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Phenotypic and genomic characterization of bacteriocin-producing lactic acid bacteria with probiotic and biotechnological potential for pathogen control in animal production

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ABSTRACT

The emergence of antibiotic-resistant pathogens has raised significant concerns in the poultry industry, driving the search for alternatives to antibiotics as growth promoters in animal feed. Probiotics, particularly those belonging to the Lactic Acid Bacteria (LAB) group, represent a promising solution by mitigating the risk of infectious disease. However, a uniform concentration of probiotic LAB is not suitable for feed additives due to varying growth kinetics. Additionally, the genomic and physiological profiles of the LAB strains involved must be thoroughly evaluated. In this study, we provide an analytical framework to comprehensively assess LAB as

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potential antibiotic alternatives in poultry farming. Three LAB strains – *Pediococcus pentosaceus* (isolated from corn silage), *Ligilactobacillus salivarius* (from the poultry gut) and *Lactococcus lactis* (from the gut of rainbow trout) – were sequenced and characterized, with a focus on evaluating their probiotic potential and safety at the genomic level. The analyses included co-culturing LAB with pathogens, assessing viable cells, and determining the minimum inhibitory concentration of bacteriocin-like inhibitory substances (BLIS). In addition to demonstrating effective antimicrobial activity against avian pathogens (*Salmonella* spp., *Clostridium* spp. and *Campylobacter coli*), the results revealed notable probiotic traits in all three LAB strains, such as tolerance to bile salts and acidic environment and adhesion to intestinal cells. In conclusion, our analytical framework and results highlighted the potential of the tested LAB strains as biotechnological tools for developing zootechnical additives. These probiotics show promise as viable alternatives to antibiotics for enhancing poultry health and productivity.

Impact statement

This study identifies three promising strains of Lactic Acid Bacteria (LAB) with potential as probiotics in animal feed to enhance health and productivity. Isolated from natural sources, they demonstrated the ability to combat pathogens such as *Listeria, Salmonella* and *Clostridium*, tolerating even challenging intestinal conditions. Through the production of bacteriocins —natural antimicrobial compounds — these LAB represent a viable alternative to antibiotics, which raise concerns about antibiotic resistance in livestock. This research establishes a framework for the development of probiotic additives that can improve animal welfare and reduce dependence on antibiotics in agriculture.

Introduction

Antimicrobial resistance is one of the most significant challenges to global public health. The use of antibiotics in animals dates back over 70 years, when chlortetracycline was found to improve growth and health [1]. Since then, these substances have been widely and systematically used in animal husbandry for diseases treatment, prevention, and growth promotion. However, the use of antibiotics in animal has been a subject of ongoing debate, particularly regarding its impact on antimicrobial resistance in humans. Some antibiotics used in animals are either the same or closely related to those used in human medicine [2], which may contribute to the emergence of resistant microorganisms, zoonotic transmission, and transfer of resistance genes from animal to human [3].

The first evidence of this correlation was reported in 1995 in Denmark, when the ban on avoparcin, a poultry growth promoter chemically related to the glycopeptide vancomycin, led to a reduction in the prevalence of vancomycin-resistant enterococci (VRE) from 72 % in 1995 to 2 % in 2005 [4,5]. Similarly, a decrease in avilamycin resistance was observed in *Enterococcus faecium* isolated from poultry following the prohibition of this growth promoter [4,6]. The restriction on colistin use was due to reports from Chinese researchers, who identified the *mcr*-1 gene in enterobacteria isolated from animals, food, and humans [7]. These *mcr*-1-carrying enterobacteria, which were subsequently detected in Europe, Asia, and Africa, also showed resistance to other polymyxin antibiotics frequently used to treat infections caused by multidrug-resistant bacteria [8]. In 2016, the *mcr*-1 gene was identified in Brazil in *Escherichia coli* strains isolated from poultry, pigs, and humans, which suggested a chain of resistance transmission starting from antibiotic use in animal feed and spreading through slaughtered animals, food products, and the environment [9,10].

As antibiotic restrictions continue to intensify within the animal production industry [11], there has been an increased focus on identifying viable alternatives to enhance the natural defense mechanisms of animal and reduce dependence [12]. Among these alternatives, probiotic microorganisms have emerged as a promising solution to combat the rise of bacterial antimicrobial resistance and correct imbalances or dysbiosis of host microbiota. Evidence indicates that probiotics can antagonize pathogens, modulate immune responses, and support overall host health by restoring balance to the gut microbiota [13].

Although numerous probiotic strains have been described, their efficacy is highly dependent on both the specific strain and the host species. A report issued by the International Scientific Association for Probiotics and Prebiotics in 2010 emphasized that the beneficial effects of probiotics are also strain-specific [14]. Furthermore, studies have shown that the effects of probiotics are host-specific [15]. As such, strains isolated from a particular host are more likely to colonize, survive, and exert similar physiological effects in individuals of the same species. This suggests that probiotics isolated from humans may not produce the same effects when administered to animals, and vice versa [16]. However, it is essential to demonstrate that these probiotics are also safe for use in humans or animals [17].

Probiotics can be administered as live microbial feed supplements, commonly referred to as Direct-Fed Microbials (DFMs) [18], offering a range of benefits including improvements in the value, texture, stability, flavor, color, alkalinity, and sourness of food or feed, while also limiting the spread of pathogens [19]. In agriculture, probiotics have been explored across various sectors of animal production as potential alternatives to antibiotics, serving both as growth promoters and as tools for controlling specific enteric pathogens [20]. Most of the bacteria used as DFMs belong to the Lactic Acid Bacteria (LAB) group due to their robust ability to tolerate the conditions of the gastrointestinal tract, as well as their natural capacity to inhibit pathogen growth by lowering pH through lactic acid production and by producing bacteriocins. LAB are also Generally Recognized as Safe (GRAS) in many regions and frequently exhibit beneficial immunomodulatory effects, making them ideal candidates for enhancing feed efficiency and promoting overall animal health. Furthermore, minimizing the genetic spread associated with antibiotic resistance is a critical factor in the selection of probiotic strains. According to the European Food and Safety Authority (EFSA) BIOHAZ Panel recommendations, probiotic strains intended for animal or human use must undergo thorough genomic and phenotypic evaluations to ensure they do not carry transmissible antibiotic resistance genes or virulence factors. This stringent evaluation process helps mitigate the spread of antimicrobial resistance while ensuring the safety and efficacy of LAB-based DFMs in animal nutrition [21,22]

Despite the widespread use of many LAB strains in the food and feed sectors, it is uncommon for a single strain to exhibit multiple beneficial effects [23]. As a result, the current market trend favors the use of combination of different probiotic strains to maximize host benefits. For example, if a LAB produces a potential antimicrobial molecule (e.g., a bacteriocin) but cannot be classified as a probiotic, its antimicrobial properties may still be effectively utilized in combination with other probiotic LAB, provided that it does not inhibit the activity of the other probiotics in the formulation [18–23].

This study hypothesizes that a uniform concentration of potential probiotic LAB is unsuitable for feed additive formulations due to the distinct features and growth kinetics of each bacterium. Furthermore, the genomic profile of each probiotic LAB must be thoroughly evaluated to ensure both their probiotic potential and safety. Therefore, an effective probiotic formulation should account for the genomic and kinetic characteristics of each microorganism involved. To test this hypothesis, this study presents an analytical framework that integrates different LAB species with comprehensive genomic, *in silico*, and *in vitro* analyses. This

framework is designed to characterize their probiotic potential and antimicrobial effects in co-cultures with pathogens relevant to agro-industry and animal husbandry. The methodology and results presented here may provide valuable insights for developing a relevant LAB mix for biotechnological applications, including direct feeding or encapsulation as microbial additives, offering a viable alternative to enhance poultry health and productivity.

Results

Genomic characterization and probiotic potential of isolates

The complete genome sequences of the potential probiotic LAB isolates *Pediococcus pentosaceus* LBM18 (Fig. 1A), *Ligilactobacillus salivarius* LBM71 (Fig. 2A), and *Lactococcus lactis* LBMB2 (Fig. 3A) were successfully obtained with high quality, encompassing both the circular main chromosomes and plasmid sequences (Table 1). *P. pentosaceus* LBM18, isolated from corn silage, harbors the penocin A gene and its immunity protein within a ribosomally synthesized and post-translationally modified peptide (RiPP-like) gene cluster on the main chromosome (Fig. 1B). A two-component system, consisting of a sensor histidine kinase and a response regulator, is located downstream of the penocin A and the immunity protein genes, suggesting a potential regulatory mechanism for bacteriocin expression. Additionally, the main chromosome of *P. pentosaceus* LBM18 contains a diverse array of multidrug efflux transporters, the *vanZ* gene (VanZ-like domain - IPR006976) linked to glycopeptide antibiotic resistance, particularly vancomycin, and two hemolysin-related genes. Furthermore, three intact tailed prophages were identified near the replication terminus of the *P. pentosaceus* LBM18 genome. The 19 Kb plasmid carries four ABC transporter-related genes, along with genes encoding relaxase, mobilization, and replication initiator proteins, suggesting potential conjugative properties. In contrast, the 18 Kb plasmid contains relaxase and *parA* gene but lacks genes associated with the type IV secretory system. Notably, no probiotic potential, virulence and pathogenicity genes were identified in either plasmid.

L. salivarius LBM71, isolated from poultry intestine, carries a RiPP-like gene cluster in its chromosome, which encodes enterolysin A, one copy of the *vanZ* gene (VanZ-like domain - IPR006976), and two hemolysin-related genes (Fig. 2B). Additionally, the RiPP-like gene cluster located on the 242 kb megaplasmid displays a complex structure, encoding at least six bacteriocins: plantaricin S-beta, plantaricin S-alpha, plantaricin NC8-alpha, lactacin-F subunit *lafA*, salivaricin P chain A, and salivaricin P chain B (Fig. 2C). This cluster also includes genes involved in bacteriocin secretion and peptide modification, as well as two copies of a two-component system, which may regulate the

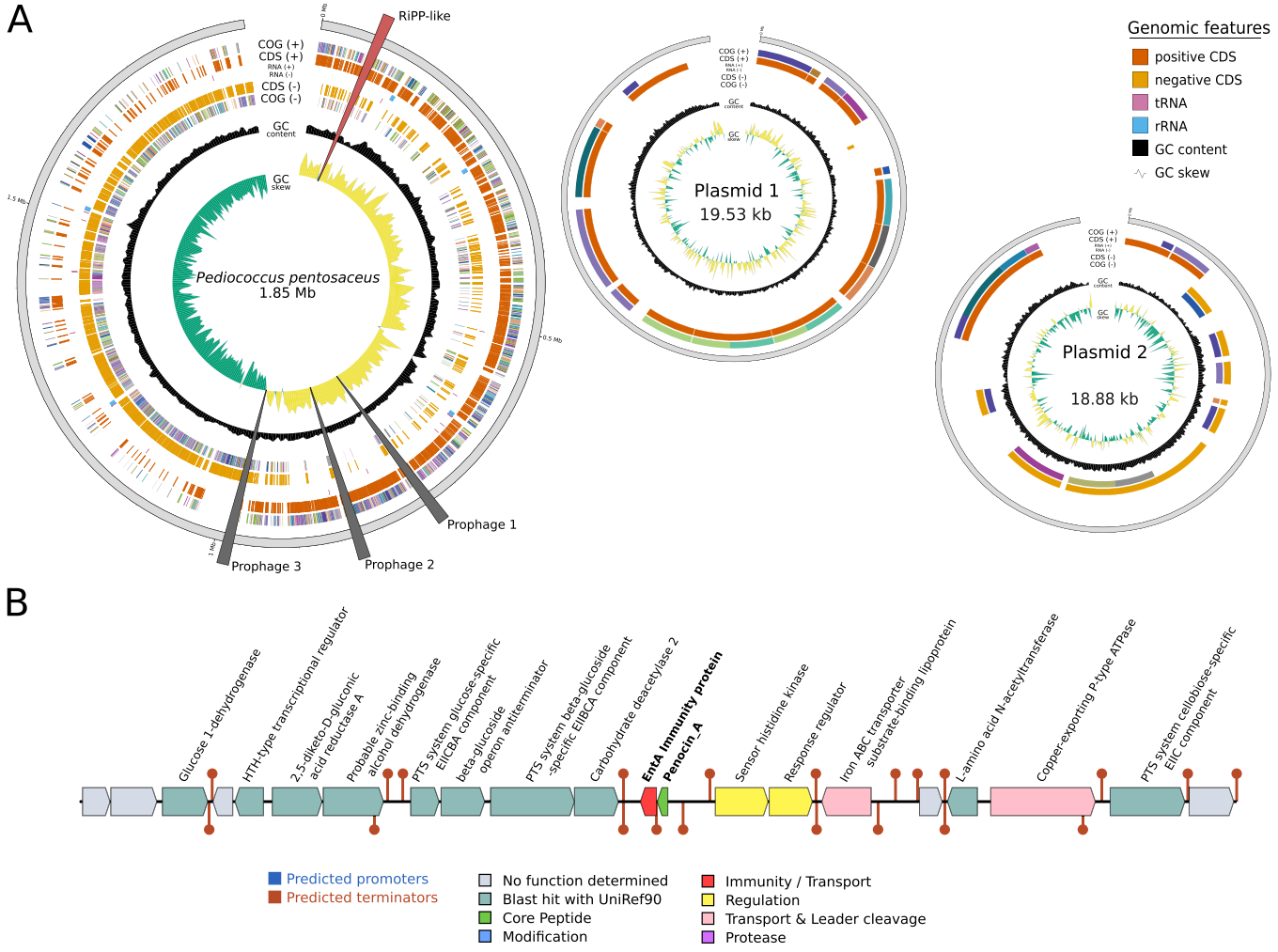

Fig. 1. Genomic feature circle maps (A) and (B) Biosynthetic Gene Clusters (BGCs) organization in the genome of *Pediococcus pentosaceus* LBM18. The outermost circle of the pie chart is the genome size marker; the third and sixth circles are the coding sequence (CDS) on the positive and negative strands; the second and seventh circles indicate the functional classification of the different Clusters of Orthologous Groups (COGs) of the CDS; the fourth and fifth circles represent the positive and negative strands for rRNA and tRNA; the eighth circle is for the GC content; the innermost circle represents the GC skew value. The arrows indicate the location of Prophages and Ribosomally synthesized and post-translationally modified peptides (RiPPs) in the genome.

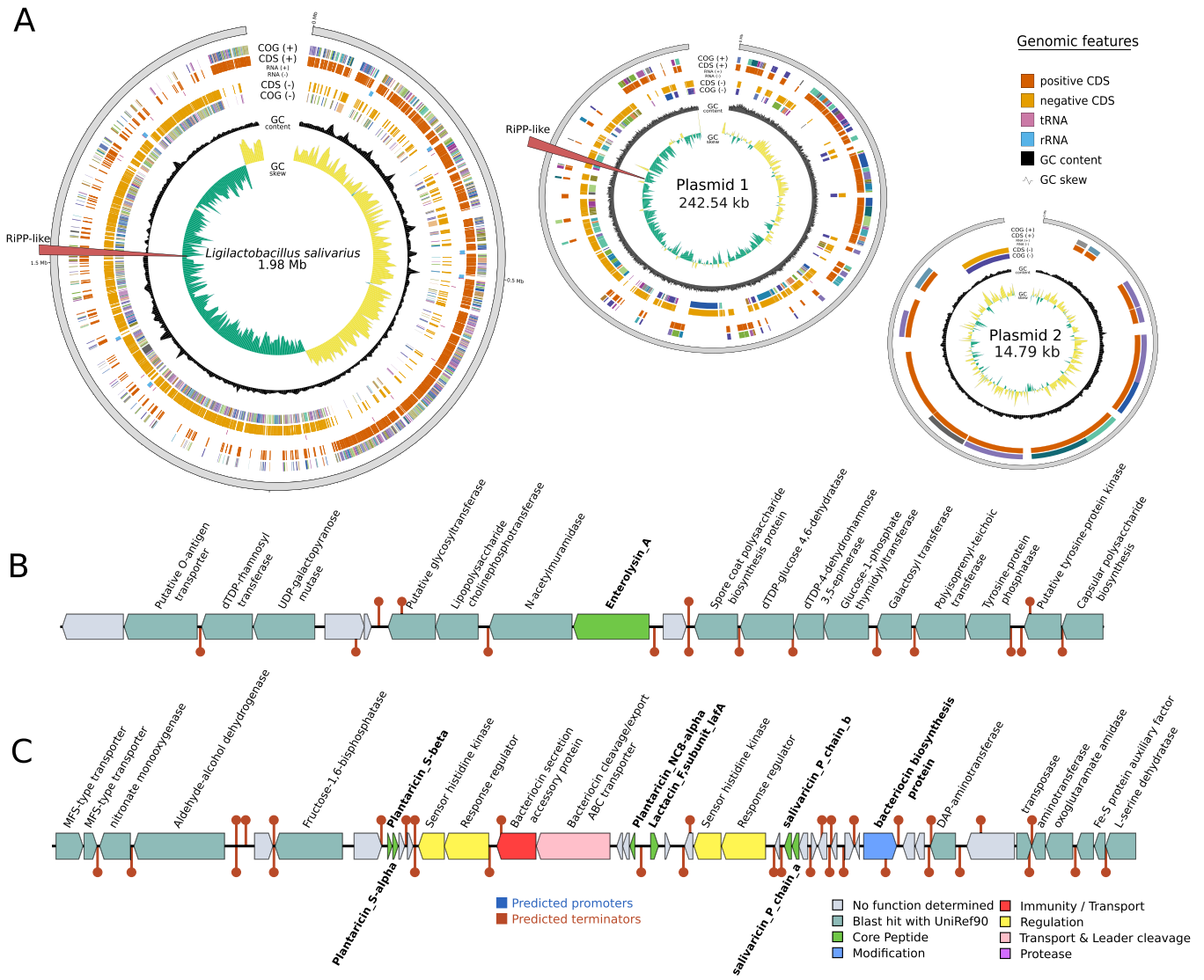

Fig. 2. Genomic feature circle maps (A) and (B and C,) Biosynthetic Gene Clusters (BGCs) organization in the genome of *Ligilactobacillus salivarius* LBM71. The outermost circle of the pie chart is the genome size marker; the third and sixth circles are the coding sequence (CDS) on the positive and negative strands; the second and seventh circles indicate the functional classification of the different Clusters of Orthologous Groups (COGs) of the CDS; the fourth and fifth circles represent the positive and negative strands for rRNA and tRNA; the eighth circle is for the GC content; the innermost circle represents the GC skew value. The arrows indicate the location of Prophages and Ribosomally synthesized and post-translationally modified peptides (RiPPs) in the genome.

expression of these bacteriocins. The megaplasmid further contains the *repA* gene, relaxase genes (*mobP2* and *mobV*), site-specific integrase, recombinases, and five ABC transporter genes. Additionally, the megaplasmid harbors conjugation-related genes, several Insertion Sequences (IS), and various metabolic genes, particularly those associated to heavy metal metabolism. Notably, it carries the lincosamide nucleotidyltransferase *lnu(A)* gene, which confers resistance to lincosamide, a *tetM/tetW/tetO/tetS* tetracycline resistance ribosomal protection protein, and a frameshifted arsenical-resistance gene. The lnu(A) gene exhibits the aminoglycoside-2''-adenylyltransferase domain (IPR019646), conferring resistance to kanamycin, gentamicin, and tobramycin [54], while the *tet* gene features the translation elongation factor EFG/EF2 domain IV (IPR005517), elongation factor EFG domain V-like (IPR000640), and tetracycline resistance protein C-terminal domain (IPR035650), which mediate tetracycline resistance. The 14 Kb plasmid contains the plasmid replication initiator protein (*repB*), site-specific integrase, recombinases, two ABC transporter genes, permeases, and a lysozyme/endolysin gene, but no conjugative machinery-related genes or probiotic potential were detected.

L. lactis LBMB2, isolated from the intestine of rainbow trout intestines, contains two RiPP-like gene clusters in its main chromosome. The first gene cluster encodes the lactococcin B bacteriocin and its associated immunity protein (Fig. 3B). The second cluster encodes the nisin Z bacteriocin along with its related proteins, including those involved in transport, regulation, modification, and immunity (Fig. 3C). Two identified tailed prophages were found to be associated with *Lactococcus* phage ul36 (37 Kb - NC_004066) and PLgT-1 (40 kb - NC_031016). Notably, *L. lactis* LBMB2 also harbors the tetracycline efflux pump responsible for tetracycline resistance (Tetracycline resistance protein TetA/multidrug resistance protein MdtG-like domain IPR001958), as well as the *lmrA*, *lmrC*, *lmrD*, and *lmrP* genes, which are associated with antibiotic resistance via efflux pumps. In addition, the APH(3′) gene encoding an aminoglycoside 3′-phosphotransferase, conferring resistance to aminoglycoside antibiotics (aminoglycoside 3-phosphotransferase domain - IPR024165), and the *vanZ* gene (VanZ-like domain – IPR006976) were identified. All of these resistance-associated genes were classified as intrinsic according EFSA BIOHAZ Panel recommendation [22]. Moreover, four hemolysin-related genes

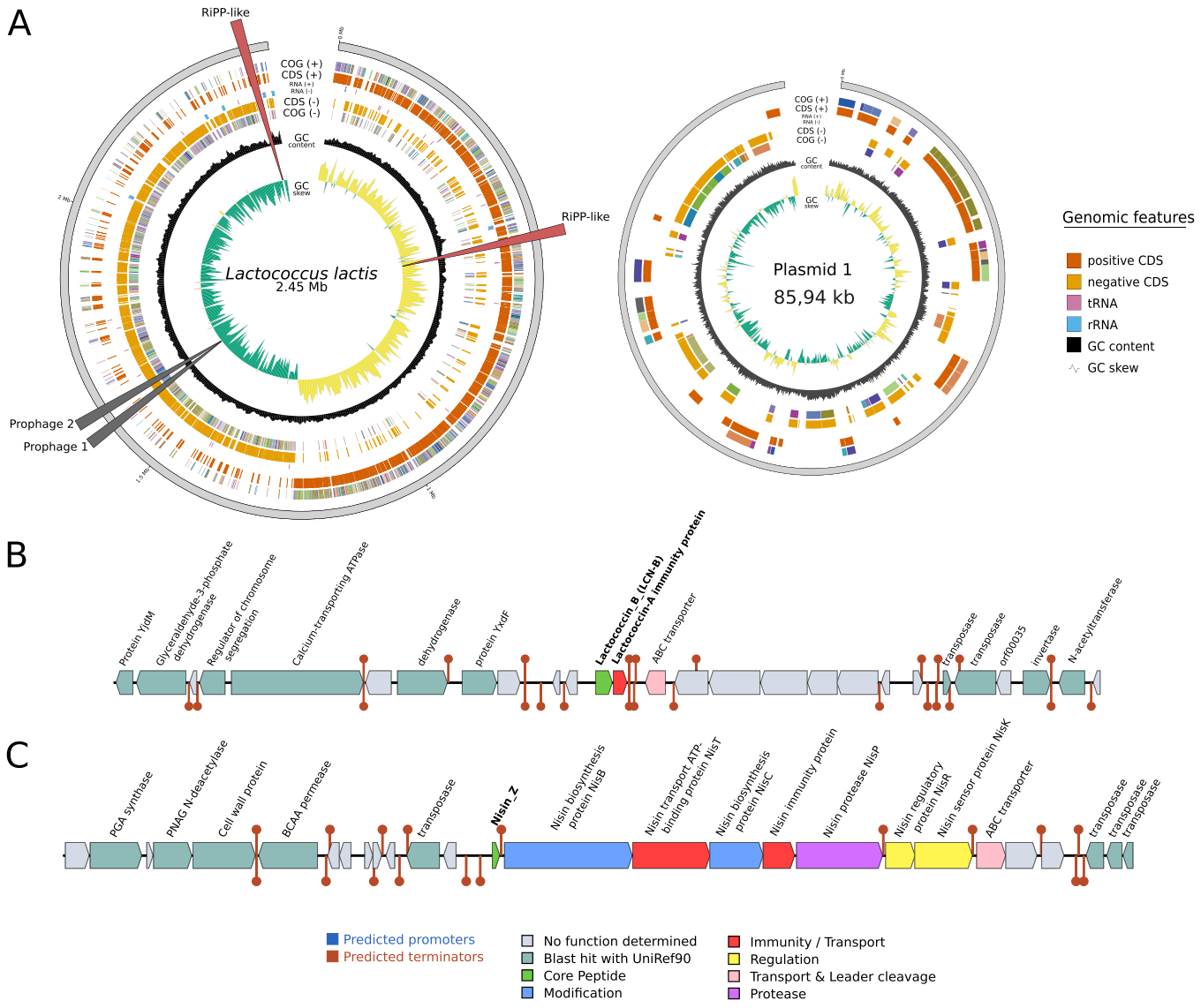

Fig. 3. Genomic feature circle maps (A) and (B and C) Biosynthetic Gene Clusters (BGCs) organization in the genome of *Lactococcus lactis* LBMB2. The outermost circle of the pie chart is the genome size marker; the third and sixth circles are the coding sequence (CDS) on the positive and negative strands; the second and seventh circles indicate the functional classification of the different Clusters of Orthologous Groups (COGs) of the CDS; the fourth and fifth circles represent the positive and negative strands for rRNA and tRNA; the eighth circle is for the GC content; the innermost circle represents the GC skew value. The arrows indicate the location of Prophages and Ribosomally synthesized and post-translationally modified peptides (RiPPs) in the genome.

were identified.

In contrast, the 85 Kb plasmid of *L. lactis* LBMB2 contains typical plasmid-associated genes, such as recombinase, plasmid mobilization relaxosome *mobC*, relaxase, *repA*, and *parB*, but no genes related to conjugative machinery were identified. Additionally, this plasmid harbors metabolic genes, including those related to heavy metal metabolism, ABC transporters, and several IS-related genes. No antibiotic resistance or probiotic-related genes were identified.

Antibiotic susceptibility test of LAB

Each isolate was tested using the disk diffusion antibiogram method (Table 2). According to the CLSI standard classification [43], *P. pentosaceus* LBM18 and *L. salivarius* LBM71 were sensitive to 11 of the 19 antibiotics tested, including ampicillin, azithromycin, bacitracin, clindamycin, chloramphenicol, erythromycin, florfenicol, gentamicin, novobiocin, penicillin, and rifampicin. In comparison, *L. lactis* LBMB2 was sensitive to 10 of these antibiotics, namely ampicillin, bacitracin, chloramphenicol, erythromycin, florfenicol, gentamicin, novobiocin, penicillin, rifampicin, and tetracycline. *P. pentosaceus* LBM18 showed resistance to only 3 antibiotics, specifically amphotericin, nalidixic acid, and vancomycin. On the other hand, L. salivarius LBM71 and L. lactis LBMB2 exhibited resistance, in addition to them, also to 2 additional antibiotics (L. salivarius LBM71: enrofloxacin and streptomycin; L. lactis LBMB2: enrofloxacin and kanamycin). Finally, *P. pentosaceus* LBM18 displayed varying intermediate sensitivity to 5 antibiotics (enrofloxacin, kanamycin, neomycin, streptomycin and tetracycline), L. salivarius LBM71 to 3 antibiotics (azithromycin, kanamycin and neomycin), and L. lactis LBMB2 to 4 antibiotics (azithromycin, clindamycin, neomycin and streptomycin).

To determine the minimum inhibitory concentration (MIC) according to the current regulations of zootechnical additives [44], an antimicrobial susceptibility test was performed using serial twofold broth dilution, with *E. coli* ATCC25922 and *S. aureus* ATCC29213 taken as quality control strains. As shown in Table 2, *P. pentosaceus* LBM18 and *L. lactis* LBMB2 were susceptible to all nine antibiotics tested, exhibiting MIC values equal to or lower than the FEEDAP cut-off. In contrast, *L. salivarius* LBM 71 was the only strain with MIC values for streptomycin

Table 1
Genomic features of the three LAB strains sequenced in this study.

Features	*P. pentosaceus* LBM18	*L. salivarius* LBM71	*L. lactis* LBMB2
Genomic size (bp) – main chromosome (bp)	1886,279 1847,863	2238,013 1980,681	2536,468 2450,530
Sequencing Depth (mean)	88x	95x	103x
GC content (%)	37	33	35
Genes (total)	1923	2155	2563
CDSs (total)	1849	2049	2475
tRNA	56	80	65
5S rRNA	5	8	7
16S rRNA	5	7	6
23S rRNA	5	7	6
ncRNA	3	4	4
Pseudogenes	48	38	41
Prophage regions (intacts) – prophage 1 (kb) – prophage 2 (kb) – prophage 3 (kb)	3 55.7 47.2 37.8	0 - - -	2 43.7 56.3 -
Plasmids – plasmid 1 (bp)– plasmid 2 (bp)	2 19,534 18,882	2 242,545 14,787	1 85,938 -
Antibiotic Resistance-related genes	**chr encoded**– 2x vanZ (glycopeptide antibiotic) – 4x multidrug efflux transporters	**chr encoded**– 2x *vanZ* (glycopeptide antibiotic) **megaplasmid encoded** – *lnu(A)* (lincosamides)– TetM/TetW/TetO/TetS family (tetracyclin)	**chr encoded**– tet efflux pump (tetracyclin) – lmrA,C, D, and P (lincosamides) – APH(3′) (aminoglycoside) – 2x vanZ (glycopeptide antibiotic) – 2x multidrug efflux transporters
Bacteriocins genes	**chr encoded**– penocin A	**chr encoded**– enterolysin A **plasmid encoded**– plantaricin S-beta – plantaricin S-alpha – plantaricin NC8-alpha – lactacin F, subunit lafA – salivaricin P chain A – salivaricin P chain B	**chr encoded**– Nisin_Z – Lactococcin_B
Virulence-related genes	**chr encoded**– hemolysin III family protein– hemolysin family protein	**chr encoded**– hemolysin III family protein– hemolysin family protein	**chr encoded**– hemolysin III family protein – 2x hemolysin family protein – hemolysin XhlA family protein
CheckM (completeness/ contamination)	88.34 / 4.84	94.83 / 11.76	99.39 / 0.29
BUSCOs (completeness)	99.2	99.2	98.4
ANI	98.56	97.27	97.97

and kanamycin exceeding the established cut-off.

Virulence factors and catalase test of LAB

The virulence factors, including catalase activity and coagulase tests, were assessed for the three isolated LAB strains, whose results are summarized in Table 2. All three LAB strains tested negative in the coagulase test, consistent with *in silico* analysis, which revealed no coagulase-related genes in their genomes. Regarding hemolytic activity, *L. salivarius* LBM71 exhibited partial hemolysis (α-hemolysis), while *P. pentosaceus* LBM18 and *L. lactis* LBMB2 displayed complete hemolysis (β-hemolysis). Notably, the intensity of hemolysis varied between these two strains, with *P. pentosaceus* LBM18 showing lower intensity (+) compared to *L. lactis* LBMB2 (++).

To gain further insight into these phenotypes, *in silico* analyses were performed. The *mecA* gene, which activates the ClpC/ClpP/ClpX protein complex responsible for disaggregating and refolding aggregated proteins, was identified in *P. pentosaceus* LBM18, *L. salivarius* LBM71, and *L. lactis* LBMB2 (Table SM01a). The ClpX protein was detected in all three strains, with *L. salivarius* LBM71 harboring four copies.

These genetic components appear to correlate with the functional characteristics of the strains. Hemolytic activity showed that all three LAB strains contained at least one gene from the hemolysin family and one from the hemolysin III family in their genomes (Table SM01b). However, the *L. lactis* LBMB2 isolate, which exhibited more intense and complete hemolysis compared to *P. pentosaceus* LBM18, also harbored two additional genes encoding proteins within the hemolysin family, including hemolysin XhlA.

Despite their robust hemolytic capabilities, all three LAB strains exhibited positive results for hemodigestion, a process linked to the metabolic by-products produced by bacteria under aerobic conditions. Notably, all three strains tested negative for both gelatinase and proteolytic activity.

Tolerance of LAB to low pH and blie salts

Although *P. pentosaceus* LBM18 exhibited greater sensitivity at low pH (2.0) than the other two strains, its viable cell count decreased by no less than 6 log units (3.48 log CFU/mL) compared to the control at pH 6.0 (9.34 log CFU/mL). In contrast, smaller reductions were observed for *L. salivarius* LBM71 (3 log units = 5.30 log CFU/mL) and *L. lactis* LBMB2 (5 log units = 4.08 log CFU/mL) compared to their respective controls at pH 6.0 (8.04 and 9.04 log CFU/mL, respectively). However, at pH 3.0 and 4.0, the reductions for *P. pentosaceus* LBM18 were less pronounced (3 log units = 6.81 and 6.65 log CFU/mL, respectively), while *L. salivarius* and *L. lactis* exhibited even greater tolerance, with reductions of only 2 log units (6.04 and 6.36 log CFU/mL for the former; 7.20 and 7.48 log CFU/mL for the latter). These results suggest that *P. pentosaceus* LBM18, while sensitive to extremely low pH, may still survive under slightly higher gastric pH levels (e.g., pH 3–4) typically encountered in the fed state. Previous reports have also demonstrated that a portion of *Pediococcus* cells can survive gastric transit when protected by feed matrices or through microencapsulation [55,56]. Additionally, under slightly alkaline conditions (pH 8.0), all strains, including *P. pentosaceus* LBM18, exhibited satisfactory growth, with reductions of only 2 log units for *P. pentosaceus* and L. lactis (7.66 log CFU/mL and 7.75 log CFU/mL, respectively) and just 1 log unit for *L. salivarius* (7.28 log CFU/mL). These results underscore the survival and adaptive potential of these strains under varying pH conditions. Animal performance following the administration of LAB-based probiotics is an effective indicator to confirm the suitability of candidate strains, demonstrating the expected *in vivo* benefits of probiotics in animal feed.

Regarding the different concentrations of bile salts (Table 3), all LAB

Table 2
Virulence profile of LAB isolates.

Antibiotics	LAB strains					
	Cut-off values and Minimal Inhibitory Concentration (μg/mL) / (result)					
	***P. pentosaceus* LBM18**		***L. salivarius* LBM71**		***L. lactis* LBMB2**	
	MIC/result	Cut-off values	MIC/result	Cut-off values	MIC/result	Cut-off values
Clindamycin	< 0.125 (S)	1	< 0.125 (S)	4	< 0.125 (S)	2
Ampicillin	< 0.125 (S)	4	< 0.125 (S)	4	< 0.125 (S)	2
Chloramphenicol	1.0 (S)	4	1.0 (S)	4	1.0 (S)	8
Erythromycin	< 0.125 (S)	1	< 0.125 (S)	1	0.125 (S)	1
Gentamicin	1.0 (S)	16	4.0 (S)	16	4.0 (S)	32
Kanamycin	16.0 (S)	64	128 (R)	64	16.0 (S)	64
Streptomycin	32 (S)	64	128 (R)	64	32.0(S)	32
Tetracyclin	4.0 (S)	8	1.0 (S)	8	< 0.25 (S)	4
Vancomycin	NI (R)	NI (R)	NI (R)	NI (R)	< 0.5 (S)	4

Antibiotics / Disc concentration **(μg or U)**	LAB strains Diameter (mm) of inhibition zone / (result)		
	***P. pentosaceus* LBM18**	***L. salivarius* LBM71**	***Lac. lactis* LBMB2**
Amphotericin (100 U)	NI (R)	NI (R)	NI (R)
Ampicillin (10 μg)	24.94 (S)	25.71 (S)	20.67 (S)
Azithromycin (15 μg)	26.21 (S)	17.73 (I)	15.64 (I)
Bacitracin (10 U)	12.50 (S)	21.70 (S)	21.00 (S)
Clindamycin (2 μg)	34.17 (S)	21.92 (IS)	20.03 (I)
Chloramphenicol (30 μg)	32.57 (S)	25.53 (S)	21.86 (S)
Enrofloxacin (5 μg)	15.92 (I)	NI (R)	NI (R)
Erythromycin (15 μg)	30.67 (S)	22.44 (IS)	21.22 (IS)
Florfenicol (30 μg)	32.69 (S)	26.72 (S)	23.36 (S)
Gentamicin (10 μg)	18.00 (S)	21.50 (S)	22.00 (S)
Kanamycin (5 μg)	13.50 (I)	16.00 (I)	12.00 (R)
Nalidixic acid (30)	NI (R)	NI (R)	NI (R)
Neomycin (10 μg)	14.50 (I)	16.40 (I)	17.00 (I)
Novobiocin (30 μg)	18.60 (S)	28.00 (S)	28.60 (S)
Penicillin (10 U)	22.50 (S)	30.50 (S)	31.00 (S)
Rifampicin (5 μg)	25.08 (S)	21.45 (S)	20.03 (S)
Streptomycin (10 μg)	15.40 (SI)	11.00 (R)	14.30 (RI)
Tetracyclin (30 μg)	16.00 (I)	25.00 (S)	19.00 (S)
Vancomycin (30 μg)	NI (R)	NI (R)	NI (R)
Virulence test			
Conjugated coagulase	-	-	-
Free coagulase	-	-	-
Hemolysis	+ (β)	+ (α)	+ + (β)
Hemodigestion	+	+	+
Gelatinase	-	-	-
Proteolysis	-	-	-
Catalase	-	-	-

NI = no inhibition; S = susceptible; I = intermediate; R = resistant. Classified according to EFSA FEEDAP Panel (2018) for MIC assay and CLSI standards for diffusion disc test. *Staphylococcus aureus* CECT239 was used as virulence test control, while *Streptococcus agalactiae* was used as control strain for hemolytic activity (β-hemolysis). Intensity of hemolysis: (+): low; (++): moderate.

Table 3
Tolerance of *Pediococcus pentosaceus* LBM18, *Ligilactobacillus salivarius* LBM71 and *Lactococcus lactis* LBMB2 to different pH values and bile salts concentrations after 20 h of cultivation. Results were expressed in log CFU/mL.

pH	Strains		
	***P. pentosaceus* LBM18**	***L. salivarius* LBM71**	***L. lactis* LBMB2**
2.0	3.48 ± 0.00^{Ec}	5.30 ± 0.16^{Ea}	4.08 ± 0.16^{Eb}
3.0	6.81 ± 0.00^{Cb}	6.04 ± 0.52^{Dc}	7.20 ± 0.71^{Da}
4.0	6.65 ± 0.52^{Db}	6.36 ± 0.19^{Cc}	7.48 ± 0.00^{Ca}
6.0	9.34 ± 0.50^{Aa}	8.04 ± 0.46^{Ac}	9.04 ± 0.08^{Ab}
8.0	7.66 ± 0.53^{Bb}	7.28 ± 0.60^{Bc}	7.75 ± 0.30^{Ba}
Bile salts (%)			
0.1	7.23 ± 0.81^{Ba}	6.51 ± 0.13^{Db}	7.23 ± 0.21^{Ea}
0.2	6.80 ± 0.15^{Ac}	7.48 ± 0.00^{Ab}	7.88 ± 0.57^{Ba}
0.3	5.88 ± 0.41^{Cc}	6.82 ± 0.52^{Bb}	7.82 ± 0.82^{Ca}
0.4	5.20 ± 0.68^{Ec}	6.56 ± 0.81^{Cb}	7.30 ± 0.82^{Da}
0.5	5.41 ± 0.43^{Db}	5.30 ± 0.41^{Ec}	7.90 ± 0.12^{Aa}

Different capital letters in each column indicate significant differences among the three LAB strains at the same pH and bile salt concentration. Different lowercase letters in each line indicate significant differences for the same strain at different pH values and bile salt concentrations.

strains exhibited notable tolerance, with a reduction of 2 log units for *P. pentosaceus* (7.23–5.41 log CFU/mL) and only 1 log unit for *L. salivarius* (6.51–5.30 log CFU/mL) between the lowest (0.1 %) and highest (0.5 %) bile salt concentrations tested. In contrast, *L. lactis* showed no statistically significant growth reduction across all bile salt concentrations, indicating superior tolerance compared to the other two strains.

LAB adherence to intestinal epithelial cells and coexistence assay

The three LAB strains were able to adhere to Caco-2 cells (Table 4). Among them, *P. pentosaceus* LBM18 exhibited the greatest adhesion stability throughout the 4-hour experiment, with no significant decrease in the number of adhered cells (1 and 2 h = 5.56 log CFU/mL; 4 h = 5.23 log CFU/mL). In contrast, *L. salivarius* LBM71 showed the poorest performance (1 h = 4.08 log CFU/mL; 2 h = 4.18 log CFU/mL), with a complete loss of adhesion by the end of the experiment (4 h). The number of adhered *L. lactis* LBMB2 cells decreased in the first two hours (1 h = 4.20 log CFU/mL; 2 h = 4.08 log CFU/mL), followed by a further reduction of 1 log unit at the end of the experiment (3.11 log CFU/mL). Notably, *P. pentosaceus* LBM18 not only demonstrated superior adhesion stability but also a higher overall number of adhered cells. Based on

Table 4
Adhesion capacity of *Pediococcus pentosaceus* LBM18, *Ligilactobacillus salivarius* LBM71 and *Lactococcus lactis* LBMB2 to Caco-2 the intestinal epithelial cells Caco-2 inat 1, 2 and 4 h of experimentation. Results were expressed in log CFU/mL.

Time (h)	Strains		
	***P. pentosaceus* LBM18**	***L. salivarius* LBM71**	***L. lactis* LBMB2**
0	9.11 ± 0.10^{Ab}	8.34 ± 0.12^{Ac}	9.28 ± 0.13^{Aa}
1	5.56 ± 0.08^{Ba}	4.08 ± 0.15^{Cc}	4.20 ± 0.10^{Bb}
2	5.56 ± 0.10^{Ba}	4.18 ± 0.13^{Bb}	4.08 ± 0.10^{Cc}
4	5.23 ± 0.12^{Ca}	-	3.11 ± 0.12^{Db}

Different capital letters in the same column indicate significant differences for the same strain at different times of the experiment. Different lowercase letters in the same line indicate significant differences among the three LAB strains at the same time of experiment.

these results, if administered to a host, *P. pentosaceus* LBM18 would likely be the strain with the greatest potential to adhere to intestinal cells and exert a probiotic effect.

Organic acids and hydrogen peroxide production by LAB

High-performance liquid chromatography (HPLC) analysis demonstrated that the three LAB strains are homofermentative (Fig. 4A). Furthermore, all three strains were able to produce hydrogen peroxide (H_2O_2), a well-established characteristic of LAB [63]. Notably, *P. pentosaceus* LBM18 exhibited the highest hydrogen peroxide production, reaching 6.07 g/L after 24 hours of cultivation.

Genomic analyses of these strains confirmed their homofermentative nature, as no genes associated with acetyl-CoA synthetase, a key enzyme in acetic acid production, were detected. The genomes of all three isolates contained several essential genes like those related to acetolactate synthase, acetolactate decarboxylase, acetate kinase, acyl transport protein, ribulose-phosphate 3-epimerase, fumarate hydratase, L-lactate dehydrogenase, pyruvate kinase, and glucose-6-phosphate dehydrogenase (Table SM01c).

Furthermore, genes associated with the production of proteins involved in the scavenging of free radicals, such as thiol peroxidase and thioredoxin, were identified (Table SM01d). *P. pentosaceus* LBM18 contained three copies of thioredoxin, one of thiol peroxidase, and one of thioredoxin-disulfide reductase, which may explain its higher *in vitro* hydrogen peroxide production compared to the other isolates.

Growth kinetics of LAB

The growth kinetics of the three LAB strains were assessed, revealing peculiarities in their growth phases. P. pentosaceus LBM18 displayed a lag phase (4 hours) twice as long as that of L. salivarius LBM71 and L. lactis LBMB2. However, the duration of the exponential growth phase was similar for all strains, lasting approximately 4 hours. As a result, L. salivarius and L. lactis entered the stationary phase after 8 hours of cultivation, while P. pentosaceus reached it after 10 hours. All strains transitioned into the decline phase after 30 hours, with peak cell death occurring after 48 hours (Fig. 4B).

Acidification kinetics of LAB

As demonstrated in the previous subsection, *P. pentosaceus* LBM18 exhibited lower growth compared to *L. salivarius* LBM71 and *L. lactis* LBMB2. To ensure that *P. pentosaceus* LBM18 could compete in terms of biomass in co-culture, a 4-fold higher starting concentration was necessary. Without this adjustment, *P. pentosaceus* LBM18 would not have reached a sufficient concentration in the co-culture due to its slower growth rate, eventually diminishing over time. This 4-fold increase in starting concentration was determined from growth kinetics

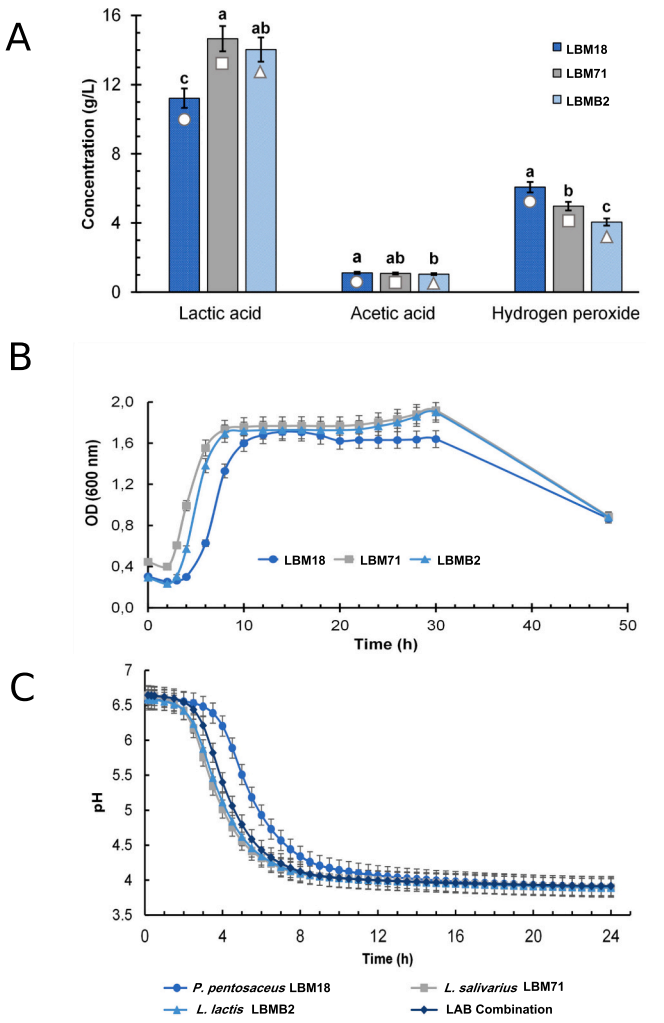

Fig. 4. Production of organic acids and hydrogen peroxide by LAB. (A) Production of lactic acid, acetic acid and hydrogen peroxide by *Pediococcus pentosaceus* LBM18 (dark cyan blue), *Ligilactobacillus salivarius* LBM71 (gray) and *Lactococcus lactis* LBMB2 (cyan blue) after 24 h of cultivation. (B) Growth kinetics of *Pediococcus pentosaceus* LBM18 (dark cyan blue), *Ligilactobacillus salivarius* LBM71 (gray) and *Lactococcus lactis* LBMB2 (cyan blue) during 48 h of cultivation. (C) Acidification profiles of *Pediococcus pentosaceus* LBM18 (dark cyan blue), *Ligilactobacillus salivarius* LBM71 (gray), *Lactococcus lactis* LBMB2 (cyan blue) and LAB combination (dark blue) during 24 h of cultivation.

data (Fig. 4 B), considering parameters such as growth rate and microbial concentration at the onset of the exponential phase. The pH decreased from 6.5 to 4.0 in approximately 12 hours in monocultures of *L. salivarius* and *L. lactis,* and consequently in their co-cultures, while *P. pentosaceus* took no less 15 hours to reach the same pH value (Fig. 4 C).

BLIS activity and minimum inhibitory concentration

To evaluate the antibacterial effect of BLIS produced by the three LAB strains against Gram-positive (Fig. 5 A) and Gram-negative (Fig. 5 B) pathogens, their activities were assessed every 2 hours by measuring optical density in 24-hour cultures of selected pathogens exposed to each cell-free supernatant (CFS). *P. pentosaceus* LBM18 showed the most potent strain-specific antimicrobial effect, exhibiting inhibition rates of 62 %, 48 %, 29 %, and 14 % against *Listeria monocytogenes* CECT934, *L. monocytogenes* NADC2045, *Clostridium perfringens* A-IB/CPDIV/Cp-A/s 01–07, and *Clostridium septicum* IB/CPDIV/Cp-A/s 01–07, respectively. In contrast, the BLIS produced by *L. salivarius* LBM71 was most effective

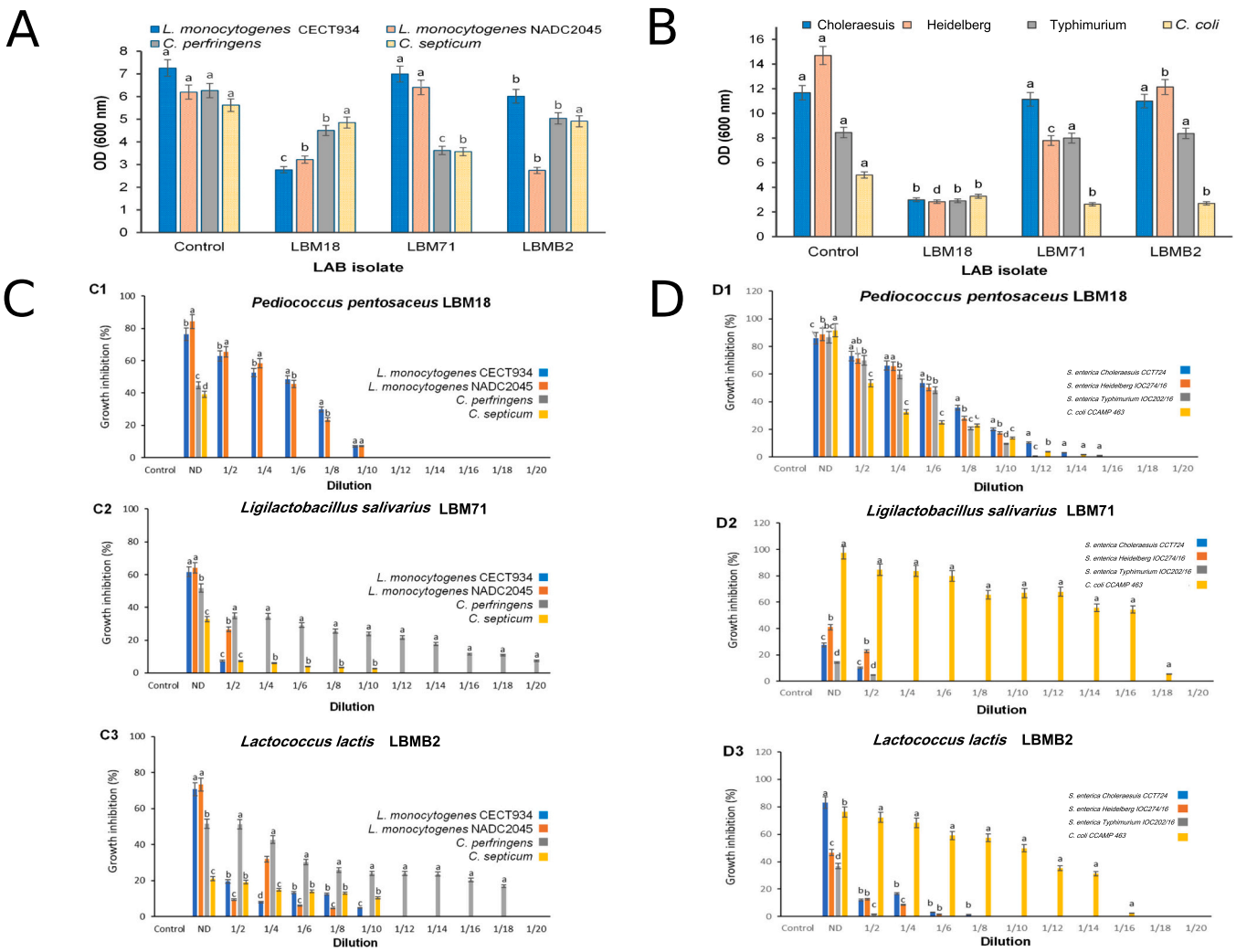

Fig. 5. BLIS activity and minimal inhibitory concentration the three LAB strains against Gram-positive and Gram-negative pathogens. Antibacterial activity of the BLIS produced by *Pediococcus pentosaceus* LBM18, *Ligilactobacillus salivarius* LBM71 and *Lactococcus lactis* LBMB2 against Gram-positive (A) and Gram-negative (B) pathogens after 24 h of cultivation. Control = absence of BLIS. (C) Inhibition of Gram-positive pathogens growth after 20 h of cultivation by BLIS produced by *Pediococcus pentosaceus* LBM18 (C1), *Ligilactobacillus salivarius* LBM71 (C2) and *Lactococcus lactis* LBMB2 (C3). Pathogens: *Listeria monocytogenes* CECT934 (dark cyan blue); *Listeria monocytogenes* NADC2045 (orange); *Clostridium perfringens* A-IB/CPDIV/Cp-A/s 01–07 (gray); *Clostridium septicum* IB/CPDIV/Cp-A/s 01–07 (yellow). (D) Inhibition of Gram-negative pathogens growth after 20 h of cultivation by BLIS produced by *Pediococcus pentosaceus* LBM18 (D1), *Ligilactobacillus salivarius* LBM71 (D2) and *Lactococcus lactis* LBMB2 (D3). Pathogens: *Salmonella enterica* serovar Choleraesuis CECT724 (dark cyan blue); *Salmonella enterica* serovar Heidelberg IOC274/16 (orange); *Salmonella enterica* serovar Typhimurium IOC202/16 (gray); *Campylobacter coli* CCAMP463 (yellow). Different letters mean statistically significant differences among results obtained using the same BLIS dilution. ND = no diluted BLIS; 1/2–1/20 = BLIS dilutions.

against clostridia, with inhibition rates of 43 % and 37 % against *C. perfringens* and *C. septicum*, respectively, while that produced by *L. lactis* LBMB2 yielded 17 %, 56 %, and 19 % inhibition against *L. monocytogenes* CECT934, *L. monocytogenes* NADC2045, and *C. perfringens* A-IB/CPDIV/Cp-A/s 01–07, respectively.

The BLIS produced by *P. pentosaceus* LBM18 also exhibited significant inhibition of Gram-negative pathogens relevant to agriculture, with inhibition rates of 74 %, 80 %, 65 %, and 34 % against *Salmonella enterica* serovar Choleraesuis CECT724, *S. enterica* serovar Heidelberg IOC274/16, *S. enterica* serovar Typhimurium IOC202/16, and *Campylobacter coli* CCAMP463, respectively (Fig. 5 A). The BLIS produced by *L. salivarius* LBM71 demonstrated effectiveness against *S. enterica* serovar Heidelberg IOC274/16 (46 %) and *C. coli* CCAMP463 (47 %), while the BLIS from *L. lactis* LBMB2 was successful exclusively against *C. coli* CCAMP463 (46 %) (Fig. 5 B).

None of the BLIS exhibited bactericidal effects, despite the significant inhibition of pathogen growth observed (Fig. 5 C and D), confirming their bacteriostatic nature. Against the two Gram-positive *L. monocytogenes* strains, the most pronounced inhibitory effects were observed with undiluted BLIS from *P. pentosaceus* (76–84 %) and *L. lactis* (71–73 %) (Fig. 5 C1 and C3). The inhibitory effect of *P. pentosaceus* BLIS persisted up to the tenth dilution (1/10; v/v), whereas that of *L. lactis* remained effective only up to the second dilution (1/2; v/v) (Fig. 5 C1 and C3).

For Gram-negative pathogens, the BLIS produced by *P. pentosaceus* exhibited greater than 85 % inhibition of all tested pathogens. Dilution significantly impacted the inhibition, with the sixth dilution (1/6; v/v) reducing *Salmonella* spp. growth by approximately 50 % and *C. coli* growth by 25 % (Fig. 5 D1). The BLIS from *L. salivarius* was less effective against *Salmonella* spp., achieving 41 % inhibition against *S. enterica* serovar Heidelberg (Fig. 5 D2), but demonstrated the highest efficacy against *C. coli* (97 %, undiluted BLIS). The BLIS from *L. lactis* inhibited *S. enterica* serovar Choleraesuis growth by 50–83 % (from 1/10 to undiluted), and *C. coli* CCAMP463 growth by 35–80 % up to the 1/14 dilution (Fig. 5 D3). Notably, BLIS from *P. pentosaceus* LBM18 and *L. lactis* LBMB2 inhibited *C. coli* CCAMP463 growth by 5 % and 2 %,

respectively, at 1/18 and 1/16 dilutions (Fig. 5 D1 and D3).

Antibacterial potential of LAB as microbial additive in agriculture

To assess the probiotic potential of the three LAB strains for formulating an animal feed additive, their antibacterial effects were also evaluated. The LAB strains, either alone or in combination, were cultured with pathogens for 24 hours, after which pathogen colonies were counted, and the results were expressed in CFU/mL. As shown in Fig. 6, the results were promising, particularly against *Salmonella* species. Notably, *L. salivarius* LBM71 not only completely suppressed the growth of *Listeria* and *Salmonella* species but also significantly inhibited those of *C. perfringens* (38 %), *C. septicum* (36 %), and *C. coli* (36 %). In contrast, despite their high antimicrobial effectiveness, *P. pentosaceus* LBM18 and *L. lactis* LBMB2 were less effective against *Listeria* pathogens (Fig. 6).

Discussion

The results of this study provide a comprehensive evaluation of the three selected LAB strains, namely *Pediococcus pentosaceus* LBM18, *Ligilactobacillus salivarius* LBM71, and *Lactococcus lactis* LBMB2. They were tested through both *in silico* and *in vitro* analyses to explore their potential as biotechnological tools for bacteriocin production or as direct feeding additive in poultry farming.

Genomic characterization and probiotic potential based on bacteriocinogenic properties

Genomic analysis confirmed the high-quality assembly and taxonomic identification of each isolate, consistent with established literature [57]. The absence of virulence factors, such as *mcr-1*, in all strains is crucial for ensuring the safety of these probiotics when used in animal feed [8–10,47]. Notably, all three LAB strains harbor multiple bacteriocin-related genes, highlighting their potential for antimicrobial activity.

For instance, P. pentosaceus LBM18 harbors the penocin-A bacteriocin operon, which is likely responsible for its broad-spectrum inhibition against both Gram-positive and Gram-negative pathogens [58]. In contrast, L. salivarius LBM71 produces four distinct bacteriocins, three of which commonly associated with the Lactobacillaceae family (plantaricin, lactacin F, and salivaricin P) and one related to Enterococcus faecalis (enterolysin A). These bacteriocins effectively target Gram-positive bacteria, such as Listeria and Staphylococcus, as well as Gram-negative bacteria like Escherichia and Pseudomonas [59]. In L. lactis LBMB2, complete operons for two bacteriocins with differing spectra of activity – nisin-Z (broad-spectrum) and lactococcin (narrow-spectrum) – were identified, further enhancing the strain's antagonistic potential against Gram-positive bacteria, including Listeria, Staphylococcus, and Enterococcus [59].

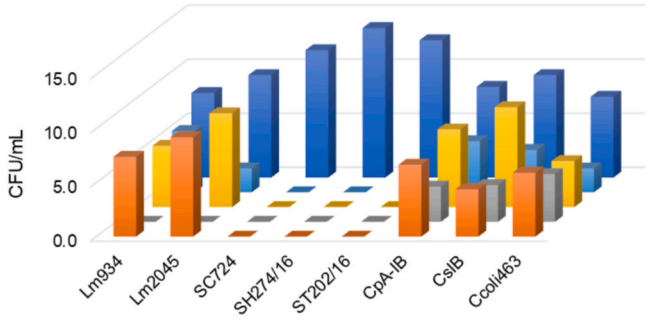

Fig. 6. Antibacterial potential of LAB as microbial additives with LAB strains alone or in combinations. Antibacterial effect of *Pediococcus pentosaceus* LBM18 (orange), *Ligilactobacillus salivarius* LBM71 (gray), *Lactococcus lactis* LBMB2 (yellow), LAB combination (cyan blue) and control (dark cyan blue) as microbial additives against Gram-positive and Gram-negative pathogens. Lm934: *Listeria monocytogenes* CECT934; Lm2045: *Listeria monocytogenes* NADC2045; SC724: *Salmonella enterica* serovar Choleraesuis CECT724; SH274/16: *Salmonella enterica* serovar Heidelberg IOC274/16; ST202/16: *Salmonella enterica* serovar Typhimurium IOC202/16; CpA-IB: *Clostridium perfringens* A-IB/CPDIV/Cp-A/s 01–07; CsIB: *Clostridium septicum* IB/CPDIV/Cp-A/s 01–07; Ccoli463: *Campylobacter coli* CCAMP463.

Importantly, the *in silico* analyses were validated by our *in vitro* assays, through which the probiotic potential of the three specific LAB strains was evaluated. These assays assessed tolerance to low pH and bile salts, production of organic acids and hydrogen peroxide, and antibacterial activity against pathogenic microorganisms.

Antibiotic resistance and safety considerations

A thorough assessment of resistance to antibiotics, particularly to those used in human and veterinary medicine, is essential [44], as additives should not contribute further to the pool of antibiotic resistance genes in the host's intestinal microbiota or the environment. Even when a bacterial species is considered to have Qualified Presumption of Safety (QPS) status by the European Food and Safety Authority (EFSA), it must not harbor antimicrobial resistance genes acquired from clinically relevant antimicrobials in order to maintain their safety status. In the present study, the presence of vancomycin resistance gene (*vanZ*) was detected in the genomes of *P. pentosaceus* LBM 18, *L. salivarius* LBM71 and *L. lactis* LBMB2. It is well established that vancomycin resistance genes are intrinsic to LAB species and it is typically chromosomally encoded, presenting a low risk for horizontal gene transfer [60,61]. Additionally, genes associated with tetracycline resistance (*lmrA*, *lmrC*, *lmrD*, and *lmrP*) and aminoglycoside resistance (*aph* (3 '')) were identified in *L. lactis* LBMB2 chromosome and classified as acquired genes based on the bioinformatic approach proposed by the EFSA BIOHAZ Panel [22].

Probiotic bacteria are known to harbor both intrinsic and mobile genetic elements that confer resistance to a broad spectrum of antibiotics [60]. Notably, *L. salivarius* LBM71 and *L. lactis* LBMB2, isolated from the intestines of poultry and fish, respectively, exhibited more similar antibiotic resistance profiles in qualitative test (disc diffusion), with a few exceptions. For example, *L. salivarius* LBM71 was classified as intermediate for kanamycin, while *L. lactis* LBMB2 was resistant; conversely, for streptomycin, *L. salivarius* LBM71 was resistant, and *L. lactis* LBMB2 was intermediate. This similarity in resistance profiles may reflect the use of antibiotics as growth promoters or pathogen inhibitors in animal husbandry. Furthermore, several LAB strains, including those from the *Ligilactobacillus*, *Bifidobacterium*, *Lactococcus*, and *Streptococcus* genera, exhibit intrinsic resistance to antibiotics such as kanamycin, gentamicin, streptomycin, and vancomycin [60], which was also observed in this study, with the exception of gentamicin, to which all three strains were sensitive. *P. pentosaceus* LBM18 displayed resistance to two antibiotics — streptomycin and vancomycin — which are known to be responsible for intrinsic resistances in this species [45]. Additionally, certain species within the Lactobacillaceae family naturally resist specific fluoroquinolone antibiotics [59]. Notably, all three isolates were sensitive to chloramphenicol and erythromycin, consistent with findings from studies on various LAB strains, including *Pediococcus acidilactici*, *P. pentosaceus*, *L. lactis*, and other Lactobacillaceae species [45].

It is important to note that the antibiogram disc diffusion is a valuable tool for establishing a preliminary antimicrobial susceptibility profile of potential probiotic bacteria. However, for use as a commercial zootechnical additive, regulatory requirements must be carefully considered. The European Food Safety Authority (EFSA) recommends that minimum inhibitory concentration (MIC) assays must be performed using antimicrobials considered relevant or critically important to both humans and animals [62]. In this study, *L. salivarius* LBM71 exhibited

values above the cutoff for streptomycin and kanamycin, despite the absence of resistance genes for these antibiotics in its genome. For the analysis, CARD, ARG-ANNOT, ResFinder datasets and the guidelines established by the EFSA Panel were utilized, indicating that no further studies are necessary.

The consistent patterns of antibiotic resistance observed across these LAB strains highlight the potential implications of these traits for the efficacy and safety of probiotic formulations, emphasizing the need for further investigation into their roles in microbial ecology and public health. However, the presence of a megaplasmid in L. salivarius LBM71 encoding antibiotic resistance genes for the lincosamide group (e.g., clindamycin) and tetracycline, as well as conjugation transfer genes, raises concerns. This megaplasmid also harbors a range of bacteriocin-related genes (plantaricin, lactacin F, and salivaricin P), highlighting its potential for antimicrobial activity. This is particularly significant because bacteria with distinct evolutionary backgrounds, coexisting in the intestine, may exchange resistance plasmids through conjugation, even across large taxonomic distances [63]. Gene transfer could also include bacteriocin genes, enhancing the probiotic capabilities and competitive advantage of recipient LAB strains by providing them with new bacteriocins and antibiotic resistance genes, potentially increasing their ability to inhibit pathogenic bacteria and promote intestinal health. However, a potential drawback is the spread of antibiotic resistance genes among different bacterial species via conjugation, exacerbating the global issue of antimicrobial resistance [63,64]. It is worth noting that these resistance genes are not flanked by insertion sequences or other mobile genetic elements, suggesting that they are unlikely to be transferred to the main chromosome via this specific mobilization system [65]. Consequently, these genes are likely stable features of the megaplasmid. Nevertheless, due to the presence of acquired antibiotic resistance genes in the *L. salivarius* LBM71 genome, this strain is not classified as QPS, warring further studies [22,44].

Conversely, metabolic genes, including those involved in heavy metal metabolism, were also identified within this megaplasmid. Previous studies on Lactobacillus pentosus MP-10 plasmids have demonstrated that plasmid-encoded metal tolerance genes are crucial for the bacterium's to survive during fermentation, thereby enhancing its probiotic properties and enabling it to thrive in diverse ecological environments [66]. Therefore, it is likely that the megaplasmid in L. salivarius LBM71 confers similar probiotic features. These findings suggests that further investigations, including plasmid-curing studies, are required to confirm the megaplasmids role in metal tolerance and biosequestration. Moreover, comprehensive risk assessments should be conducted to evaluate the presence of antibiotic resistance genes before L. salivarius LBM71 is considered for widespread application as a probiotic.

Alternatively, the bacteriocins identified on the L. salivarius LBM71 megaplasmid can be utilized independently. For instance, these bacteriocins may be encapsulated using various techniques, such as microencapsulation, nanoencapsulation, or polymer-based encapsulation, to improve their stability throughout the gastrointestinal tract and ensure controlled release. Previous studies have demonstrated that encapsulated bacteriocins are effective in inhibiting microbial growth [67,68].

Furthermore, to mitigate potential risks, several measures may be considered, including labeling probiotics with antibiotic susceptibility profiles, which could be a viable approach for the use of *L. salivarius* LBM71 [69]. In this context, the proposed formulation containing this isolate could be effectively integrated with therapies combining both antibiotics and probiotics. Another measure may be utilizing the cell-free supernatant from *L. salivarius* LBM71 to offer a safer alternative for harnessing the antimicrobial potential of this strain [70]. This strategy of using bacteriocins avoids the potential horizontal transfer of antibiotic resistance genes linked with megaplasmids, thereby reducing the risk of resistance dissemination. These approaches provide a promising strategy to exploit the antimicrobial properties of *L. salivarius* LBM71 while minimizing the associated risks of antibiotic resistance.

In contrast, due to the stable nature of their resistance mechanisms and the chromosomally encoded bacteriocins, *P. pentosaceus* LBM18 and *L. lactis* LBMB2 can be considered safe probiotics strains.

Assessment of virulence factors, catalase activity, and proteolytic-hemolytic properties in LAB strains

The negative coagulase test results for the three LAB strains demonstrate their inability to convert fibrinogen into fibrin, a key virulence factor commonly associated with various pathogens. This process allows the formation of clots, which "camouflage" bacteria from the host's immune system. These findings are consistent with the genomic data, as no coagulase genes were identified in the genomes of the strains. However, it is important to note that, despite the absence of clot formation, several granules were observed in the coagulase-free test. This could be attributed to the action of alternative proteases, potentially those activated by the *mecA* gene. Certain proteases in LAB are known to contribute to protein coagulation effects, even in the absence of traditional coagulases [71–73]. The identification of the *mecA* gene in all three LAB strains supports this hypothesis, as it is linked to the activation of the ClpC/ClpP/ClpX protein complex, which plays a critical role in protein metabolism and may compensate for the lack of conventional coagulase genes [73,74].

The hemolytic activity observed in these strains is consistent with their genomic profiles, which include genes from the hemolysin and hemolysin III families. Notably, the complete hemolytic activity exhibited by *L. lactis* LBMB2, along with the presence of additional hemolysin-related genes, particularly the XhlA domain, is significant. Hemolysin XhlA is known to contribute to the virulence of the Gram-negative bacterium *Xenorhabdus nematophila*, where it plays a role in immune cells lysis [75]. However, the *xhl*A gene in *L. lactis* LBMB2 is not closely related to the *xhl*A gene in *X. nematophila*, as indicated by the low sequence coverage (<10 %) and the absence of the hemagglutinin repeat domain (Table SM01e). This suggests that *L. lactis* LBMB2 is unlikely to pose a safety risk and can be considered safe for use as a probiotic additive.

Moreover, hemolysin-like proteins may contribute to the survival and colonization of probiotic bacteria in the gastrointestinal tract. For instance, studies involving *Bifidobacterium longum* BBMN68 and recombinant *L. lactis* strains have demonstrated that enahnced resistance to bile stress can improve colonization capabilities [75]. These findings align with the characteristics of LAB, which frequently exhibit proteolytic and hemolytic activities as a result of their metabolic and genetic profiles.

Although *P. pentosaceus* LBM18, *L. lactis* LBMB2 and *L. salivarius* LBM71 exhibited hemolytic characteristics in both *in vitro* and *in silico* analyses, all three species are classified as QPS by the EFSA. In accordance with the principles established in the Zootechnical Additives Regulation (EC) no 1831/2003 [44], assays for toxins and virulence factors are not required, as these species have been extensively documented as safe. Nevertheless, it remains crucial to assess hemolytic activity for LAB intended for use as probiotics during product development and *in vivo* trials.

In addition to evaluating virulence risk factors, the absence of gelatinase and proteolytic activity in these strains suggests a reduced risk of tissue degradation, which supports their potential use as safe probiotics. Furthermore, to assess their ability to utilize mucus, we screened all the genomes of all LAB strains for mucin-degrading GH families, including GH33 (sialidase), GH29/95 (fucosidases), GH20 (N-acetylglucosaminidase), GH2/35/42 (galactosidases), and GH16 (endo-O-glycanase). Although GH20 and GH1 were identified in *L. lactis* LBMB2, these enzymes alone are insufficient for mucin degradation due to the absence of GH33, GH29, GH95, and other critical hydrolases required for the removal of terminal glycans. This confirms that our LAB strains lack the necessary enzymatic repertoire to degrade intestinal mucus, further mitigating the risk of mucosal damage and reinforcing

their safety as probiotic candidates.

Production of organic acid and hydrogen peroxide

The HPLC analysis confirms that these LAB strains are homofermentative, which is beneficial for intestinal health, as acetic acid can be harmful [77,78]. The homofermentative nature of these strains is further supported by the genomic analysis, which revealed the presence of key genes in the EMP pathway, while genes associated with acetyl-CoA synthetase were absent. This ensure that these strains do not produce acetic acid, enhancing their suitability as probiotics.

The production of hydrogen peroxide by LAB is a well-estabilised antimicrobial mechanism that creates an inhospitable environment for pathogenic bacteria [77]. The notably high hydrogen peroxide production by *P. pentosaceus* LBM18 suggests that this strain may have enhanced antimicrobial properties, which, when combined with its bacteriocin production, could significantly contribute to its probiotic potential [79].

While H_2O_2 contributes to pathogen inhibition, its accumulation can also lead to the formation of other reactive oxygen species (ROS). However, the host possesses a complex set of antioxidant mechanisms (e.g., superoxide dismutase – SOD; catalase – CAT; glutathione peroxidase – GPx; glutathione – GSH), immune responses (e.g., dendritic cells, macrophages), and cellular repair systems (e.g., intestinal epithelial barrier, production of intestinal mucus) that help protect the intestine and the organism as a whole from the potentially harmful effects of probiotics and the ROS they generate. The key to maintain a healthy interaction lies in balance: while excessive ROS can lead to oxidative stress and inflammation, the host's mechanisms are designed to neutralize these effects, promoting a healthy gut microbiota and a controlled immune response [80,81].

The presence of multiple genes involved in free radical scavenging, particularly in *P. pentosaceus* LBM18, suggests an enhanced capacity to protect against oxidative stress, further reinforcing its potential as a robust probiotic strain. These genetic traits likely contribute to the its high hydrogen peroxide production and, in turn, its increased antimicrobial activity, positioning *P. pentosaceus* LBM18 a promising candidate for promoting gut health.

Acidification kinetics and adherence properties of lactic acid bacteria

The growth kinetics analysis indicates that *P. pentosaceus* LBM18 exhibits a slower growth rate compared to *L. salivarius* LBM71 and *L. lactis* LBMB2, which may affect its competitiveness in mixed cultures. However, starting with a higher initial concentration of *P. pentosaceus* LBM18 could help maintain its levels at significant concentrations, enabling it to effectively contribute to a mix LAB culture. Although *P. pentosaceus* LBM18 demonstrates slower acidification, which may limit its immediate impact on pH reduction, its prolonged lag phase may offer sustained probiotic benefits over time.

The combination of these strains in a probiotic formulation provide a balanced approach: *L. salivarius* and *L. lactis* offer rapid acidification and early stabilization of gut pH, while *P. pentosaceus* LBM18, with its superior adhesion stability, slower growth and sustained activity, ensures prolonged colonization and ongoing probiotic effects. This strategy aligns with the findings of Sarkar et al. [82], which demonstrated the complementary effects of different LAB strains in mixed fermentation, thereby enhancing overall efficacy and stability in probiotic applications.

Antagonistic potential of LAB strains against pathogenic microorganisms

In co-culture assays with avian pathogens, the LAB strains exhibited strong antagonistic activity, with *L. salivarius* LBM71 showing the highest efficacy. This strain effectively inhibited the growth of *Listeria* and *Salmonella* species and completely suppressed those of *Clostridium* spp. and *Campylobacter coli*, findings that aligns with the well-documented antagonistic properties of *L. salivarius* species [59,83–86].

Effective control of these pathogens is crucial in poultry farming due to their substantial impact on both animal and human health. For instance, *Salmonella* spp. is a significant threat, being one of the leading causes of foodborne illness worldwide, particularly for humans consuming contaminated poultry products [83]. *L. monocytogenes* poses a serious concern because it can cause listeriosis, a severe infection, and has the ability to persist in a wide range of environmental conditions, including refrigeration [84]. The inhibition of *Clostridium* spp., particularly *C. perfringens*, is vital to prevent necrotic enteritis, a debilitating disease that can decimate poultry flocks [85]. Additionally, *C. coli*, a globally distributed zoonotic pathogen, is the primary cause of bacterial gastroenteritis in humans, often associated to the consumption of poultry [86].

The LAB strains tested, particularly *L. salivarius* LBM71, exhibited significant antimicrobial potential, positioning them as valuable biocontrol agents in poultry farming. Their ability to effectively reduce pathogen load highlights their role in enhancing food safety by mitigating contamination risks, improving poultry intestinal health in poultry, and ultimately contributing to improved public health outcomes.

BLIS production and their antimicrobial properties

The pronounced antimicrobial activity exhibited by the BLIS produced by P. pentosaceus LBM18, particularly against Gram-negative pathogens such as Salmonella spp. and C. coli, emphasizes the potential of this strain in formulations designed to improve poultry health [82–85]. Similarly, L. salivarius LBM71 demonstrated potent BLIS production against Listeria and Clostridium species, thereby positioning it as a valuable candidate in strategies aiming at preventing bacterial infections in poultry [83–86]. Furthermore, *L. lactis* LBMB2 exhibited substantial bacteriostatic effects, particularly against Gram-positive bacteria such as *L. monocytogenes*. The presence of nisin-Z and lactococcin operons in this strain further enhances its capacity to inhibit the growth of pathogenic bacteria, reinforcing its potential as an effective feed additive [26]. Moreover, the broad-spectrum activity of nisin-Z significantly bolsters the strain's role as part of a comprehensive strategy to control the proliferation of detrimental microorganisms.

In pursuit of developing an effective probiotic feed additive, the high efficacy of P. pentosaceus LBM18 in inhibiting a broad spectrum of pathogens is particularly promising. Additionally, the rapid growth of *L. lactis* LBMB2 may offer immediate benefits, while the slower, more sustained activity of *P. pentosaceus* LBM18 is likely to ensure prolonged colonization and continued probiotic effects, especially against resistant and persistent pathogens [76]. The strain's ability to effectively inhibit harmful pathogens, including Listeria spp., Salmonella spp., and Campylobacter spp., supports its incorporation into a synergistic combination with *P. pentosaceus* LBM18 and L. lactis LBMB2. The complementary properties of these strains offer the potential for a robust defense against pathogenic microorganisms while simultaneously promoting overall gut health in poultry [21–26,82].

L. salivarius LBM71 harbors four distinct bacteriocins in its genome, namely plantaricin, lactacin F, salivaricin, and enterolysin A, which confer the strain the ability to effectively suppress the growth of pathogens such as *Listeria* spp., *Salmonella* spp., *Clostridium* spp., and *Campylobacter* spp. However, its megaplasmid carries antibiotic resistance genes, which may pose a risk of horizontal gene transfer. To mitigate this potential risk while preserving its antimicrobial efficacy, an alternative strategy would be to encapsulate each bacteriocin individually or utilize the cell-free supernatant [45,66,67], as previously suggested.

This study provides compelling evidence supporting the use of *P. pentosaceus* LBM18 and *L. lactis* LBMB2 as direct-fed microbial zootechnical additive. Furthermore, *L. salivarius* LBM71 demonstrated

potential as a bacteriocin producer, through the development of probiotic products that are assessed for antibiotic susceptibility. These LAB strains exhibit essential probiotic characteristics, including potent antimicrobial activity, resilience to acidic and bile environments, and strong adhesion to intestinal cells.

In summary, a potential combination of these LAB, along with encapsulated bacteriocins, offers a promising strategy for achieving both immediate and long-term benefits in animal production. This approach has the potential to reduce bacterial infections and reduce reliance on antibiotics, which is crucial in addressing the growing concern of antibiotic resistance in animal husbandry. Further research, including *in vivo* studies, is needed to validate these findings and optimize the formulation for practical application, potentially enhancing efficacy through the synergistic effects of bacteriocins.

Comparison with other feed additives

Probiotics, phytobiotics, organic acids, and prebiotics represent distinct but often synergistic strategies for antibiotic replacement. Phytobiotics are plant-derived compounds with potential anti-inflammatory and antimicrobial properties [87], while LAB-based probiotics confer additional benefits such as gut microbiota modulation and direct immune stimulation [55]. Although not tested here, combining bacteriocin-producing LAB with mild organic acidification or essential oil feed additives may offer complementary pathogen control. Future research should systematically compare these approaches under standardized farm conditions to establish the most cost-effective and biologically robust alternatives to antibiotics.

Potential risks and mitigation

Although our results suggests an overall safe profile — characterized by coagulase-negative status, the absence of known virulence factors, and predominantly intrinsic resistance genes — concerns remains regarding the presence of transferable elements in the megaplasmids of *L. salivarius* LBM71. While horizontal gene transfer has been infrequently reported in *Ligilactobacillus* spp., it cannot be entirely excluded in the complex environment of the gut microbiota. Comprehensive in vivo safety assessment, including histopathological and immunological endpoints, is necessary to further confirm that these LAB strains do not induce inflammatory or dysbiotic changes in poultry [88].

While we adhered to EFSA (EU) antibiotic susceptibility standards, other regions have parallel, yet not identical frameworks for probiotic approvals (e.g., U.S. FDA, Canadian CFIA, Brazilian MAPA). Harmonizing safety assessments and labeling guidelines across region is a crucial step toward the global acceptance of LAB-based zootechnical additives.

The large-scale application of these LAB may influence manure microbiomes or nutrient cycling in poultry litter. Preliminary evidence suggests that probiotic-driven shifts could have environmental beneficial, such as reduced ammonia emissions or decreased pathogen shedding [89,90]; however, systematic field monitoring is needed to confirm the overall ecological impacts.

Materials and methods

Lactic acid bacteria and target pathogens

Potentially probiotic LAB were isolated from various production animals. *Pediococcus pentosaceus* LBM18 was isolated from corn silage [24], *Ligilactobacillus salivarius* LBM71 from the poultry intestine following the methodology described by Sabo et al. [25], and *Lactococcus lactis* LBMB2 from the intestine of rainbow trout (*Oncorhynchus mykiss*) [26]. The LAB strains were cultured in De Man, Rogosa, and Sharp (MRS) broth (Difco Laboratories, Detroit, MI, USA) at 37 °C under anaerobic conditions using an anaerobic jar.

Different pathogenic strains commonly found in poultry farming were selected for this study. *Listeria monocytogenes* CECT934 and *Salmonella enterica* serovar Choleraesuis CECT724, obtained from the Spanish Type Culture Collection (Valencia, Spain), as well as *Listeria monocytogenes* NAD2045, *Salmonella enterica* serovar Heidelberg IOC 274/16, and *Salmonella enterica* serovar Typhimurium IOC 202/16, generously provided by Fiocruz (Rio de Janeiro, Brazil), were cultured in Brain Heart Infusion (BHI) broth (Kasvi, São José dos Pinhais, PR, Brazil) at 37 °C under aerobic conditions using a Biochemical Oxygen Demand incubator. *Clostridium perfringens* A-IB/CPDIV/Cp-A/s 01–07 and *Clostridium septicum* IB/CPDIV/Cp-A/s 01–07, provided by Fiocruz, were cultured in thioglycolate broth (Kasvi) at 37 °C under anaerobic conditions using an anaerobic jar. *Campylobacter coli* CCAMP 463, also provided by Fiocruz, was cultured in MacConkey broth (Kasvi) supplemented with 5 % defibrinated sheep's blood and 5 % oxygen-reducing solution (ferrous sulfate 0,1 g/L, sodium bisulfate 0,1 g/L, and sodium pyruvate 0,5 g/L) at 42 °C under microaerophilic conditions (5 % CO_2).

Genome sequencing of the LAB isolates

DNA isolation and PacBio HiFi REVIO™ sequencing for genome analysis

Genomic DNA was extracted from each bacterial isolate using the PureLink™ Genomic DNA Mini Kit (Invitrogen™, Thermo Fisher Scientific, Wilmington, DE, USA), following the manufacturer's protocols. The quantity and purity of the extracted DNA were assessed using spectrophotometry (NanoDrop One/One^c - Thermo Fisher Scientific, USA) and fluorometry (Qubit 2.0™ - Thermo Fisher Scientific). DNA libraries were prepared using the PacBio HiFi protocol and sequenced on the PacBio HiFi REVIO™ platform. All library preparation and sequencing procedures were carried out at the Arizona Genomics Institute.

Quality assessement of raw reads, genome assembly, and taxonomic identification

The raw reads were assembled using HiFiAsm-meta (v0.3-r073) [27]. The complete circular microbial genomes were reoriented using the DNAapler tool (v0.7.0) [28]. Assembled genomes were analyzed for taxonomic identification and quality assessment using the GTDB-Tk tool (v2.3.2) and the Genome Taxonomy Database (GTDB vR214) [29], as well as CheckM [30], and BUSCO (v5.6.1) (lactobacillales_odb10) [31]. The Average Nucleotide Identity (ANI) was calculated by comparing the genomes to the reference genomes of *P. pentosaceus* DSM 20336 (GCF_001437285.1), *L. salivarius* DSM 20555 = ATCC 11741 (GCF_001435955.1), and *L. lactis* ATCC 19435 (GCF_900099625.1).

Structural and functional genome annotation

Structural and functional annotation of the DNA sequences was performed using PGAP (v2023–10–03.build7061) [32] and eggNOG-mapper [33]. The identification of prophages within bacterial genomes was carried out using the PHASTEST (v3.0) [34]. Circular genome representations were generated using the GenoVi pipeline (v0.4.3) [35]. To identify antibiotic resistance genes (ARGs), protein-coding nucleotide sequences from each genome were compared against different databases, including NCBI AMRFinderPlus [36], CARD [37], ResFinder [38], ARG-ANNOT [39], MEGARES [40], and PlasmidFinder [41] using the ABRICATE tool (v1.0.1) (https://github.com/tseemann/abricate). Additionally, genomes were analyzed for the presence of Biosynthetic Gene Clusters (BGCs) potentially involved with antimicrobial activity using BAGEL (v.4.0) [42]. All figures were edited with Inkscape (https://inkscape.org).

In vitro evaluation of potential probiotic properties of LAB isolates

Antibiotic susceptibility testing. Antibiotic susceptibility testing was carried out following the standard Kirby-Bauer disk diffusion method,

recommended by the Clinical and Laboratory Standards Institute (CLSI) [43]. The following antibiotics were tested: amphotericin (AP, 100 U), ampicillin (AMP, 10 μg), azithromycin (AZI, 15 μg), bacitracin (B, 10 U), alindamycin (CLI, 2 μg), chloramphenicol (CHL, 30 μg), enrofloxacin (ENO, 5 μg), erythromycin (ERI, 15 μg), florfenicol (FFC, 30 μg), gentamicin (GEN, 10 μg), kanamycin (K, 5 μg), nalidixic acid (NA, 30 μg), neomycin (NEO, 10 μg), novobiocin (NV, 30 μg), penicillin (P, 10 U), rifampicin (RIF, 5 μg), streptomycin (STR, 10 μg), tetracyclin (TE, 30 μg) and vancomycin (VAN, 30 μg).

Minimum Inhibitory Concentration (MIC) of ampicillin, clindamycin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin, tetracyclin and vancomycin was determined using a serial twofold dilution method in broth, as described by the ESFA [44]. Briefly, the prepared working solutions of antibiotics were subjected to double dilutions and distributed into the appropriate wells of microtiter plates, each containing 5×10^5 CFU (Colony Forming Units)/mL of bacterial suspension in Cation-Adjusted Mueller-Hinton Broth (CAMHB, 90 %) supplemented with MRS (10 %) culture medium for LAB [45]. The plates were incubated at 37 ± 1 °C for 12–18 hours. Strains were classified as resistant or susceptible to each antibiotic tested according to microbiological cut-off values established by the FEEDAP Panel (Table 2). Quality control was carried out by assessing MIC of *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC29213 reference strains, in accordance with the recommendations of CLSI. This experiment was performed in triplicate to ensure the accuracy and reproducibility of the results.

Coagulase test for identification and characterization

Two types of coagulase tests were performed: bound and free coagulase. For the bound coagulase test, isolated LAB strains were streaked onto the surface of MRS agar (1.5 %, w/v) and incubated at 37 °C for 24 hours. *S. aureus* CECT239, a known positive coagulase producer, was used as a control and streaked onto the surface of Tryptic Soy Broth (TSB) (Difco) agar (1.5 %, w/v), followed by incubation at 37 °C for 24 hours. To detect the presence of bound coagulase or clumping factor, a drop of sterile water was added to a microscope slide, followed by the addiction of bacterial colonies. A drop of human plasma was then mixed, and the presence of positive coagulase reaction was indicated by the clumping of plasma fibrinogen on the slide. For the free coagulase test, 500 μL of rabbit plasma were mixed with 100 μL of an overnight LAB culture and incubated at 37 °C for 24 hours. Both tests were performed in triplicate, and the results were compared to the known coagulase-positive control.

Hemolytic activity assessment

The hemolytic activity was assessed on Petri dishes containing Columbia-Agar Basis (Roth®) medium supplemented with 5 % sheep blood (Columbia-Blood). After overnight culture, LAB strains were streaked onto the surface of the Columbia-Blood agar plates using a loop, and the plates were incubated aerobically at 37 °C for 48–72 hours.

Gelatinase production assay

The gelatinase test was performed according to Domingos-Lopes et al. [46]. A gelatin agar medium was prepared by mixing gelatin (3 %, w/v), peptone (0.5 %, w/v), yeast extract (0.3 %, w/v), and bacteriological agar (1.7 %, w/v). An aliquot of 10 μL from each overnight LAB culture grown in MRS broth, incubated at 37 °C for 24 hours under anaerobic conditions, was inoculated into 3 mL of gelatin agar medium contained in sterile glass tubes. The tubes were initially incubated at 37 °C for 48 hours under anaerobic conditions, followed by incubation at 4 °C for 1 hour.

Proteolytic activity assay

Proteolytic activity was assessed following the growth of LAB in MRS broth (Difco) at 37 °C for 24 hours under anaerobic conditions. An aliquot of the LAB cultures was streaked onto MRS agar (1.5 %, w/v) supplemented with lactose-free skim milk (10 %, w/v) using a microbiological loop to obtain isolated colonies. The plates were then incubated at 37 °C for 48 hours under anaerobic conditions. After the incubation period, the plates were washed with 1.0 N HCl to visualize proteolytic activity.

Catalase activity assay

To perform the catalase test, a microscope slide was used. A small amount of LAB colonies was mixed with one drop of 3 % hydrogen peroxide (H_2O_2) (Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany).

Tolerance to low pH and bile salts of LAB

The tolerance of the LAB strains to low pH and varying concentrations of bile salts was assessed following the methodology described by Tan et al. [47]. After overnight cultivation in MRS broth (Difco) at 37 °C, a suspension with an optical density of 1.0 at 600 nm ($OD_{600\ nm}$), corresponding to 10^8 CFU/mL of each LAB strain, was centrifuged at 4470 ×g for 10 minutes at 4 °C and washed with sterile 0.85 % (w/v) saline solution. The resulting pellet was then resuspended in MRS broth adjusted to different pH values (2.0, 3.0, 4.0, and 6.0), with the pH 6.0 medium serving as the control. The pH was adjusted using 1.0 N HCl solution (Labsynth, Diadema, São Paulo, Brazil), and the media were subsequently autoclaved at 121 °C for 15 minutes. For the tolerance test, 1 mL of each LAB suspension ($OD_{600\ nm} = 1.0$) was inoculated in 5 mL of pH-adjusted MRS broth and incubated at 37 °C for 20 hours. Serial dilutions (10^{-1} to 10^{-6}) of each culture were then performed and plated onto Petri dishes containing MRS agar (1.0 %, w/v).

To evaluate the effect of different concentrations of bile salts, the procedure described above for the cultivation and preparation of LAB inoculum was followed. Briefly, 1 mL of each LAB suspension ($OD_{600\ nm} = 1.0$) was inoculated in 5 mL of MRS broth supplemented with varying concentrations of bile salts (0.1 %, 0.2 %, 0.3 %, 0.4 %, and 0.5 %) and incubated at 37 °C for 20 hours. After incubation, serial dilutions (10^{-1} to 10^{-6}) of cultures were performed and plated onto Petri dishes containing MRS agar (1.0 %, w/v).

Adhesion capacity of LAB to intestinal epithelial cells

To assess the adhesion capacity of LAB to intestinal epithelial cells, the Caco-2 cell line (human colon adenocarcinoma) was employed. For this, 6-well culture plates were used to seed 1 mL of Caco-2 cells (2×10^5 cells/well) in 5 mL of low-glucose DMEM medium (Thermo Fisher Scientific) supplemented with 10 % fetal bovine serum (FBS) and antibiotics (10,000 units/mL penicillin, 10,000 μg/mL streptomycin, and 25 μg/mL amphotericin) (Thermo Fisher Scientific). The plates were incubated at 37 °C with 5 % CO_2 for approximately three days to allow for sufficient cell growth (monolayer formation) prior to the experiment.

To perform the adhesion assay, a suspension of each LAB strain at 10^7 CFU/mL ($OD_{600\ nm} = 0.9$) was prepared from an overnight culture in MRS broth (Difco) at 37 °C, followed by centrifugation (10,000 ×*g* for 10 minutes) to obtain a bacterial pellet. The pellet was then resuspended in the same culture medium used for growing the Caco-2 cells (low-glucose DMEM with 10 % FBS), but without antibiotics. The Caco-2 cell monolayer was washed twice with sterile 1X PBS to thoroughly remove any residual antibiotics from the medium.

Subsequently, 1 mL of each LAB inoculum (10^7 CFU/mL) was added to the Caco-2 cells monolayer, and the plates were incubated at 37 °C with 5 % CO_2 for 1, 2, and 4 hours. After incubation, the Caco-2 cell monolayer was washed twice with 1X PBS to remove non-adhered

bacterial cells and then lysed by adding 0.1 % Triton X-100 (Sigma®) to the wells. The resulting suspension, containing viable bacterial cells adhered to Caco-2 cells, was serially diluted, plated onto Petri dishes containing MRS agar (1 %, w/v), and incubated at 37 °C for 24 hours. The experiment was performed in duplicate.

Coexistence assay

The coexistence test assesses the potential for co-cultivation between bacteria, following the method described by Guo et al. [48]. Briefly, LAB were grown in MRS broth at 37 °C for 24 hours. Forty microliters of each LAB culture were pipetted onto the surface of MRS agar (1.5 % w/v) with the plate positioned vertically, arranging the cultures in a grid pattern. After 24 hours of incubation at 37 °C, growth inhibition between the isolates was visually evaluated.

Production of organic acids and hydrogen peroxide

The cell-free supernatants (CFS) of LAB cultures were analyzed for the concentrations of lactic acid, acetic acid, and hydrogen peroxide using High-Performance Liquid Chromatograph (HPLC, Shimadzu, Kyoto, Japan). The system was equipped with a 300 × 7.8 mm Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and a UV-Vis detector (SPD-M20A, Shimadzu) set to 210 nm for organic acids and 254 nm for hydrogen peroxide detection [49]. The quantification of the compounds was carried out under isocratic separation conditions, with a flow rate of 0.6 mL/min, using a 5 mM sulfuric acid solution in Milli-Q water as the mobile phase. The sample injection volume was 10 μL, and the total analysis time was 28 minutes.

Growth kinetics of LAB

The growth kinetics of the LAB strains were determined by optical density measurements ($OD_{600\ nm}$) using a microplate reader (BioTek, Winooski, VT, USA). The initial concentration of each LAB culture was adjusted to an OD of 1.0, corresponding to 10^8 CFU/mL. The cultures were incubated in MRS broth at 37 °C in sterile 96-well plate (TPP, Trasadingen, Switzerland), and OD measurements were automatically recorded every 2 hours for 24 hours. To construct the growth kinetic curve, samples were taken at 48-hour intervals from the microplate reader for colony counting.

Acidification kinetics of LAB

The acidification profile of the LAB strains was assessed using the Cinétique d'Acidification System (Cinac 14, Ysebaert, Frépillon, France) at 37 °C for 24 hours. MRS broth was used to culture the three selected LAB strains, and the acidification profile was determined for each LAB individually and in combination, with data analysis performed using Cinac System software (Ysebaert).

Production, activity, and minimum inhibitory concentration (MIC) of bacteriocin-like inhibitory substances (BLIS)

After cultivation of each LAB strain in MRS broth at 37 °C for 24 hours under anaerobic conditions, the cultures were centrifuged at 4470 ×*g* for 10 minutes at 4 °C to recover the cell-free supernatant (CFS). The pH of the CFS was adjusted to 6.0–6.5 using 1.0 M NaOH to stabilize the organic acids produced by the LAB. The CFS was then exposed to 80 °C for 3 minutes to inactivate dissolved enzymes [50]. To sterilize, the CFS was filtered through 0.22 μm pore size filters, and its antimicrobial activity was assessed at different growth stages of selected pathogens using $OD_{600\ nm}$ measurements [51].

The antimicrobial activity of BLIS against each pathogen was assessed in sterile 96-well plates (TPP) containing 100 μL of the respective broth, 50 μL of overnight cultures of each pathogen, and 50 μL of BLIS. The final mixture (200 μL) was thoroughly mixed, and the microplate was incubated inside the microplate reader (BioTek) at pathogen-specific temperatures: 37 °C for *Listeria* spp., *Salmonella* spp., and *Clostridium* spp., and 42 °C for *Campylobacter* spp. Pathogen growth was monitored automatically every hour for 24 hours by measuring absorbance ($OD_{600\ nm}$). The BLIS evaluation was performed in triplicate.

The broth microdilution method was employed to determine the Minimum Inhibitory Concentration (MIC) of BLIS, defined as the lowest concentration of BLIS that completely inhibits the growth of a pathogen in the microdilution wells [52]. The experimental procedure is illustrated in Figure S1.

Antibacterial activity of LAB in co-culture with pathogens strains

To directly evaluate the antimicrobial effect of each LAB strain, the colony count methodology described by Piazentin et al. [53] was employed. Specifically, 3 mL of pathogen-specific culture medium were added to 15 mL Falcon® tubes, along with 1 mL of each LAB suspension (OD600nm = 1.0, corresponding to approximately 10^8 CFU/mL), either alone or in combination, and 1 mL of overnight diluted pathogen culture ($\sim 10^6$ CFU/mL) in the same culture medium used for its growth. The control samples consisted of the same volume of culture medium without LAB suspension. The tubes were incubated for 20 hours at the optimal temperature for each pathogen. Serial dilutions of both control and treated samples were performed, followed by plating onto Petri dishes containing the respective specific agar medium for each pathogen. Incubation was then carried out at the optimal temperature for each pathogen. Each experiment was performed in triplicate.

Statistical analysis

The results, expressed as mean values ± standard deviations, were subjected to one-way analysis of variance (ANOVA) using Statistica Software 12 (TIBCO Software Inc., Palo Alto, CA, USA). Post-hoc omparisons were made using Tukey's test with a significance level set at $p < 0.05$. Viable bacteria counts were log-transformed prior to analysis. The data from tolerance tests were also compared using Statistica 12 [26].

Nucleotide sequence accession numbers

The complete genome sequences of the three LAB strains were submitted to the National Center for Biotechnology Information (NCBI). The accession numbers for *P. pentosaceus* LBM18 are CP160877 (chromosome genome), CP160878 (plasmid 1), and CP160879 (plasmid 2). For *L. salivarius* LBM71, the accession numbers are CP160868 (chromosome genome), CP160869 (plasmid 1), and CP160870 (plasmid 2). The accession numbers for *L. lactis* LBMB2 are CP162855 (chromosome genome) and CP162856 (plasmid 1).

CRediT authorship contribution statement

Gierus Martin: Writing – review & editing. **de Medeiros Oliveira Mauro:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Converti Attilio:** Writing – review & editing. **de Azevedo Pamela Oliveira de Souza:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **de Souza Oliveira Ricardo Pinheiro:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Matajira Carlos Emilio Cabrera:** Conceptualization. **Varani Alessandro M.:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Formal analysis, Data curation, Conceptualization. **Kuniyoshi Taís Mayumi:** Writing – review & editing, Methodology. **Meireles Piazentin Anna Carolina:** Validation, Methodology, Formal analysis, Data curation. **Santos Pessoa**

Amanda Romana: Conceptualization. **Pereira Wellison Amorim:** Methodology. **Domínguez José Manuel:** Writing – review & editing, Visualization, Methodology. **Mendonça Carlos Miguel Nóbrega:** Methodology. **Dias Meriellen:** Conceptualization. **Frota Elionio Galvão:** Conceptualization. **Bermúdez-Puga Sebastián Armando:** Conceptualization.

Ethics statement

This study has no animal or human experiments. There are no ethical issues involved.

Declaration of Competing Interest

Authors declare that they have no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2025.04.008.

Data availability

All data generated or analyzed during this study are included in this article and its supporting information files.

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