with nST145, which, as previously mentioned, is linked to recent and impactful outbreaks in North America. This division presents a strong geographical signal of diversification among trout isolates. Our isolates are not phylogenetically related to these clades, indicating they evolved from different ancestors. In contrast, two of our *L. petauri* strains (AM-LG02 and AM-LG03), despite some phylogenetic distance, share a common ancestor with isolates associated with disease outbreaks in Nile tilapia in Brazil. Both isolates were obtained from the intestine of *Colossoma macropomum*. In an experimental infection study in this same aquatic host, the AM-LG02 strain did not cause any macroscopic or microscopic alterations in the challenged animals [50]. Therefore, future studies should use this and other LCBs isolates in challenge experiments with tilapia to verify their pathogenic potential for this species.

In conclusion, this study provides new insights into the genetic diversity of Brazilian *Lactococcus* spp. strains isolated from different fish species, using an MLST approach. The analysis revealed that LCB isolates constitute a genetically diverse population based on their STs. Specifically, *L. garvieae* and *L. formosensis* exhibited greater heterogeneity compared to *L. petauri*, for which the majority of isolates belonged to a single clonal complex (CC29). MLST and phylogenetic analysis demonstrated genetic relatedness between the *L. garvieae* and *L. formosensis* isolates from this study and those from other aquatic animal species deposited in the PubMLST database. Regarding the *L. petauri* strains, all the STs identified in this study were unrelated to the MLST lineages responsible for outbreaks in Brazilian Nile tilapia and North American rainbow trout. This suggests that piscine *L. petauri* populations in the Americas evolved from distinct ancestral origins. However, phylogenetic analysis and MLST data showed that, although they belong to different CC, two isolates from *Colossoma macropomum* are genetically closely related to isolates from Nile tilapia in Brazil. Therefore, future studies, particularly those employing a whole-genome sequencing approach, are necessary to better elucidate the ancestral relationship between these strains.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. **Table S1**: *Lactococcus* ssp. strains isolated from aquatic animals with publicly available genome sequences in GenBank databases included in this study. **Table S2**: *Lactococcus* spp. strains

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isolated from diverse animal aquatic species with previously reported alleles and sequence type included in this study to phylogenetic analyses.

Author Contributions: GCT, FP, SUG, CAGL and HCPF conceived and designed the experiments. SPC, ACCB, AECdR, HCM, CRMdSM, HLC, RCE performed the microbiological analyses, DNA extraction, and PCR amplification. JCCR conducted the Sanger sequencing. FLP developed the *L. garvieae* MLST scheme for inclusion in PubMLST database. GCT and LFFN performed analyses and visualization of the data. GCT wrote the manuscript and coordinated all analyses of the project. FLP, FP, ES, CAGL and HCPF contributed substantially to data interpretation and to revisions of the manuscript. All authors read, critically reviewed, and approved the final manuscript.

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