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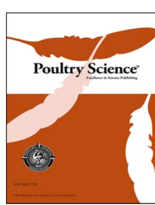

Full-Length Article

Distinct phenotypes of salivaricin-producing *Ligilactobacillus salivarius* isolated from the gastrointestinal tract of broiler chickens and laying hens

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ABSTRACT

Ligilactobacillus salivarius harbors bacteriocin genes in its *repA*-type megaplasmid, specifically salivaricin P (**salP**), a class IIb bacteriocin. This study aimed to differentiate 25 *salP*-positive *Lig. salivarius* strains isolated from the gastrointestinal tract (**GIT**) of broilers and laying hens. Results showed that 12 isolates were classified as Type A, with active bacteriocins, while the rest were Type B, with no active bacteriocins. *In vitro* and *in silico* characterization of salP bacteriocins revealed narrow-spectrum antibacterial activity against *Listeria monocytogenes* and *Enterococcus faecalis*. SalP bacteriocins were predicted as positively charged, hydrophobic, small molecular weight (α, 4.097 kDa; ß, 4.285 kDa) bacteriocins with characteristic GXXXG motif. Investigation of the salP gene cluster based on genomic data revealed that Type B strains lacked the *lanT* and *hlyD* genes that encode export proteins dedicated to the modification and extracellular transport of mature salP peptides. However, two Type B strains (B4311 and B5258) showed inhibitory activity against *L. monocytogenes* ATCC19114. Multiplex PCR analysis and synteny mapping analysis revealed that B4311 and B5258 strains harbored the *lanT* gene, highlighting the importance of LanT protein in the cleavage of leader peptide and excretion of mature peptides. Further analysis revealed that the resistance of Type B strains to salP was attributable to the presence of a dedicated immunity protein, blurring the evolutionary significance of producing active bacteriocins for competitive advantage. Additionally, the loss of export proteins occurred in a polyphyletic manner, consistent with the genetic plasticity of the *repA*-type megaplasmid. This suggests that the loss of *lanT* and *hlyD* is likely in the presence of limited nutritional competitors. In conclusion, the observed differences in salivaricin production of *Lig. salivarius* exist independent of isolation host and that Type A and Type B strains can coexist in the same environment. Finally, the functional characterization of active salP allows for a better understanding of its potential to control specific bacteria in human food and animal production.

Introduction

Ligilactobacillus salivarius (formerly *Lactobacillus salivarius*) are Gram-positive, facultative-anaerobic, non-spore-forming, homofermentative lactic acid bacteria (Oberg et al., 2022; Oren and Garrity, 2020). They are commensal bacteria commonly found in the oral cavity, gastrointestinal tract (**GIT**), and urogenital tract of humans and animals (Guerrero Sanchez et al., 2022; Yang et al., 2024; Zheng et al., 2020). Certain strains of *Lig. salivarius* exhibit probiotic qualities and are widely acknowledged to have beneficial effects on the GIT health by regulating the immune system and inhibiting harmful bacteria by producing antimicrobial compounds (Gupta et al., 2021; Quilodran-Vega et al., 2020; Yao et al., 2021). For example, *Lig. salivarius* CGMCC17718 (Yang et al., 2023) and *Lig. salivarius* Erya strain (Chen et al., 2022) were reported to improve the productive performance of broilers by modulating the gut microbiota and improving antioxidative capacity, and protecting against aflatoxin B1 infection, respectively. Furthermore, the genus is commonly used as a non-starter ingredient, particularly in the biopreservation of meat products directed towards the control of *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* (Barcenilla et al., 2022).

In terms of probiotic safety, *Lig. salivarius* was assigned a Qualified Presumption of Safety (**QPS**) status by the European Food Safety Authority (EFSA Panel on Biological Hazards) (BIOHAZ et al., 2023). Numerous strains with various beneficial effects on animal health, such as antibacterial activity, immune stimulation, and regulation of the microbial community in the GIT, have been reported (Jiang et al., 2022; Yadav and Chauhan, 2022; Zamojska et al., 2021). Moreover, *Lig. salivarius* has potential applications for microbial control in food and

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disease models (i.e., inflammatory bowel disease, periodontitis, and bacterial infections) as well as for promoting animal production (Yang et al., 2024). Additionally, the production of bacteriocin by *Lig. salivarius* will be beneficial as an alternative to conventional antibiotics, given the growing concerns regarding the emergence of multidrug-resistant bacteria. Moreover, the good auto-aggregation capacity of *Lig. salivarius*, which demonstrates resistance to gastrointestinal conditions, is crucial for probiotic applications. However, only a limited number of strains have been used for industrial applications (Barcenilla et al., 2022; Li and Gänzle, 2020). This limitation is brought about by the need to fully understand the role of these potential probiotic candidates, necessitating further research and characterization of these strains, including their bacteriocin production.

Bacteriocins, which are ribosome-synthesized proteins or peptides with inhibitory activities against related taxa, are gaining attention as potential alternatives to antibiotics in various industries (Darbandi et al., 2022; Perez et al., 2022). Several *Lig. salivarius* strains have been reported to produce the two-peptide bacteriocin salivaricin P (**salP**), which was originally discovered from intestinal isolates (strain UCC118) and is highly active against the pathogenic *L. monocytogenes* (Barrett et al., 2007). The genetic component for salP has been found in the *repA*-type megaplasmid (Barrett et al., 2007; Han et al., 2023), and was observed to be a common feature exclusive to this species (Abramov et al., 2023; Harris et al., 2017). Bacteriocins have been used in diverse food and feed systems following extensive characterization and genetic investigation because of their effectiveness and safety (Bastos et al., 2015; Kjos et al., 2011). They can be used as purified or semi-purified additives, bacteriocin-based compounds derived from fermented foods, or via bacteriocin-producing starter cultures (Schillinger et al., 1996; Sobrino-López and Martín-Belloso, 2008). To date, nisin and pediocin PA-1 are the only commercially available bacteriocins licensed for biopreservative applications by the Food and Drug Administration (**FDA**) (Siddiqui et al., 2023; Sobrino-López and Martín-Belloso, 2008). Therefore, it is crucial to accurately and clearly characterize new bacteriocins in terms of safety and functionality using traditional *in vitro* characterization and recent bioinformatic techniques, as this will contribute to the expanding repertoire of antimicrobial alternatives. Furthermore, it's important to take into account the compatibility between the strain and its host, as a strain naturally adapts to its isolated environment. Thus, the coevolution of the probiotic candidate and its host may provide key evidence on the factors affecting the successful colonization of the target niche and subsequent manifestation of the beneficial effects on the host (Johnson et al., 2023).

In the present study, 25 strains of *Lig. salivarius* harboring the structural *salP* genes, isolated from the GIT of broilers and laying hens, were studied. The genetic architecture of the *salP* gene cluster was reconstructed to identify the genes involved in the production of active bacteriocins. The evolutionary importance of salP production was also inferred through the combined phylogenetic analysis and the reconstructed *salP* operon of *Lig. salivarius* strains. Finally, the active salP bacteriocins were characterized based on temperature and pH stability, and spectrum of activity to provide valuable insights on how salP-producing *Lig. salivarius* can be utilized with a focus on the potential biopreservation of food materials and antimicrobial application in poultry.

Materials and methods

Isolation and screening of Ligilactobacillus salivarius

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (**IACUC**) of Chung-Ang University. A total of 233 *Lactobacillus* spp. was isolated using *Lactobacillus* selective agar (BD Difco) from the GIT of Ross 308 broiler chickens (n = 8) and Hy-Line Brown laying hens (n = 6), collected over five different timepoints. The strains were identified by 16S rRNA gene sequencing using 27F (5′- AGA GTT TGA TCC TGG CTC AG -3′) and 1492R (5′- GGT TAC CTT GTT ACG ACT T -3′) universal primers. The *salP*-positive *Lig. salivarius* isolates (n = 25) were classified by amplifying the *salP* structural genes using the primers for Type A (F: 5′- GCA CTC GAG AAA AGA AAA CGT TAT CCT AAT -3′; R: 5′- GTC ATC TCT AGA TTA ACG ACA ACT TGC AAA -3′) and Type B (F: 5′- TCG CAT ATG ATG AAG GAA TTT AC -3′; R: 5′- TGC CTC GAG ATG GCA ACT TGC AAA TC -3′).

The isolates were routinely cultured in 5 mL de Man, Rogosa, and Sharpe (**MRS**) broth (BD Difco, Sparks, MD, USA) supplemented with 0.05 % L-cysteine (**cys-MRS**) (Sigma-Aldrich, Steinheim, Germany) and incubated at 37°C for 24 h. The cell-free culture supernatant (**CFS**) of each cultured strain was prepared via centrifugation at 8000 × *g* and 4°C for 10 min, followed by filtration through a 0.45 μm syringe filter (Pall Corporation, New York, USA) to completely remove all cells (Elnar and Kim, 2024). Bacteriocin activity was determined via the spot-on-lawn assay (Han et al., 2014) using the CFS of isolates against *L. monocytogenes* ATCC19114 grown on Tryptic soy agar (**TSA**, BD Difco). Strain classification was complemented by inhibitory activity against the test organism (Table 1).

Bacteria, growth media, and culture conditions

Lig. salivarius strains were routinely cultured in cys-MRS at 37°C. All test organisms used in this study and their respective culture conditions are listed in Table 2. All strains were cultured twice in their respective culture media and incubation conditions prior to the experiment. Glycerol stocks were kept in 10 % skim milk + glycerol (3:1, v/v) and stored at -80°C.

Antimicrobial activity

Representative strains of *Lig. salivarius* (B4112, B4311, B5258, B5121, and L5301) were selected to evaluate the inhibitory activity of salP against the test organisms listed in Table 2. Briefly, the *Lig. salivarius*

Table 1
Origin and respective typing of *Ligilactobacillus salivarius* based on *salP* amplification and inhibitory activity against *Listeria monocytogenes* ATCC19114.

			Salivaricin P		
	Strain	Isolation Host	Type	Activity[a]	Reference
1	L4301	Laying Hen	A	+	This study
2	L4049	Laying Hen	A	+	This study
3	L5306	Laying Hen	A	+	This study
4	L5304	Laying Hen	B	-	This study
5	L4072	Laying Hen	A	+	This study
6	B4112	Broiler	A	+	This study
7	B4206	Broiler	A	+	This study
8	B4210	Broiler	A	+	This study
9	B4304	Broiler	A	+	This study
10	B4305	Broiler	A	+	This study
11	B4307	Broiler	A	+	This study
12	B4404	Broiler	A	+	This study
13	B4311	Broiler	B	+	(Han et al., 2023)
14	B5102	Broiler	B	-	This study
15	B5208	Broiler	B	-	This study
16	B5337	Broiler	B	-	This study
17	B5258	Broiler	B	+	This study
18	B5269	Broiler	B	-	This study
19	B5121	Broiler	B	-	This study
20	L5213	Laying Hen	B	-	This study
21	L5301	Laying Hen	B	-	This study
22	L5302	Laying Hen	B	-	This study
23	L5204	Laying Hen	B	-	This study
24	L5322	Laying Hen	B	-	This study
25	L3302 N	Laying Hen	A	+	This study
26	GJ-24	Human (Adult)	A	+	(Cho et al., 2011)

[a] Activity is expressed in terms zone of inhibition (ZOI); +, positive and -, negative.

Table 2
Activity spectrum of *Ligilactobacillus salivarius* bacteriocins.

Bacterial Strain	Growth conditions[a]	Activity[b]
Streptococcus mutans KCTC5356	BHI, 37°C	-
Strep. mutans KCTC3065[c]	BHI, 37°C	-
Staphylococcus aureus ATCC33591	LB, 37°C	-
Lactococcus lactis subsp. *lactis* DOME6301	cys-MRS, 37°C	-
Ligilactobacillus salivarius GJ24	cys-MRS, 37°C	-
Lig. salivarius 301	cys-MRS, 37°C	-
Lactiplantibacillus plantarum KCTC3018	cys-MRS, 37°C	-
Lactobacillus acidophilus 214	cys-MRS, 37°C	-
Lacticaseibacillus casei MCL	cys-MRS, 37°C	-
Lcb. casei ATCC9029	cys-MRS, 37°C	-
Lcb. casei ATCC2782	cys-MRS, 37°C	-
Enterococcus faecalis CAUM157	cys-MRS, 37°C	+
Listeria monocytogenes ATCC19111	TSB, 37°C	+
L. monocytogenes ATCC19114	TSB, 37°C	+
L. monocytogenes ATCC19115	TSB, 37°C	+

[a] BHI, Brain-Heart Infusion media (BD Difco); LB, Luria-Bertani media (BD Difco); TSB, Tryptic Soy Broth (BD Difco).
[b] Activity is expressed in terms of the presence of visible zone of inhibition (ZOI); +, positive and -, negative.
[c] Type strain of the species.

strains were cultured in cys-MRS at 37°C overnight. CFS was prepared as described previously and divided into two equal volume fractions. One fraction was neutralized with 1 N NaOH, while the other was left untreated. The spot-on-lawn assay was performed by spotting 10 μl of the untreated and neutralized CFS onto the lawn of test organisms. The activity was determined based on the presence of an inhibition zone. Bacteriocins from *Lig. salivarius* GJ-24 and *Enterococcus faecalis* CAUM157 served as reference controls.

Multiplex PCR analysis

A multiplex PCR analysis for salP core peptides (α and ß chains) and export proteins (LanT and HlyD) genes was performed using the primers listed in Table 3. The primers were designed using the conserved consensus sequences of the salP core peptides and two export proteins (LanT and HlyD) derived from the NCBI BLASTn search. PCR was conducted using the H-star Taq polymerase (BioFact, Daejeon, South Korea) with the following temperature ramp: initial denaturation (95°C, 15 min), 30 cyc les of denaturation (95°C, 1 min), annealing (55°C, 1 min), and elongation (72°C, 1 min), and final elongation (72°C, 10 min). The amplicons were electrophoresed on a 1 % agarose gel with EcoDye Nucleic Acid Staining Solution (BioFact) at 100 V for 20 min and viewed under UV light. The 1 kb Plus DNA Ladder (BioFact) was used as a size marker.

Genomic and phylogenetic analyses

Five representative strains (B4112, B4311, B5258, B5121, and L5301) were selected to investigate the genetic architecture of the *salP* gene cluster. The BAGEL4 online tool (http://bagel4.molgenrug.nl) was used to detect the putative *salP* gene in the sequence data of the strains (van Heel et al., 2018). The resulting bacteriocin gene clusters were subjected to synteny mapping analysis using ProgressiveMauve v. snapshot-2015-02-25 (Darling et al., 2010). The *salP* gene cluster was then reconstructed for Type A and Type B strains based on the results of synteny mapping and multiplex PCR.

A phylogenetic tree was constructed using the MEGA 11 software (v. 11.0.13) to infer the evolutionary relevance of export proteins. The 16S rRNA gene, three housekeeping genes (*gyrB, rpoB,* and *groEL*), and the megaplasmid marker genes *repA* and *parA* of the 20 strains identified in Fig. 4 were obtained from NCBI through a BLASTn search, selected based on the availability of gene markers used. The BLASTn search set was limited to *Ligilactobacillus salivarius* (taxid: 1624) and optimized for highly similar sequences (Megablast). A maximum likelihood tree, with bootstrap analysis of 1000 replicate datasets, was created from concatenated sequences of housekeeping genes and megaplasmid marker genes to visualize the evolutionary relationship of selected *Lig. salivarius* strains. *Clostridium perfringens* B20 was selected as an outgroup. Putative genes associated with the *salP* gene cluster (putative immunity protein, *comC* 1, *salP*A, *salP*B, *hisK, abpR, abpIM, comC* 2, *lanT* 1, *lanT* 2, *lanT* 3, and *hlyD*) were searched against the strains of interest using BLASTn. A heatmap based on the percent identity (% ID) of BLAST hits was created using GraphPad Prism (v. 9.5.1) and merged with the tree map to correlate the genetic architecture of the *salP* operon with the evolutionary lineage of the species.

In silico and in vitro characterization of Salivaricin P

The amino acid sequences of salP peptides were used for *in silico* predictions of their physicochemical properties using the ProtParam online tool (https://web.expasy.org/protparam/) (Gasteiger et al., 2005). The tertiary structure of each chain was determined *ab initio* using AlphaFold2 v. 1.5.5 (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb) (Jumper et al., 2021). The resulting structure was validated using the Ramachandran plot in SAVES v. 6.0 (https://saves.mbi.ucla.edu) (Laskowski et al., 1993). All PDB files were visualized using USCF Chimera v. 1.16 (Pettersen et al., 2004).

The physicochemical characteristics of salP, including stability under various temperature and pH conditions as well as sensitivity to proteases, were also investigated. Briefly, the neutralized CFS was exposed to various temperature (-80, -20, 4, 37, 60, 80, and 100°C for 30 min, and 121°C for 15 min) and pH (2 to 10; one-unit increments) conditions and treated with hydrolytic enzymes (proteinase K, pepsin, lipase, and catalase; 5 mg/mL) (Hwang et al., 2018). The residual bacteriocin activity was determined using a spot-on-lawn assay against *L. monocytogenes* ATCC19114. Untreated neutralized CFS was used as the control. All assays were performed in triplicate. Statistical analysis was performed using one-way ANOVA test with Dunnett's multiple comparison test (α, 0.05) using GraphPad Prism (v. 9.5.1).

Table 3
Primer sequence used for multiplex PCR amplification of salivaricin P genes.

Primer[a]	Target Gene	Product Size (bp)	Sequence (5′ – 3′)	Tm[b] (°C)
Type A (F)	*salP* Type A	425	GCACTCGAGAAAAGAAAACGTTATCCTAAT	62.7
Type A (R)			GTCATCTCTAGATTAACGACAACTTGCAAA	62.7
Type B (F)	*salP* Type B	425	TCGCATATGATGAAGGAATTTAC	55.3
Type B (R)			TGCCTCGAGATGGCAACTTGCAAATC	64.8
LanT (F)	*LanT*	806	GACCACAAGTAGATGAATCTG	55.9
LanT (R)			CCAGGAGCATCTATAATCTTAC	56.5
HlyD (F)	*HlyD*	1119	CGCATATGGAAGATAAATTTTTAG	54.2
HlyD (R)			GAGCTCAAAAAATGTTGTTTTC	52.8

[a] F, forward primer; R, reverse primer.
[b] Tm, melting temperature.

Results

Isolation of bacteriocinogenic *Ligilactobacillus salivarius*

A total of 25 *Lig. salivarius* strains, isolated from the GIT of chickens, harbors the structural gene for salivaricin P bacteriocin. Based on PCR analysis of *salP*, 12 isolates were classified as Type A, while the rest were Type B. All Type A strains inhibited the growth of *L. monocytogenes* ATCC19114. Among the 13 Type B strains, B4311 and B5258 exhibited antimicrobial activity against the test organism, and the other 11 strains showed no inhibitory activity. The control Type A strain, *Lig. salivarius* GJ-24 (Cho et al., 2011), also inhibited the test organism.

Reconstruction of Salivaricin P Operon

Multiplex PCR analysis and synteny mapping of bacteriocin operons from B4112, B4311, B5258, B5121, and L5301 strains revealed evident differences in the genetic architecture between Type A (active bacteriocins) and Type B (inactive bacteriocins). Multiplex PCR analysis was performed with the specific primers (Table 3). The primary difference between Type A and Type B strains lies in the presence of *lanT* and *hlyD* genes, as depicted in Fig. 1. All Type A strains showed bands corresponding to the three target genes, whereas Type B strains only showed bands corresponding to the *salP* core peptides. The *salP* operon was reconstructed from the available sequence data of B4112, B4311, B5258, B5121, and L5301 strains using the BAGEL4 online tool. Generally, Type B strains lack the export protein genes, except for strains B4311 and B5258, which showed an open reading frame (**ORF**) corresponding to *lanT*. This observation was consistent with the multiplex PCR analysis.

The nucleotide sequences of B4311 and B5258 *lanT* were highly similar to those of B4112 (99.91 % sequence homology), except for three differences: 222T>C, 576A>G, and 739C>T. The observed differences resulted in two missense substitutions, 193Ile>Val and 247Thr>Ile. Synteny mapping of the *salP* operon was also conducted to assess overall sequence homology among the five tested strains. The *salP* operon for Type A and Type B strains was reconstructed as depicted in Fig. 2. The type A *salP* operon showed 15 potential ORFs with genes encoding core peptides, immunity, regulation, leader sequence cleavage, and export. In contrast, Type B only showed nine ORFs, lacking the two export proteins (*lanT* and *hlyD*) and several ORFs upstream until the *abpIM* gene (Fig. 3).

Phylogenetic analysis

Three distinct clusters correlated with the isolation source (pig, chicken, or human). Cluster 1 primarily comprised strains isolated from pigs (JCM 1046, ZSA5, H1, BNS11, and SS-258); cluster 2 consisted of human isolates (UCC118, LPM01, AR612, CECT 5713, and 2102-15); and cluster 3 consisted of strains from chickens (CICC23174, B4311, and SNK-6), humans (VHProbi A17, AR809, and GJ-24), and other vertebrates. The phylogenetic analysis of 20 *Lig. salivarius* strains suggested that the absence of *lanT* and *hlyD* export proteins does not correspond to the evolutionary lineage of the species (Fig. 4).

Activity spectrum

The spectrum of activity of salP bacteriocins was determined using the spot-on-lawn antimicrobial assay. Neutralized salP showed strong activity against *L. monocytogenes* ATCC19114 (ZOI, 13.08 ± 1.29 mm), ATCC19115 (10.54 ± 0.92 mm), and ATCC19111 (9.36 ± 1.41 mm), and comparatively weaker against *E. faecalis* M157 (7.79 ± 1.20 mm) as depicted in Fig. 5. The growth of the remaining test organisms was not inhibited (Table 2). On the other hand, when the culture supernatant was directly used without pH adjustments (pH 4.3 to 4.5), it showed inhibitory activity against *Escherichia coli* ATCC 43888 and ATCC 25922, *Pseudomonas aeruginosa* KCTC 1750^T and KCTC 2651, and *Staphylococcus aureus* RN6390. The observed activity, however, was not observed when the pH was adjusted to pH 7.0, suggesting that the inhibition of these strains was due to the presence of organic acids rather than salP bacteriocins.

Bacteriocin characterization

The physicochemical characteristics of pre- and mature salP bacteriocin peptides are summarized in Table 4. Both the mature α and ß chains are cationic and hydrophobic in nature, with mature peptides having 4.10 kDa and 4.28 kDa molecular masses, respectively. The amino acid sequences are shown in Fig. 2B, and the two-peptide bacteriocins were initially produced as precursor peptides with a double-glycine N-terminal leader peptide. The core peptides contain the $G_{17}XXXG_{21}$ motif (Oppegård et al., 2008; Acedo et al., 2018). ***In vitro*** characterization assays revealed that the salP bacteriocin had structural stability, retaining 97.65 ± 2.54 % and 94.74 ± 2.92 % of its activity after exposure to a wide range of temperature and pH conditions, respectively (Fig. 6). Moreover, salP was rendered inactive by proteinase K and was partially inhibited by pepsin (pH 2.0).

Discussion

Ligilactobacillus salivarius is a lactic acid bacteria commonly associated with dairy products and various vertebrates. In this study, the salP production by 25 *Lig. salivarius* isolates from the GIT of broilers and laying hens was studied, with an emphasis on the genetic architecture of the bacteriocin gene cluster and phylogeny of the species. At least one strain of *Lig. salivarius* was isolated from each individual chicken, and all of the isolates were differentiated into two distinct phenotypes: Type A with active bacteriocins and Type B with inactive bacteriocins. This observation was attributed to differences in the genetic architecture of the *salP* operon, specifically due to the presence of at least one export gene that can cleave the leader sequence from the pre-peptide and

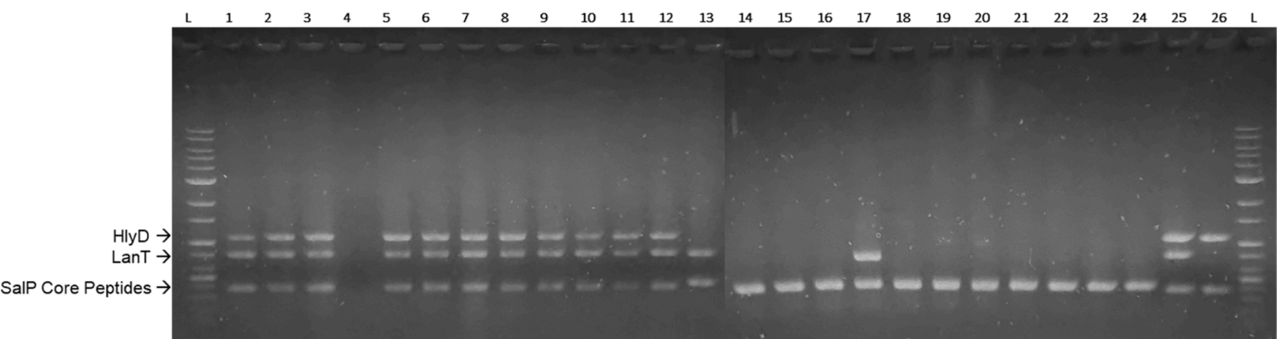

Fig. 1. Multiplex PCR analysis of 26 *Ligilactobacillus salivarius* strains for salivaricin P core peptides (425 bp), *LanT* (806 bp), and *HlyD* (1,119 bp) genes; L – 1 kb DNA size ladder.

(A)

Putative Immunity Protein · Histidine Kinase · AbpR (Regulator) · AbpIM (Immunity) · LanT (Export) · HlyD (Export)

comC · Core Peptides (α,ß) · comC

* - not present in B4112

Putative Immunity Protein · Histidine Kinase · AbpR (Regulator) · AbpIM (Immunity) · LanT (Export) · HlyD (Export)

comC · Core Peptides (α,ß) · comC

* - not present in L5301

(B)

	Leader Sequence	Mature Peptide Sequence
Chain α	MIIMMKEFTVLTECELAKVDGG	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL
Chain ß	MKNLDKRFTIMTEDNLASVNGG	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH

(C)

Strain	Chain α	Chain ß
B4311	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH
LS7274	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFTSCR
UCC118	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFTSCR
DPC6005	KCGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	KNGYGGSGNRWVHCGAGIVGGALIGTIGGPWSAVAGGISGGFISCR
DPC6027	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH
DPC6189	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH
M7.2	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH
7.3	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH
CICC23174	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH
AR809	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH
SNK6	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH
JCM1046	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH
S96	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFTSCH
S40	KRGPNCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL	KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFTSCH
	* ***	*************************:**************** **:

Fig. 2. Salivaricin P operon analysis: (A) Reconstructed salivaricin P operon (top, Type A; bottom, Type B); (B) Salivaricin P amino acid sequences; (C) Amino acid sequence alignment of salivaricin P peptides with other *Lig. salivarius* strains.

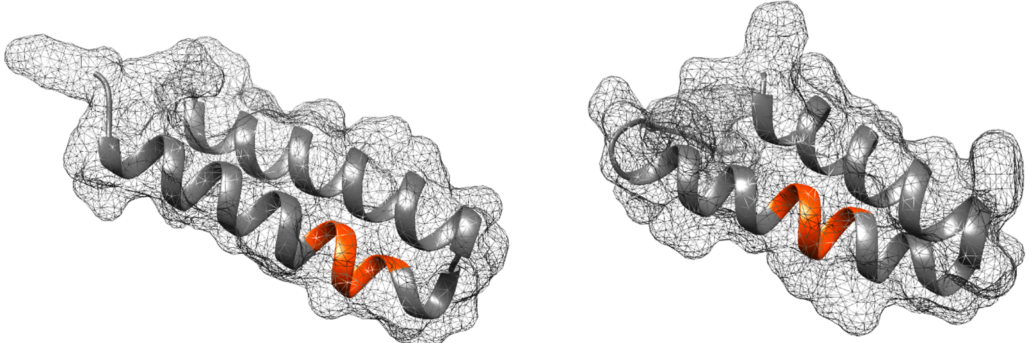

Fig. 3. Structural conformation of salivaricin P chain α (left) and chain ß (right). The highlighted orange regions represent the GXXXG motif.

secrete mature bacteriocins extracellularly (Singh and Sareen, 2014). The *salP* operon analysis showed that the gene clusters of Type A and B strains are not the same. Type B does not have any genes that code for the transport proteins LanT and HlyD. Contrastingly, two *Lig. salivarius* Type B strains (B4311 and B5258) inhibited *L. monocytogenes* ATCC19114. The multiplex PCR analysis confirmed that both strains harbored the *lanT* gene. This highlights the importance of LanT in cleaving the leader peptide and exporting the mature peptide out of the cell. These results are consistent with other reconstructed *Lig. salivarius salP* operon (O' Shea et al., 2012; Niu et al., 2024), illustrating a similar genetic architecture for the bacteriocin operon.

Strains that lacked either or both of the export proteins were classified as Type B based on the BLASTn search for *salP* operon components. It is possible that *Lig. salivarius* is constantly undergoing a

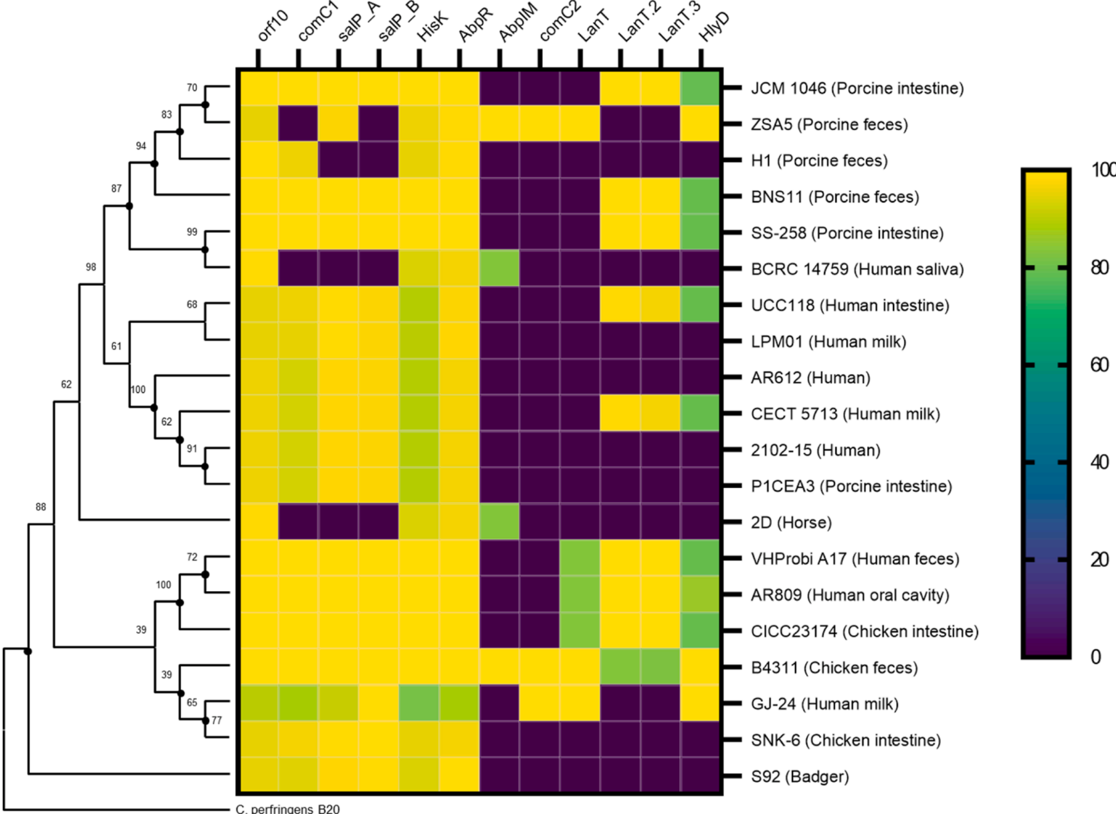

Fig. 4. Phylogenetic tree of 26 *Ligilactobacillus salivarius* strains based on 16S rRNA gene, *gyrB, rpoB, groEL, repA*, and *parA*, correlated with salivaricin P genes. Color scale represents percent identity (%) of genes.

significant evolutionary step involving the loss of both *lanT* and *hlyD* genes. Numerous factors affect the evolution of a species, including the immediate environmental conditions of the niche (absence of competitors for nutrients), which may directly influence the expression of bacteriocin genes and the plasticity of the plasmid, which may lead to the modification, acquisition, or loss of genes (Koskiniemi et al., 2012). The *salP* operon revealed that the *salP* structural genes and regulatory proteins are flanked by two *comC* genes, which are believed to be part of the *com* operon in streptococcal species (Whatmore et al., 1999). A BLASTp search of the two *comC* genes revealed that the *comC* genes are related to bacteriocin production, specifically as a bacteriocin-type signal sequence. Previous studies have identified Plantaricin A as the inducer peptide pheromone for the synthesis of two-peptide bacteriocins (Hauge et al., 1998; Sand et al., 2010).

Downstream of the *salP* structural genes were the regulatory genes (AbpK and AbpR) and export protein genes (LanT and HlyD). The LanT protein contains the peptidase C39 family motif (Pfam: PF03412), designated as the maturation protease which cleaves the N-terminal signal sequences and directs peptide bacteriocins across the cytoplasmic membrane via the Sec pathway (Havarstein et al., 1995). LanT is predicted to function as an AbpT bacteriocin export accessory protein, and it is responsible for the cleavage of the double-glycine leader sequence to produce the active peptide (Singh and Sareen, 2014; Walsh et al., 2015). Meanwhile, HlyD has been proposed to function as an AbpD bacteriocin-export accessory protein, particularly as an efflux transporter periplasmic adaptor (Walsh et al., 2015). The absence of these two export proteins in the Type B strains substantiates their role in the synthesis and excretion of mature salP.

In this study, it was confirmed that strains B4311 and B5258 harbor a functional *lanT* gene. It has been reported that some bacteriocins, including ABP-118, are transcriptionally regulated through a three-component regulatory system (Barrett et al., 2007), including a peptide pheromone, a membrane-associated histidine protein kinase (AbpK), and a response regulator (AbpR) (Nissen-Meyer et al., 2010). The production of the pre-peptide is followed by simultaneous cleavage of the leader sequence and secretion through a dedicated transport system. The results of this study corroborate previous observations highlighting the role of export proteins in the synthesis of active bacteriocins. More specifically, the need for post-translational modification (i.e., cleavage of the leader sequence) necessitates the presence of a functional LanT protein.

Meanwhile, despite the inability to produce active bacteriocins, it is important to note that Type B strains remain unaffected by salP. There are several mechanisms by which bacteria resist the activity of bacteriocins, including self-resistance by ABC transporters, self-resistance by immunity proteins, and immune mimicry (Ahmad et al., 2020). The putative immunity protein located upstream of the core peptides was verified to confer resistance to salP (O' Shea et al., 2012) whereas the *abpIM* located downstream of the core peptides, although present in Type A strains, is most likely associated with other types of bacteriocins (Sevillano et al., 2023b). This notion is consistent with our observation that Type B strains are resistant to salP despite the absence of *abpIM* gene. The immunity of Type B *Lig. salivarius* strains can be explained by the conserved putative immunity proteins. This also provides insights into how closely related strains and other microorganisms are affected by bacteriocins, thereby influencing overall interactions in the microbiome. Ríos Colombo et al. (2023) investigated the effects of lantibiotics and pediocin-like bacteriocin production on bacterial community composition using a simplified human intestinal microbiota model. Generally, bacteriocins directly reduce the population of sensitive species. However, indirect relationships between bacteriocins and non-susceptible species have also been documented. For example, the growth of *Bifidobacterium longum* was adversely affected by lacticin 3147 despite having a positive correlation with pediocin-like bacteriocins.

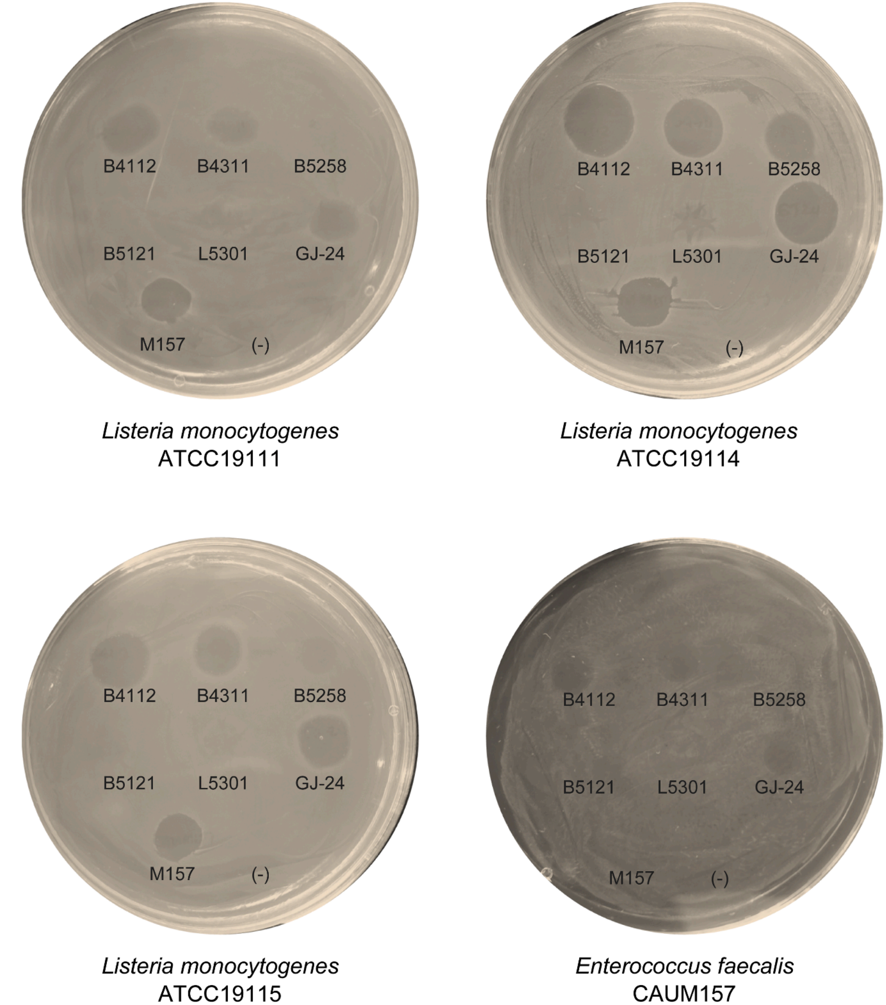

Fig. 5. Spot-on-lawn assay showing inhibition zones produced by active salivaricin P bacteriocins against *Listeria monocytogenes* strains and *Enterococcus faecalis* CAUM157.

Table 4
Physicochemical properties of salivaricin P pre-peptides and mature bacteriocins predicted by ProtParam.

Parameters	α Chain		ß Chain	
	Pre-peptide	Mature Peptide	Pre-peptide	Mature Peptide
Number of residues	67	45	68	46
Positively-charged residues	4	2	5	2
Negatively-charged residues	4	0	3	0
Molecular weight (Dalton)	6537.81	4096.84	6721.58	4284.80
Isoelectric point (pI)	6.01	8.96	8.80	8.90
Instability Index (II)	18.46	13.57	27.71	25.63
Grand Average of Hydropathicity	0.797	0.880	0.084	0.376

This was attributed to the indirect consequence of the inhibition of *Enterococcus faecalis* (Ríos Colombo et al., 2023). Similarly, numerous studies have also shown the complex effect of bacteriocins on various microbial communities in the gut (Guinane et al., 2016; Anjana and Tiwari, 2022; Teng et al., 2023) and food systems (Mills et al., 2017; Todorov et al., 2022; Zhang et al., 2022), supporting the effective role of bacteriocins in modulating microbial communities.

Phylogenetic analyses corroborated previous findings that the *salP* operon was located in the *repA*-type megaplasmid, a feature common to *Ligilactobacillus salivarius* (Barrett et al., 2007; Harris et al., 2017). The absence of *lanT* and *hlyD* genes (Karlyshev and Gould, 2023; Sevillano et al., 2023a) does not follow the evolutionary lineage of the species. Thus, alterations in the *salP* operon occurred independently during the species evolution and were likely influenced by the immediate environment. Harris et al. (2017) conducted a phylogenomic and comparative genomic study, demonstrating that the *salP* gene had no strong association with a particular isolation source, but its distribution was associated with several sub-clades. Thus, *salP* genes are conserved in *repA*-type megaplasmids and can be considered a species-specific trait. In contrast, the loss of export proteins might be due to selection-driven gene loss (Koskiniemi et al., 2012). Similar observations were made in the phylogenetic tree of *Lig. salivarius* strains presented in this study, in which all *Lig. salivarius* strains had *salP* structural genes, but 9 of the 20 strains lacked *lanT* or *hlyD*. In this regard, investigation of the co-evolution of the host and *Lig. salivarius* in relation to the observed loss of export proteins should be further investigated.

A closer examination of the phylogenetic tree showed that the loss of export proteins did not exhibit a distinct linear pattern but was rather observed in polyphyletic groupings. Moreover, there was no association between the isolation host and *salP* genetic architecture, as loss of transport proteins was observed in strains isolated from different hosts. Particularly, 44 % of the strains of human origin (BCRC14759, LPM01, AR612, and 2102.15) and 33 % of the strains of porcine (H1 and P1CEA3) and chicken origin (SNK6) lacked export proteins. The strains

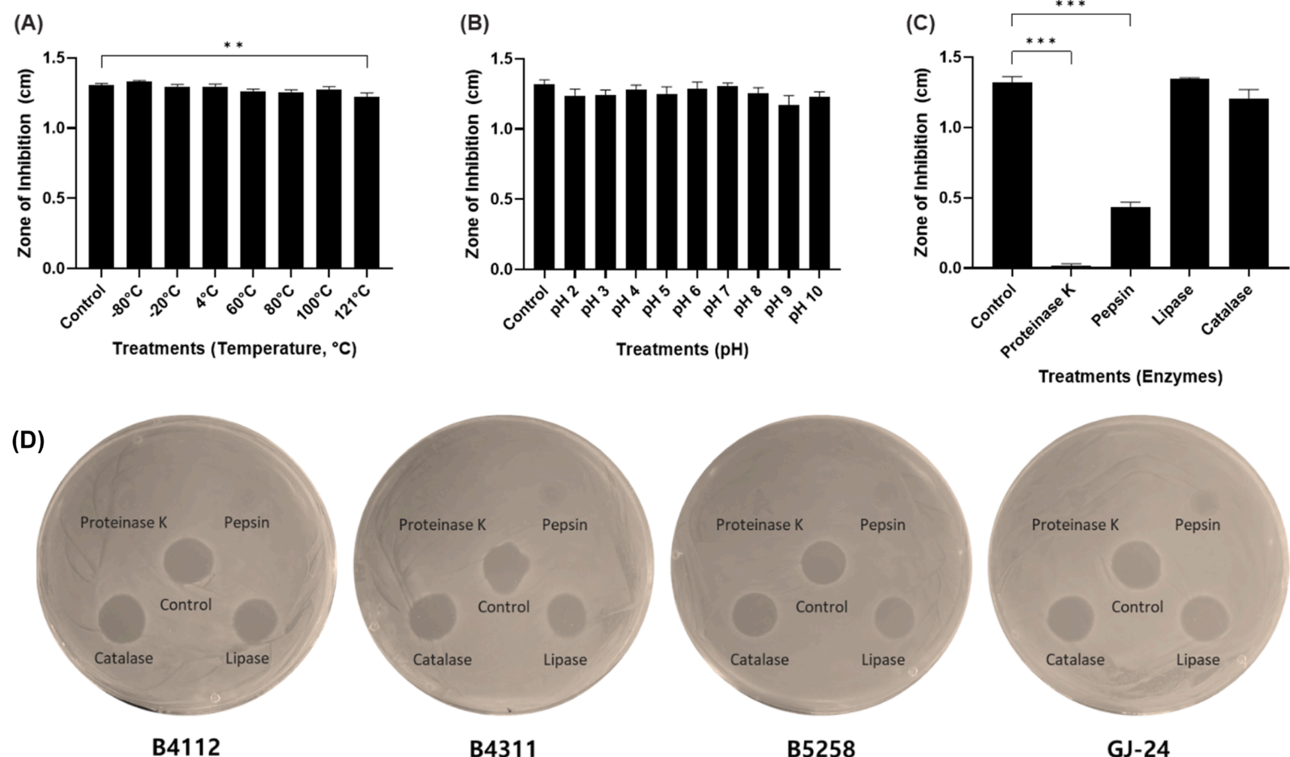

Fig. 6. *In vitro* characterization of physicochemical properties of salivaricin P: (A) effects of temperature; (B) effects of pH; (C) effects of hydrolytic enzymes showing (D) inhibition of bacteriocin activity by proteinase K treatment and pepsin via spot-on-lawn assay. Asterisks denote significant difference among means ($p < 0.05$).

isolated from horses (2D) and badgers (S92) lacked both the export protein genes. However, several strains were reported to exhibit inhibitory activity, particularly ZSA5 (LS2 bacteriocin) (Niu et al., 2024), H1 (24 kDa protein) (Sandoval-Mosqueda et al., 2023), CECT 5713 (H_2O_2) (Martin et al., 2006), 2102-15 (nisin F or LS7247 bacteriocin) (Karlyshev and Gould, 2023), P1CEA3 (nisin S) (Sevillano et al., 2023a), and S92 (anti-*Salmonella* factor) (Wang et al., 2023).

The presence of genes involved in the synthesis of multiple bacteriocins suggests the potential to produce multiple bacteriocin types that require precise mechanistic control to optimize energy investment in an efficient production system (Perez et al., 2022). However, when a bacterium is unable to handle the metabolic demands of producing multiple bacteriocins, it may be more advantageous to focus the production of only one inhibitory compound. In this regard, the loss of genes encoding other bacteriocins may occur. For *Lig. salivarius*, the rapid evolutionary rate of the *repA*-type megaplasmid with large variations in size and functional properties coincided with the loss of dedicated export protein genes. Dec et al. (2021) reported that the *repA*-type megaplasmid and genes for Abp118 (salP) bacteriocins were found in 94 % and 51.5 % of the 33 strains studied, respectively. Moreover, the evolutionary variability of *Lig. salivarius* strains of avian origin is evident in their carbohydrate fermentation profiles (Dec et al., 2021). The documented genetic plasticity of *repA*-type megaplasmids suggests a higher probability of genetic modification, acquisition, or loss, which could be a key factor influencing the conservation of *lanT* and *hlyD*.

Another factor that may explain the divergence of Type B strains is their persistent immunity to salP bacteriocins. In this scenario, Type B strains hardly produced active salP bacteriocins, yet they remained unaffected by the active bacteriocins because of the immunity genes located upstream of the salP core peptide genes. Such a phenomenon occurs when bacteriocin production does not confer a competitive advantage, such as when no other microorganisms compete for niches or nutrients. Thus, the metabolic burden associated with bacteriocin production can be reduced if the process is terminated, resulting in the redirection of energy to other metabolic processes or biomass production. The bacteriocins from Type A strains then act as selection pressure for Type B strains to conserve the immunity proteins.

The narrow range of activity (Barrett et al., 2007; Vera Pingitore et al., 2009; O' Shea et al., 2012) makes it suitable to address food-related problems where the mentioned harmful microorganisms are the primary contributing agents, i.e., *L. monocytogenes* as the causative agent of listeriosis (Osek et al., 2022), and enterococci in food intoxication as potential opportunistic pathogens (Braïek and Smaoui, 2019). From an evolutionary perspective, the development of narrow-spectrum toxins ensures the efficient inhibition of the most significant competitors. Narrow-spectrum bacteriocins are employed to specifically target and control infections, exerting efficient inhibition of the pathogen while reducing or eliminating any negative effects on commensal microorganisms (Rea et al., 2013). This is the advantage of using narrow-spectrum antimicrobials in contrast to broad-spectrum compounds that may have a negative impact on commensal microorganisms with unexpected side effects (Anjana and Tiwari, 2022; Ríos Colombo et al., 2023).

In silico characterization of salP bacteriocins revealed that the α and ß chains shared high sequence homology. The predicted structural conformations of the two peptides revealed the position of the GXXXG motif, which was hypothesized to play a crucial role in the orientation and mechanism of action of two-peptide bacteriocins. Additionally, salP bacteriocins are positively charged hydrophobic bacteriocins with high thermal and pH stability. Along with its narrow spectrum of activity and susceptibility to digestive proteases, the use of salP in human and animal diets seems compatible. The structural analysis of salP α and ß chains revealed that the peptides are conformed as helices, with the GXXXG motifs located on the same side of the α-helix, hypothesized to facilitate the inter-helical interactions, inducing the formation of the transmembrane-spanning helix-helix structure and ultimately leading to pore formation (Acedo et al., 2018). This mode of mechanistic action has been proposed for many Class II two-peptide bacteriocins (Nissen-Meyer

et al., 2010), where the pore formation is highly dependent on the structural homology of the two peptides. Additionally, the bacteriocins exhibited high thermal and pH stability under various conditions. This highlights the ability of salP bacteriocins to withstand a wide range of temperature and pH conditions and maintain their activity.

Conclusions

The study focused on the differences in bacteriocin production of *Lig. salivarius* based on two distinct genotypes. The results highlight the role of individual genetic components of the *salP* operon. *In vitro* experiments evidenced the effectiveness of salP in controlling *Listeria monocytogenes* and *Enterococcus faecalis*. The high temperature stability and pH resistance, as well as the susceptibility to digestive proteases, substantiate the safety and potential of *Lig. salivarius* salP for human food and animal feed industries. These characteristics ensure that bacteriocins can contribute to food safety and preservation or remain active after administration and exert antagonistic effects against the target microorganisms. Moreover, understanding the coevolution of the isolates with their hosts might elucidate specific interactions to maximize their potential application as probiotics. Further efforts should be directed towards evaluating the salP-producing strains for potential probiotic applications. Alternatively, the large-scale production of salP for pathogen control in food and animal systems and as alternatives to common antibiotics used in the animal industry may be sought.

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Declaration of competing interest

The authors of the study entitled “Distinct phenotypes of salivaricin-producing *Ligilactobacillus salivarius* isolated from the gastrointestinal tract of broiler chickens and laying hens” hereby confirm that the submitted manuscript for publication on *Poultry Science* is entirely original and has not been previously published or is currently under consideration for publication elsewhere.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2024.104537.

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