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Selection of Beneficial Bacterial Strains With Potential as Oral Probiotic Candidates

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Abstract

This study aimed to select beneficial strains from the oral cavity of healthy volunteers and to evaluate these as potential oral probiotic candidates. The selection process was based on the isolation, differentiation, identification, and safety assessment of LAB strains, followed by a series of experiments for the selection of appropriate candidates with beneficial properties. In the screening procedure, 8 isolates from the oral cavity of a Caucasian volunteers were identified as *Streptococcus* (*Str.*) *salivarius* ST48HK, ST59HK, ST61HK, and ST62HK; *Lactiplantibacillus plantarum* (*Lb.*) (*Lactobacillus plantarum*) ST63HK and ST66HK; *Latilactobacillus sakei* (*Lb.*) (*Lactobacillus sakei*) ST69HK; and *Lactobacillus* (*Lb.*) *gasseri* ST16HK based on 16S rRNA sequencing. Physiological and phenotypic tests did not show hemolytic, proteinase, or gelatinase activities, as well as production of biogenic amines. In addition, screening for the presence of *efa*A, *cyt*, *IS*16, *esp*, *asa*1, and *hyl* virulence genes and vancomycin-resistant genes confirmed safety of the studied strains. Moreover, cell-to-cell antagonism indicated that the strains were able to inhibit the growth of tested representatives from the genera *Bacillus*, *Enterococcus*, *Streptococcus*, and *Staphylococcus* in a strain-specific manner. Various beneficial genes were detected including *gad* gene, which codes for GABA production. Furthermore, cell surface hydrophobicity levels ranging between 1.58% and 85% were determined. The studied strains have also demonstrated high survivability in a broad range of pH (4.0–8.0). The interaction of the 8 putative probiotic candidates with drugs from different groups and oral hygiene products were evaluated for their MICs. This is to determine if the application of these drugs and hygiene products can negatively affect the oral probiotic candidates. Overall, antagonistic properties, safety assessment, and high rates of survival in the presence of these commonly used drugs and oral hygiene products indicate *Str. salivarius* ST48HK, ST59HK, ST61HK, and ST62HK; *Lb. plantarum* ST63HK and ST66HK; *Lb. sakei* ST69HK; and *Lb. gasseri* ST16HK as promising oral cavity probiotic candidates.

Keywords Beneficial · *Streptococcus salivarius* · *Lactiplantibacillus plantarum* · *Latilactobacillus sakei* · *Lactobacillus gasseri* · Oral cavity · Bacteriocins

Introduction

According to the World Health Organization, probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer benefits to the health of the host" [1]. With positive effects on human health, probiotics can contribute to the prevention or treatment of different clinical conditions [2]. Positive health benefits of bacterial species on humans were initially suggested by Ilya Ilyich Mechnikov and his collaborator Stamen Grigorov. This concept has since been developed over the last century [3–5] and became well established as a powerful tool and even as an adjunct to western medicine in the prevention and treatment of some diseases [6–8]. Although the initial concept for the application of probiotics targeted the gastro-intestinal tract (GIT) microbial balance [9], their role and impact on the immune system, skin health, brain, kidney, and liver functions has also been progressively explored in the past decades [10, 11].

The oral cavity, serving as a principal access to the digestive tract, plays an essential role in the wellness of humans and other animals [12]. The oral cavity is a complex structure composed of several elements, such as the teeth, tongue, palate, and buccal mucosa, that are involved in a functional

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complex. These serve as principal habitats for diverse microorganisms involved in and supporting the normal functional *status quo* of macroorganisms [13]. It was suggested that more than 700-1000 bacterial species are permanently or transiently colonize the human oral cavity and play a role in the functional balance. Additionally, the biodiversity in the human oral cavity also includes representatives of some viruses, fungi, and even members of the Archaea [2, 14]. The oral cavity of humans and other animals is a habitat for different representatives of lactic acid bacteria (LAB), which comprise a large group of bacteria from the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Enterococcus*, and *Weissella*, some of which plays a significant role in oral health [12]. LAB are Gram-positive bacteria belonging to the phylum Firmicutes, class Bacilli, and order Lactobacillales, with a low G + C content (less than or equal to 55 mol %) in their DNA. In the human oral cavity, LAB are mainly from the genera *Lactobacillus* (now subdivided into 25 new genera) and *Streptococcus*, which account for 29.2% of the whole oral microbiome. LAB are widely spread in nature and are known to be autochthonous, but several representatives are specifically considered as beneficial microorganisms and are described and applied as probiotic cultures [8, 15].

The conditions in the human mouth provide an optimal setting for the growth and colonization of microorganisms. However, the specificity of the saliva composition and presence of different enzymes and bioactive peptides serve as selection pressure for microbial presence and dominance in the mouth [16, 17]. The environmental conditions in the oral cavity also allow successful niching in for various microorganisms due to the optimal temperature, ambient moisture, available nutrients, minerals, vitamins, and various tissue surfaces that enable successful microbial attachment, which ultimately facilitates colonization in this environment. This exemplifies the diverse ecological niches associated with living systems, which are innately equipped with deterrents and mechanisms for effective reduction and/or control of different pathogens. Furthermore, it has been confirmed that the saliva of several animals was found to be highly antiseptic [18, 19].

The role of LAB in the oral cavity needs to be evaluated as either beneficial or detrimental. Several LAB can be considered as beneficial, especially those that are adapted to the mouth, since they promote microbial balance, which subsequently results in good oral health [12, 15]. Some specific LAB or oral pathogens, however, may contribute to oral diseases such as dental caries and periodontitis [20]. Previous studies have shown the relationship between oral pathogens and precancerous lesions of gastric cancer (PLGC), rheumatoid arthritis, and type-2 diabetes, indicating that oral health is an important component of overall human health [8, 21, 22]. The beneficial properties of LAB (and generally for all probiotics) are strain-specific [23]. In the selection process of appropriate probiotic candidates, both for application in the oral cavity and/or other purposes, careful evaluation and consideration of their association with specific bacterial strain(s) is necessary, especially concerning the environment in which they are intended to be applied. Some of the key factors in the evaluation process of new probiotic strains are their interactions with microorganisms typical of the specific ecological niche, its safety, and appropriate identification.

LAB, particularly representatives of the former genus *Lactobacillus*, were evaluated and applied as potential probiotics in numerous studies [24, 25]. There have been reports of LAB with beneficial effects on the control of dental problems, such as dental caries, which is frequently associated with the oral pathogen *Streptococcus mutans*, and halitosis, which is commonly caused by Gram-negative bacteria such as *Porphyromonas gingivalis* [20, 26]. Oral probiotics strains, developed for dental health, have been investigated in clinical studies. Some of these include the application of *Streptococcus salivarius* strains M18 and K12 as reported by Wescombe et al. [27] and He et al. [29]. It was demonstrated that *Str. salivarius* M18 is effective in inhibiting the oral pathogen *Str. mutans*, thereby reducing the risk of dental caries [27, 28]. In the other study, *Str. salivarius* K12 was found to have a beneficial effect on dental health and supported halitosis treatment post-removal of tongue coating [29].

The aim of this study was to select possible beneficial strains from the oral cavity of healthy volunteers and to evaluate these as potential oral probiotic candidates. The selection process was based on isolation of LAB strains, differentiation, identification, safety assessment, and a series of experiments leading to the selection of potential candidates for the application as oral probiotics.

Materials and Methods

Isolation, Differentiation, Identification, and Selection of LAB Strains with Antimicrobial Activity

For the isolation of potential oral probiotic candidates with antagonistic properties against common oral cavity pathogens, saliva swabs from 26 healthy volunteers of Caucasian and Asian ethnicities were obtained and processed. The modified triple agar layer method was adapted from dos Santos et al. [30] and performed on M17-lactose (Difco, Detroit, MI, USA) and MRS (Difco) supplemented with 1% agar (Difco). Saliva swabs were surface spread on the solid media (M17-lactose and MRS) in triplicates and covered with 10 mL of 1% agar. Plates were incubated aerobically

at 37 °C for 24–48 h and examined for bacterial growth. Plates with distinct individual colonies were selected and overlaid with an additional layer of BHI (Difco), supplemented with 1% agar and the test (indicator) organisms (*Str. mutans* KACC 16833, *Streptococcus gordonii* KACC 13829, and *Listeria monocytogenes* ATCC 15313) at 10^5 CFU/mL (final concentration). Plates were incubated for an additional 24 h at 37 °C and evaluated for the presence of inhibition zones around the previously formed colonies. Individual colonies were isolated and grown in M17-lactose or MRS broth. Preliminary identification of the isolates was done based on the morphology observed via phase contrast microscopy, catalase reaction, and reactions to other physiological and biochemical tests indicated in Bergey's Manual of Systematic Bacteriology of Archaea and Bacteria [31]. Stock cultures were kept in M17-lactose or MRS broth supplemented with 20% (v/v) glycerol at −20 °C.

The isolates were initially screened for the production of antibacterial metabolites against *Str. mutans* KACC 16833, *Str. gordonii* KACC 13829, and *L. monocytogenes* ATCC 15313 by determining bacteriocin production and cell-to-cell interaction according to dos Santos et al. [30]. Cell-free supernatants (CFS) of the cultures (MRS, 24 h, 37 °C) obtained after centrifugation (12,000 × *g*, 10 min, 20 °C) were heat-treated for 10 min at 80 °C and spotted on the surface of previously prepared solid BHI medium, supplemented with 1% agar and 10^5 CFU/mL of *Str. mutans* KACC 16833, *Str. gordonii* KACC 13829, or *L. monocytogenes* ATCC 15313. Plates were incubated for 24 h at 37 °C and evaluated for the presence of inhibition zones. Zones larger than 2 mm were considered as positive. In a parallel experiment, deferred antagonism assay was carried out by spotting 10 µL of exponentially growing cultures of the LAB isolates on the surface of similarly prepared plates. Plates were incubated following the same conditions and examined for the presence of clear inhibition zones. Isolates confirmed to be potentially antagonistic were selected for subsequent experiments.

Putative LAB isolates were cultured in MRS broth for 24 h at 37 °C and subjected to DNA extraction using ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's recommendations. The obtained DNA was quantified in SPECTROstar Nano nanodrop (BMG LABTECH, Ortenberg, Germany). Differentiation of the isolates was performed by rep-PCR DNA fingerprinting with the primer $(GTG)_5$ (Table 1) according to de Castilho et al. [32]. PCR reactions were performed in a Veriti 96-well Thermal Cycler, Applied Biosystems (Thermo Scientific, Waltham, MA, USA). Profiling of the generated amplicons was done via gel electrophoresis, run on 1.5% (w/v) agarose gels stained with 0.02 µL/mL of SYBR®Safe (Thermo Scientific) in 1 × TAE buffer at 100 V for 1 h (GH-200 Genera Biosystems, Victoria, Australia; Elite 300 Plus Power Supply, Wealtec Bioscience Co., Ltd., Taiwan). The gel was visualized using Omega Lum™G gel documenter (Aplegen, Inc., Pleasanton, CA, USA). Isolates were grouped according to the generated profiles and representatives of each group were subjected to 16S rRNA sequencing with primers 8F and 1492R (SolGent Analysis Service, Daejeon, Republic of Korea) as commercial service.

Safety Evaluation of the Selected Strains

Phenotypic Safety Test

The investigated strains were subjected to selected phenotypic tests to determine their hemolytic activity and gelatinase production according to Colombo et al. [23] and biogenic amine production following the recommendations of Bover-Cid and Holzapfel [33]. For hemolytic activity, the selected strains were streaked on the surface of trypticase soy agar supplemented with 5% (v/v) defibrinated sheep blood (Synergy Innovation, Seongnam-si, Republic of Korea). Plates were incubated at 37 °C for 24 h and observed for evidence of possible hemolytic activity of each strain. Clear halos around the colonies were considered as β-hemolysis, greenish halos were noted as partial or α-hemolysis, and no change was recorded as γ-hemolysis. Cultures of *Bacillus cereus* ATCC 27438 was applied as β-hemolysis, *Escherichia coli* ATCC 25922 as α-hemolysis, and *Lb. plantarum* ATCC 14197 as γ-hemolysis controls [23].

Gelatinase production was performed according to Colombo et al. [23] with some modifications. Ten-microliter aliquots were deposited on the surface of Luria Bertani (LB) agar (Difco), supplemented with 3% (w/v) gelatin (Difco). Plates were incubated for 48 h at 37 °C and then maintained at 4 °C for 4 h. A positive result for gelatin hydrolysis was exhibited by transformation of the solid agar medium to liquid phase. *Bacillus amyloliquefaciens* ST109 and *Lb. plantarum* ATCC 14197 served as positive and negative controls, respectively.

The ability to produce biogenic amines was performed according to Bover-Cid and Holzapfel [33]. The strains were cultured at 37 °C for 24 h for at least five consecutive times in MRS broth supplemented with 0.1% (w/v) of the respective amino acid precursors for the production of biogenic amines. After the last transfer, the strains were streaked in duplicate on a modified MRS agar supplemented with the biogenic amine precursors described above (1%, w/v). The plates were incubated for up to 4 days at 37 °C and change in color from yellow to purple was considered as positive result for the production of a biogenic amine. *E. coli* ATCC 25922 and *Lb. plantarum* ATCC 14197 served as positive and negative controls, respectively.

Table 1 Primers used for detecting virulence, vancomycin resistance, adhesion, gamma aminobutyric acid (GABA) production, bile salt deconjugation, and biogenic amine production-related genes

Genes	Description	Primer sequence (5′-3′)	PCR product size (bp)	Reference
Virulence genes				
hyl	Hyaluronidase	hyl F: 5′-ACAGAAGAGCTGCAGGAAATG-3′ hyl R: 5′-GACTGACGTCCAAGTTTCCAA-3′	276	[35]
esp	Enterococcal surface protein	esp14 F: 5′-AGATTTCATCTTTGATTCTTGG-3′ esp12 R: 5′-AATTGATTCTTTAGCATCTGG-3′	510	[35]
IS16	*Pathogenicity island*	IS16 F: 5′-CATGTTCCACGAACCAGAG-3′ IS16 R: 5′-TCAAAAAGTGGGCTTGGC-3′	547	[36]
efaA	Endocarditis antigen	efa-A F: 5′-GCCAATTGGGACAGACCCTC-3′ efa-A R: 5′-CGCCTTCTGTTCCTTCTTTGGC-3′	688	[37]
cyt	Cytolysin	cyt I F: 5′-ACTCGGGGATTGATAGGC-3 cytIIb R: 5′-GCTGCTAAAGCTGCGCTT-3′	688	[35]
asa1	Aggregation substance	asa1 F: 5′-GCACGCTATTACGAACTATGA-3′ asa1 R: 5′-TAAGAAAGAACATCACCACGA-3′	375	[35]
Vancomycin-resistant genes				
*van*A	Vancomycin resistance	vanAB F: 5′-GTAGGCTGCGATATTCAAAGC-3′ vanA R: 5′-CGATTCAATTGCGTAGTCCAA-3′	230	[38]
*van*B	Vancomycin resistance	vanAB F: 5′-GTAGGCTGCGATATTCAAAGC-3′ vanB R: 5′-GCCGACAATCAAATCCTC-3′	300	[38]
*van*C	Vancomycin resistance	vanC F: 5′-ATCCAAGCTATTGACCCGCT-3′ vanC R: 5′-TGTGGCAGGATCGTTTTCAT-3′	360	[38]
*van*D	Vancomycin resistance	vanD F: 5′-TGTGGGATGCGATATTCAA-3′ vanD R: 5′-TGCAGCCAAGTATCCGGTAA-3′	500	[38]
*van*E	Vancomycin resistance	vanE F: 5′-TGTGGTATCGGAGCTGCAG-3′ vanE R: 5′-GTCGATTCTCGCTAATCC-3′	510	[38]
*van*G	Vancomycin resistance	vanG F: 5′-GAAGATGGTACTTTGCAGGGCA-3′ vanG R: 5′-AGCCGCTTCTTGTATCCGTTTT-3′	250	[38]
Beneficial genes				
map	Mucus adhesion	map F: 5′-TGGATTCTGCTTGAGGTAAG-3′ map R: 5′-GACTAGTAATAACGCGACCG-3′	200	[43]
mub	Mucus adhesion	mub F: 5′-GTAGTTACTCAGTGACGATCAATG-3′ mub R: 5′-TAATTGTAAAGGTATAATCGGAGG-3′	200	[43]
eftu	Adhesion-like factor	EFTu F: 5′-TTCTGGTCGTATCGATCGTG-3′ EFTu R: 5′-CCACGTAATAACGCACCAAC-3′	200	[43]
msa	Adhesion protein	msa F: 5′-GCTATTATGGGGATTACGTTG-3′ msa R: 5′-CTGTCTTGACAATAGCCATATA-3′	1740	[41]
*prg*B	Aggregation substance	prgB F: 5′-GCCGTCGACTCGAGGAGAATGATA CATGAAT-3′ prgB R: 5′-CCTGCGGCCGCGTCCTTCTTTTCG TCTTCAA-3′	3917	[38]
*EF*2662	Choline-binding protein	EF2662 F: 5′-GGCGTCGACCACTTAAACTGATAG AGAGGAAT-3′ EF2662 R: 5′-CGCGCCGCAATTAATTATTAACTA GTTTCC-3′	1121	[38]
*EF*1249	Fibronectin-binding protein	EF1249 F: 5′-GCGGTCGACAAACGAGGGATT TATG-3′ EF1249 R: 5′-CTGGCGGCCGCGTTTAATACAATT AGGAAGCAGA-3′	1712	[38]
*EF*2380	Membrane-associated zinc metalloprotease	EF2380 F: 5′-GCGGTCGACATCTATGAAAAC AAT-3′ EF2380 R: 5′-TCCGCGCCGCCTTAAACTTTCTCC TT-3′	1268	[38]

Table 1 (continued)

Genes	Description	Primer sequence (5′-3′)	PCR product size (bp)	Reference
Folate production-associated genes				
*pab*B	Para-aminobenzoate synthase component I, ADCS	pabB F: 5′-CCTCAATTCATACAACCCTCTCAC A-3′ pabB R: 5′-CAGACAAATCTTCACTCACGCCAT AA-3′	1666	[30]
*pab*C	4-Amino-4-deoxychorismate lyase, ADCL	pabC F: 5′-CGGACAAGCATAATGAATACTCGG AAT-3′ pabC R: 5′-GGATTGATAACCGCTTCTATTGCC GA-3′	1850	[30]
*fol*KQ	*fol*K, 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine-diphosphokinase, HPPK; *fol*Q, dihydroneopterin-triphosphate-pyrophosphohydrolase	folK-Q F: 5′-CACTAGTGTCTATTGACTCAAATA TTTT-3′ folK-Q R: 5′-CGTTTTTATGGCTATCACGGGGCT -3'	790	[30]
*fol*PE	*fol*P, dihydropteroate synthase; *fol*E, GTPCyclohydrolase I	folP-E/F: 5′-GAGATAGTCTTAACGACATCACGA TT-3′ folP-E/R: 5′-GCAGTCTATCAATTATTGGAAGCT TT-3′	1350	[30]
GABA production-associated gene				
gad	Glutamate decarboxylase production	cor F: 5′-CCTCGAGAAGCCGATCGCTTAGTT CG-3′ cor R: 5′-TCATATTGACCGGTATAAGTGATG CCC-3′	540	[38]
Bile salt deconjugation				
bsh	Bile salt hydrolase	bsh F: 5′-CGTATCCAAGTGCTCATGGTTTAA-3′ bsh R: 5′-ATGTGTACTGCCATAACTTATCAA TCT-3′	919	[41]
Biogenic amine production genes				
*hdc*1	Histidine decarboxylase	hdc1 F: 5′-AGATGGTATTGTTTCTTATG-3′ hdc1 R: 5′-AGACCATACACCATAACCTT-3′	367	[39]
*hdc*2	Histidine decarboxylase	hdc2 F: 5′-AAYTCNTTYGAYTTYGARAARGAR G-3′ hdc2 R: 5′-ATNGGNGANCCDATCATYTTRT-GNCC-3′	534	[39]
tdc	Tyrosine decarboxylase	tdc F: 5′-GAYATNATNGGNATNGGNYTNGAY CARG-3′ tdc R: 5′-CCRTARTCNGGNATAGCRAARTC-NGTRTG-3′	924	[39]
odc	Ornithine decarboxylase	odc F: 5′-GTNTTYAAYGCNGAYAARCANTAY TTYGT-3′ odc R: 5′-ATNGARTTNAGTTCRCAYTTYTCNGG-3′	1446	[39]

Screening for Presence of Virulence, Biogenic Amines, and Vancomycin Resistance-Associated Genes

As a safety criterion, the DNA previously obtained from the studied strains were evaluated by PCR for the presence of the selected virulence genes (*efa*A, *cyt*, *IS*16, *esp*, *asa*1, and *hyl*) originally recommended by EFSA for *Enterococcus* spp. (Table 1) [34], Vankerckhoven et al. [35], Leavis et al. [36], and Martín-Platero et al. [37]. In addition, the presence of genes associated with vancomycin resistance (*van*A, *van*B, *van*C, *van*D, *vanE*, and *van*G) was investigated according to Valledor et al. [38], as well as the genes involved in the production of biogenic amines (*hdc1*, *hdc*21, *tdc*, *odc*) (Table 1) as indicated by de la Rivas et al. [39]. PCR reactions were performed in a Veriti 96-well Thermal Cycler, Applied Biosystems (Thermo Scientific) and the products were separated on 2.0% (w/v) agarose gels in 1 × TAE and visualized as described before.

Antibiotic Resistance

The selected cultures were subjected to phenotypic analysis of antibiotic resistance by using antibiotic disks (Oxoid Ltd., Basingstoke, England). The following antibiotics were used: ampicillin (10 µg/disk), erythromycin (10 µg/disk), gentamicin (10 µg/disk), penicillin (1 U/disk), streptomycin (10 µg/disk), daptomycin (10 µg/disk), vancomycin (10 µg/disk), and tobramycin (10 µg/disk). The strains were supplemented to MRS agar at 10^5 CFU/mL (final concentration) and the disks placed on the surfaces. Plates were incubated at 37 °C for 24 h and inhibition zone diameters around antibiotic disks were measured in millimeters. The isolates were interpreted as either resistant (R) or sensitive (S) according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing [40]. The presence of intermediate resistance was considered as resistant.

Evaluation of Beneficial Properties

β-*Galactosidase Production*

The expression of β-galactosidase was evaluated using ONPG disks (Sigma-Aldrich, St. Louis, MO, USA) following manufacturer's instructions. The strains were grown on solid MRS for 24–48 h at 37 °C until the formation of distinct individual colonies. A suspension of each strain was prepared by homogenizing 3–4 colonies in 0.2 mL of sterile saline solution (0.85% NaCl, *w/v*) in a test tube with an ONPG disk. The tubes were incubated at 37 °C for 4–6 h and regularly observed for yellow color formation as evidence of ONPG hydrolysis via the action of β-galactosidase to orthonitrophenol. The experiment was performed in duplicates in at least 2 independent occasions.

Bile Salt Deconjugation

To evaluate the ability of the probiotic candidates to perform bile salt deconjugation, bacterial cultures previously grown in MRS broth at 37 °C for 24 h were streaked individually on MRS agar plates supplemented with 0.5% (*w/v*) sodium taurodeoxycholate hydrate, taurcholate acid sodium salt hydrate, glycocholic acid hydrate, sodium glycocholate hydrate, sodium glycochenodeoxycholate, or sodium taurochenodeoxycholate (Sigma-Aldrich). The presence of an opaque halo around the colonies after incubation at 37 °C for 72 h in anaerobic conditions (GasPak System, Oxoid) indicated a positive result for bile salt deconjugation following the protocol of de Maraes et al. [41]. The test was performed in two independent occasions, twice for each strain.

Screening for Presence of Beneficial Genes

The DNA previously obtained were evaluated for the presence of selected beneficial genes related to adhesion (*msa*, *map*, *mub*, and *eftu*), folate production (*pab*B, *pab*C, *fol*KQ, and *fol*PE), GABA production (*gad*), and bile salt hydrolase enzymes (*bsh*) (Table 1) by PCR according to de Moraes et al. [41], dos Santos [30], and Bajic et al. [42] (Table 1). PCR reactions were performed on a Veriti 96-well Thermal Cycler, Applied Biosystems (Thermo Scientific) and the amplicons were separated on 1.0–2.0% (w/v) agarose gels in 1 × TAE and visualized as described before.

Evaluation of Antagonistic Properties

The ability of the strains to produce antagonistic metabolites was explored in two directions: production of antimicrobial peptides (bacteriocins) and cell-to-cell interaction as described previously and according to dos Santos et al. [30]. Test organisms, including different oral cavity-related pathogens, opportunistic pathogens, and beneficial strains from culture collections of HEM Inc. (Human Effective Microbes, Pohang, Republic of Korea), ProBacLab (Handong Global University, Pohang, Republic of Korea), KCTC (Jeongeup, Republic of Korea), KACC (Jeollabuk-do, Republic of Korea), and ATCC (American Type Culture Collection, Manassas, VA, USA) (Table 2), were used in a test panel to evaluate the antagonistic properties of the strains. Inhibition zones greater than 2 mm were considered evidence of potential production of antimicrobial metabolites, including bacteriocins. All experiments were performed in triplicate in two independent occasions.

Hydrophobicity

The ability of the selected strains to adhere to hydrocarbons as a criterion of hydrophobicity was determined according to Todorov and Dicks [43]. The strains were grown in MRS broth at 37 °C until stationary phase and cells were harvested (12,000 × *g*, 5 min, 4 °C), washed twice in 50 mM potassium phosphate buffer (pH 6.5), and re-suspended in the same buffer. Cell suspensions were adjusted to $OD_{560\,nm}$ 1.0 (UV/VIS Spectrophotometer, Optizen™ POP) using 50 mM potassium phosphate buffer (pH 6.5), and mixed with 0.6 mL of *n*-hexadecane (proportion 5:1) and vigorously vortexed for 2 min. The two phases were allowed to separate for 1 h at 37 °C and the OD of the aqueous phase was determined at 560 nm. The percentage cell surface hydrophobicity was calculated as $[(A0 - A)/A0] \times 100$, where A0 and A are the optical density readings before and after extraction with *n*-hexadecane, respectively.

Table 2 Cell-to-cell antagonistic properties of the selected potential probiotic strains (*Streptococcus salivarius* ST48HK, ST59HK, ST61HK, and ST62HK; *Lactiplantibacillus plantarum* ST63HK and ST66HK; *Latilactobacillus sakei* ST69HK; and *Lactobacillus gasseri* ST16HK) against different pathogens and beneficial bacterial cultures

Test organisms	Strains evaluated in cell-to-cell inhibitory test							
	ST16HK	**ST48HK**	**ST59HK**	**ST61HK**	**ST62HK**	**ST63HK**	**ST66HK**	**ST69HK**
Bacillus cereus	1/3*	0/3	0/3	0/3	0/3	2/3	0/3	2/3
Bacillus pumilus	0/1	1/1	0/1	1/1	0/1	1/1	0/1	1/1
Enterococcus avium	0/3	1/3	0/3	1/3	1/3	2/3	1/3	1/3
Enterococcus durans	0/1	0/1	0/1	0/1	0/1	1/1	0/1	0/1
Enterococcus faecalis	0/2	0/2	0/2	0/2	0/2	2/2	0/2	2/2
Enterococcus faecium	1/6	1/6	1/6	1/6	2/6	6/6	0/6	0/6
Enterococcus gallinarum	0/1	0/1	0/1	0/1	0/1	1/1	0/1	0/1
Lactobacillus brevis	0/3	0/3	0/3	0/3	0/3	3/3	1/3	0/3
Lactobacillus curvatus	1/2	0/2	0/2	0/2	0/2	1/2	0/2	0/2
Lactobacillus fermentum	0/2	0/2	0/2	0/2	0/2	0/2	1/2	1/2
Lactobacillus gasseri	0/1	0/1	0/1	0/1	0/1	1/1	0/1	0/1
Lactobacillus paracasei	2/5	1/5	0/5	0/5	1/5	1/5	0/5	0/5
Lactobacillus plantarum	1/12	0/12	1/12	0/12	0/12	1/12	1/12	1/12
Lactobacillus rhamnosus	0/3	0/3	0/3	0/3	0/3	3/3	0/3	0/3
Lactobacillus sakei	2/4	1/4	0/4	0/4	0/4	3/4	1/4	0/4
Lactobacillus salivarius	0/3	0/3	0/3	0/3	0/3	1/3	0/3	0/3
Leuconostoc mesenteroides	1/5	0/5	1/5	0/5	0/5	3/5	2/5	2/5
Listeria innocua	1/2	1/2	0/2	1/2	1/2	1/2	0/2	0/2
Listeria monocytogenes	1/3	0/3	0/3	0/3	0/3	1/3	0/3	1/3
Pediococcus acidilactici	0/1	0/1	0/1	0/1	0/1	1/1	0/1	1/1
Pediococcus pentosaceus	0/4	0/4	0/4	0/4	0/4	2/4	2/4	1/4
Staphylococcus carnosus	1/1	0/1	0/1	1/1	1/1	1/1	0/1	1/1
Staphylococcus carnosus subsp. *carnosus*	0/2	0/2	0/2	0/2	1/2	0/2	0/2	1/2
Staphylococcus delphini	1/2	0/2	0/2	1/2	0/2	0/2	0/2	0/2
Staphylococcus lentus	0/1	0/1	0/1	0/1	0/1	1/1	0/1	0/1
Staphylococcus simulans	1/3	0/3	0/3	1/3	1/3	1/3	0/3	1/3
Staphylococcus warneri	1/2	0/2	0/2	0/2	1/2	0/2	0/2	1/2
Streptococcus gordonii	0/3	0/3	1/3	1/3	1/3	1/3	1/3	1/3
Streptococcus mutans	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
Streptococcus salivarius	2/2	0/2	0/2	1/2	0/2	0/2	0/2	0/2
Streptococcus thermophilus	1/2	0/2	0/2	0/2	0/2	1/2	0/2	0/2
Vancomycin-resistant *Enterococcus* strains (VRE)	0/5	1/5	0/5	0/5	1/5	5/5	0/5	1/5

*Number of test organisms inhibited by presence of the evaluated potential probiotic strains/total number of test strains from the same species evaluated in this study. Note that tested representatives from the following bacterial species were not affected by the presence of the evaluated potential probiotic strains in cell-to-cell interaction test: *Bacillus subtilis*, *Enterococcus thailandicus*, *Escherichia coli*, *Lactobacillus sanikiri*, *Lactococcus lactis*, *Staphylococcus arlettae*, *Staphylococcus auricularis*, *Staphylococcus capitis* subsp. *capitis*, *Staphylococcus cohnii* subsp. *cohnii*, *Staphylococcus epidermidis*, *Streptococcus mitis*, *Streptococcus sanguinis*, and *Weissella cibaria*

Growth in Different Initial pH of the Media

Fifteen microliters of the strains initially grown in MRS broth (37 °C, 24 h) was transferred to 135 µL modified MRS broth (Difco) adjusted to pH 2.0, 4.0, 6.0, 8.0 10.0, and 12.0 with 1 M HCl or 1 M NaOH before autoclaving. All tests were conducted in sterile 96-well flat-bottom microtiter plates (SPL, Pocheon-si, Gyeonggi-do, Republic of Korea). Optical density readings at 600 nm were recorded every hour for 24 h in SPECTROstar Nano (BMG LABTECH). Cultures grown in MRS broth without pH adjustment served as control. All experiments were performed in duplicate in two independent occasions.

Effect of Commercial Drugs on Bacterial Survival

The strains were tested for susceptibility to commercial drugs from different commonly used groups and oral hygiene products (Table 3). Strains were grown in MRS broth (Difco) at 37 °C for 24 h and imbedded into MRS soft

Table 3 Susceptibility of the selected potential probiotics strains (*Streptococcus salivarius* ST48HK, ST59HK, ST61HK, and ST62HK; *Lactiplantibacillus plantarum* ST63HK and ST66HK; *Latilactobacillus sakei* ST69HK; and *Lactobacillus gasseri* ST16HK) to substances in commercially available medicaments and oral hygienic products

Drug type	Commercial name/company	Active substance (mg)	Applied concentration (mg/mL)	Minimum inhibitory concentration (MIC, mg/mL)							
				ST16HK	ST48HK	ST59HK	ST61HK	ST62HK	ST63HK	ST66HK	ST69HK
Analgesic	Nurofen/Reckitt Beuckiser healthcare	Ibuprofen 200 mg	40	40	40	40	40	40	40	40	40
Antispasmodic	Buscopan/Boehringer Ingelheim	Scopolamine butylbromide 10 mg/Dipyrone 250 mg	2/50	-	-	20/50	-	-	20/50	20/50	-
Analgesic, antipyretic	Analgin/Sopharma	Metamizole sodium monohydrate 500 mg	100	-	-	-	-	-	-	-	-
Analgesic	Doloran/Ekofarm	400 mg	80	80	20	40	80	40	80	40	80
Analgesic, antipyretic	Empeace/Progen	Acetaminophen 300 mg/ Chlorzoxazone 250 mg	60/50	-	30/25	-	60/50	30/25	-	-	-
Urinary antiinfectives	Nutrofurantiona/Biofarma	Nitrofurantoin 100 mg	20	5	0.16	2.5	0.16	0.63	1.25	0.63	0.31
Nonsteroidal antiinflammatory (NSAID)	Motrin/Pfizer	Ibuprofen 800 mg	160	-	160	80	160	20	-	-	160
Antidiarrheal, intestinal antiseptic	Treda/Sanfe	Neomycin 129 mg /Kaolin 280 mg/Pectin 30 mg	25.8/56/6	-	0.81/1.75/0.19	1.61/3.5/0.38	0.40/0.88/0.09	0.81/1.75/0.19	0.81/1.75/0.19	6.45/14/1.5	0.81/1.75/0.19
Mouth wash	Listerine (sample 2)/Listerine	-		25%	50%	25%	50%	100%	25%	100%	50%
Mouth wash	Sandrin/Sandrin	-		-	-	-	100%	-	-	25%	-
Mouth wash	Listerine (sample 4)/Listerine	-		12.5%	12.5%	-	12.5%	50%	-	25%	6.25%
Mouth wash	Listerine (sample 5)/Listerine	-		50%	-	-	-	-	-	-	-
Antacids, antireflux agents, and antiulcerants	Kremil-S/Unilab	Aluminium hydroxide 178 mg /Mg hydroxide 223 mg/Simeticone 30 mg	35.6/44.6/6	0.56/0.70/0.09	-	-	-	-	-	-	-

*Note 1: The following drugs (active substance (mg/mL) / company) had no effect on growth of tested LAB: Allergosan (Chloropyrazine hydrochloride, 5 mg/mL/Sopharma), Panadol (Paracetamol, 100 mg/mL / Galxo Smith Claine), Valeriana (Valerian, 6 mg/mL / Zydas-Nikkho-Colombia), Tylenol (Paracetamol, 150 mg/mL / Johnson & Johnson, Brazil), Paracetamol (Paracetamol, 100 mg/mL / Sopharma, Bulgaria), Lopedium (Loperamide hydrochloride, 0.4 mg/mL / Hexal), Valeriane (Valerian dry extract, 6 mg/mL / Sopharma, Bulgaria), Idea Cap (Acetaminophen, 65 mg/mL, Dichloralphenazone, 20 mg/mL, Isometheptenemucate, 13 mg/mL / Pharvis, Korea), Lipitor (Atorvastatin, 2 mg/mL / Pfizer, Korea), Mobic (Meloxicam, 1.5 mg/mL / Boehringer Ingelheim, Korea), Aspirina (Acetylsalicylic acid, 100 mg/mL / Bayer, Germany), Nutrofurantiona (Nitrofurantoin, 20 mg/mL / Biofarma), Clotracemy (Tramadol, 6.54 mg/mL, Paracetamol, 65 mg/mL / Novag), Neozep Forte (Phenylephrine HCl, 2 mg/mL, Paracetamol, 100 mg/mL, Chlorpheniraminemaleate, 0.4 mg/mL / Unilab, Mexico), Omeprazol (Omeprazole, 4 mg/mL / Apotex), Buscapina (Hyoscinebutyl bromide, 2 mg/mL / Sanofi), Lyrica (Pregabalin, 10 mg/mL / Pfizer), Tafirol flex (Paracetamol, 60 mg/mL, Chlorzoxazone, 50 mg/mL / Emifarm Mexico), Restasis (Cyclosporine ophthalmic emulsion, 0.05% / Allergan), GyeBoRin (Acetaminophen, 60 mg/mL, Propyphenozone, 30 mg/mL / Samjin, Korea), Biogesic (Paracetamol, 100 mg/mL / Unilab), Alexan FR (Paracetamol, 40 mg/mL, Ibuprofen, 50 mg/mL / Unilab), Bioflu (Phenylephrine HCl, 2 mg/mL, Chlorpheniramine Maleate, 0.4 mg/mL, Paracetamol, 100 mg/mL / Unilab), Imodium (Loperamide HCl, 0.4 mg/mL / Johnson & Johnson, Philippines), Sinecod Forte (Butamirate citrate, 2 mg/mL / Glaxo Smith Cline), Vitamin Complex (Vitamins from B group, 172 mg/mL / Lemon box), Men's Ultivite Multivitamin (Vitamin complex, 320 mg/mL / Swisse), Premium Multivitamin Gold (Vitamin complex, 100 mg/mL / Nutri D-Day), Calcium+Vitamin D (Calcium, Vitamin D, Manganese, 200 mg/mL / Nutri D-Day), Goyamin Active (60 mg/mL / IGC Korea), Lutein Zeaxanthin (100 mg/mL / Life Care), Vegetable Omega3 (EPA+DHA, 150 mg/mL / New Origin), Mouth wash (different samples 10 µL from the original products / Listerine), Vitamin E (Vitamin E, 36 mg/mL / Kirkland), Vitamin C (Ascorbic acid, 100 mg/mL / Actimed), Sulfate supplement (Salbutamol sulfate, 0.4 mg/mL / Actimed), Symdex-D (Decongestant, 5 mg/mL, Antihistamine, 0.4 mg/mL, Analgesic/Antipyretic, 65 mg/mL / Allied Pharmaceutical Laboratories, Inc.), Myra d-alpha-Tocopherol (Vitamin E, 400 IU (53.6 mg/mL) / UL Skin Sciences, Inc.), Myrefen (Mefenamic acid, 100 mg/mL / Myrex), Cetirizine (Cetirizine hydrochloride, 2 mg/mL / Glaxo Smith Kline), Cisflem (Carbocisteine 100 mg/mL / The Generics Pharmacy (TGP)), Nutrawell (Sodium ascorbate, 100 mg/mL /Actimed)

*Note 2: The following commercial pharmaceutical oral cleaning powders and their ingredients (Ingredients (g/mL) / Company) had no activity on the growth of tested LAB: Cleaning time ocean mint (first- and second-order processed enzyme salt, Balloon flower, Lemon, Green tea, Quince, Mint, Propolis all at 0.5 g/mL / Cleaning Time), Coolush powder Pour-swish-swallow (Monk flower extract, first- and second-order processed enzyme salt, Balloon flower, Lemon, Green tea, Quince, Mint, Propolis, all at 0.5 g/mL / Coolush)

agar (1.0%, *w/v*, Difco) at 10^5 CFU/mL final concentrations. One tablet from each of the commercial drugs was solubilized in 5 mL sterile distilled water under aseptic conditions, with the final concentration as specified in Table 3. Ten microliters of each drug solution was spotted onto the surface of the previously described MRS agar plates. The plates were incubated for 24 h at 37 °C and examined for the presence of inhibition zones. The drugs generating inhibition zones larger than 2 mm were considered for further determination of the minimal inhibition concentration (MIC) by applying two-fold serial dilutions of the drugs.

In addition, two commercial pharmaceutical oral cleaning powders, Cleaning Time Ocean Mint (Bareun, LLC, Chun-cheon, Gang-wondo, Republic of Korea) and Coolush powder (CO-FOODS, LLC, Suwanee GA, USA), and their individual ingredients were evaluated for possible inhibitory effects against the studied strains. One commercial sachet of Cleaning Time Ocean Mint and Coolush powder were diluted in 5 mL sterile distilled water under aseptic conditions. All ingredients were dissolved in sterile distilled water under aseptic conditions to final concentrations of 0.1 g/mL (Table 3). As with the test done on commercial drugs, 10 µL of the prepared solution was spotted on the surface of MRS plates, which were incubated at 37 °C for 24 h and evaluated for the presence of inhibition zones.

Results and Discussion

Isolation and Identification of Beneficial LAB

Numerous and diverse microorganisms colonize the oral cavity, including beneficial bacteria that could exert positive effects on human and oral health. Several LAB in the oral cavity contribute to the prevention of oral diseases and numerous species have been reported for their probiotic features [12]. In this study, putative beneficial LAB strains have been isolated from the oral cavity, and have been identified as *Lactobacillus gasseri* ST16HK; *Streptococcus salivarius* ST48HK, ST59HK, ST61HK, and ST62HK; *Lactiplantibacillus plantarum* (*Lactobacillus plantarum*) ST63HK and ST66HK; and *Latilactobacillus sakei* (*Lactobacillus sakei*) ST69HK. These showed potential for further development as oral probiotics based on the characterization of their antimicrobial activity and properties.

Presumptive LAB were isolated from the saliva of healthy volunteers of different ethnicities with the aim of evaluating them as potential probiotics for oral cavity application. More than 120 bacterial colonies showed inhibition zones in the triple-level approach and were isolated and selected as promising candidates based on their antimicrobial activity against the test microorganisms (*Str. mutans* KACC 16833, *Str. gordonii* KACC 13829, and *L. monocytogenes* ATCC 15313). The isolates were tested for antimicrobial activity, with the results serving as basis for further evaluation. Differentiation of the promising isolates based on rep-PCR revealed 9 unique clusters, with representatives from each group subjected to partial 16S rRNA gene sequencing. Grouping the isolates based on their antimicrobial activities, representatives from Cluster 1 revealed a 99% similarity to *Str. mutans*, a well-known opportunistic pathogen typically isolated from the oral cavity of humans [44]. Representatives from the other 8 clusters were identified as *Lb. gasseri* strain ST16HK; *Str. salivarius* strains ST48HK, ST59HK, ST61HK, and ST62HK; *Lb. plantarum* strains ST63HK and ST66HK; and *Lb. sakei* strain ST69HK. All 8 strains (*Lb. gasseri* ST16HK; *Str. salivarius* ST48HK, ST59HK, ST61HK, and ST62HK; *Lb. plantarum* ST63HK and ST66HK; and *Lb. sakei* ST69HK) showed inhibitory activity against the pathogens *Str. mutans* KACC 16833, *Str. gordonii* KACC 13829, and *L. monocytogenes* ATCC 15313 with cell-to-cell interaction. While LAB from the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Enterococcus*, and *Weissella* can be found in the oral cavity [45], *Lactobacillus* and *Streptococcus* were mostly isolated in this study.

As the gateway to the digestive system, the oral cavity is where mechanical and initial enzymatic digestion occurs. These enzymes, although primarily aid in the digestion processes, also play a crucial role in the elimination and serve as an initial barrier-function of the entry of potential pathogens to the rest of the digestive tract. In addition to this, the oral cavity is considered a rich ecological niche, with more than 700–1000 bacterial species that are permanently or temporarily associated with it [2, 14]. The dynamic state of the microbial population in the oral cavity is dependent of the age, social economic status of the individual, and the type of diet and lifestyle [2, 46]. It has been clearly demonstrated that during different life stages, specific bacterial groups may colonize the oral cavity [47], with *Str. salivarius* being typically associated with the human oral cavity. This was suggested by Andrewes and Horder [48] and this association has been frequently confirmed [49, 50]. *Lb. plantarum*, *Lb. gasseri*, and *Lb. sakei* have also been reported as inhabitants of the human oral cavity [47, 49]. However, the isolation of different LAB from the oral cavity cannot support the postulate that these species are part of the residual microbiota of this ecosystem. Several LAB isolated from the oral cavity may be temporally introduced by foods. However, their presence in the oral cavity should be evaluated, specifically their stability in this ecological niche while also investigating their potentially beneficial, negative, or a hitherto undefined role in host health.

Safety Evaluation of the Selected Strains

Antibiotic Resistance, Hemolysis, Gelatinase, and Biogenic Amine Production

The safety of the 8 selected strains was evaluated as part of the approach "safety is priority," an essential step for the application of beneficial strains as oral probiotics. Antibiotic resistance, hemolytic activity, gelatinase production, and biogenic amine production were determined as part of physiological testing.

Str. salivarius ST48HK, ST59HK, ST61HK, and ST62HK; *Lb. plantarum* ST63HK and ST66HK; *Lb. sakei* ST69HK; and *Lb. gasseri* ST16HK responded in different ways when tested for antibiotic resistance (Table 4). Special attention should be given to tobramycin (primary antibiotic treatment for Gram-negative infections, *Pseudomonas*-related infections and tuberculosis) [51] resistance exhibited by *Lb. gasseri* ST16HK. Moreover, from all the tested strains, only *Str. salivarius* ST59HK was clearly inhibited by vancomycin, while all other strains were resistant. According to EFSA, vancomycin resistance is intrinsic to most LAB species, and strains of *Lb. plantarum* and *Lb. sakei* showing resistance can still be considered as safe. On the other hand, the resistance of *Str. salivarius* strains ST48HK, ST61HK, and ST62HK to some of tested antibiotics can be considered as a safety concern.

The antibiotic resistance of the strains evaluated as probiotic candidates is considered a key issue. The uncontrolled application of antibiotics, especially during the last 3 decades of the twentieth century in health care and farming practices, resulted in the wide distribution of antibiotic resistance, not only among pathogens, but also among beneficial organisms [52]. Moreover, a serious concern is the possible scenario of antibiotic resistance transfer from beneficial bacterial species to other inhabitants of the GIT, including opportunistic and effective pathogens [52]. EFSA (https://www.efsa.europa.eu/en/topics/topic/antimicrobial-resistance) is regularly updating information and recommendations for the safety criteria regarding antibiotic resistance in different bacterial groups.

Evaluation of the hemolytic activity of the 8 selected strains revealed that they all exhibited γ-hemolytic activity. The presence of α- and particularly β-hemolysis can be considered as virulence factors, and are observed in different pathogenic species, including *E. coli*, *B. cereus*, and some *Enterococcus* spp. This is of significance, especially to *Str. salivarius* strains since the genus *Streptococcus* is known as human and other animal pathogen [30, 53]. According to the Bulletin of the International Dairy Association [54], only a few species of the genus *Streptococcus* (*Streptococcus gallolyticus* subsp. *macedonicus*, *Str. salivarius* subsp. *salivarius*, *Str. salivarius* subsp. *thermophilus*, and *Streptococcus thermophilus*) are considered as safe. Although *Str. salivarius* has a disputable reputation, MacDonald et al. [55] and Wescombe et al. [27] suggested that strains belonging to this species can be applied as beneficial strains. On the contrary, Wilson et al. [56] reported that *Str. salivarius* can be associated with meningitis cases.

The evaluation for gelatinase production demonstrated that all the strains gave negative results. Gelatinase production, although not a common feature among representatives of the genus *Lactobacillus*, is frequently reported in some representatives of the genus *Streptococcus*, especially for strains with a clear pathogenic profile [57]. The assessment for biogenic amine (BA) production also generated negative results. The production of biogenic amines is considered crucial for the safety application of beneficial organisms, especially for those intended to be applied as starter or adjunct cultures in food fermentation processes. BAs may be produced by amino acid decarboxylation in diverse foods, especially during fermentation, and their accumulation may be considered as a potential health hazard. Specific thresholds for different BAs are regulated for different fermented food products and normally are subjected to strict control [58]. This characteristic has been shown to be a strain-specific property [33]. Different studies have demonstrated that BA productions such as histamine, recorded for a strain of *Lb. plantarum* [59], and tyramine, for *Lb. curvatus* strains, are typically observed [33] and thus require close monitoring. This has been further highlighted by Barbieri [60], who reviewed the link between biogenic amine production and the presence of LAB in different food products, and emphasized the significant role of different strains of *Lactobacillus* spp.

Detection of Virulence and Vancomycin-Resistant Genes

In addition to physiological tests, safety evaluation of the selected strains was also done based on PCR screening for virulence and vancomycin-resistant genes. In this study, although detection of virulence genes (*asa*1) and vancomycin-resistant genes (*van*B and *van*D) were observed in some of the strains evaluated, the majority of the selected beneficial strains are still considered safe to be applied as oral probiotics. However, some concerns about the presence of *van*B and *van*D need to be looked at as these can be potential risks [38]. Transferable antibiotic-resistant genes, especially to vancomycin, are considered to pose a threat on the safety of LAB strains. In this study, 7 out of the 8 evaluated strains showed resistance to vancomycin based on the performed antibiotic susceptibility tests. However, for all hetero-fermentative and some homo-fermentative lactobacilli, vancomycin resistance is considered by EFSA [34] as an intrinsic feature. The phenotypic demonstration of vancomycin resistance by three *Str. salivarius* strains (ST48HK, ST61HK, and ST62HK) is a crucial point for

risk assessment for the possible applications of these strains. Based on PCR assays carried out for the detection of different vancomycin genes (*van*A, *van*B, *van*C, *van*D, *van*E, and *van*G), only *van*D was detected in *Str. salivarius* ST48HK, while *van*B was detected in *Lb. plantarum* ST63HK and ST66HK. The presence of vancomycin-resistant genes needs to be evaluated, as well as the localization of these associated genes, for possible horizontal gene transfer. It has been highlighted that the presence of *van*A and *van*B genes are much more concerning than the rest of the *van* genes. This is due to their plasmid localization, which favors horizontal gene transfer [38]. Thereby, it was suggested by EFSA that testing for presence of various key virulence genes, *efa*A, *cyt*, *IS*16, *esp*, *asa*1, and *hyl*, is necessary for safety evaluation of some LAB species. Screening for these aforementioned genes demonstrated their absence across the evaluated strains, except for *Str. salivarius* ST61HK and ST62HK, where *asa*1 was detected. This observation is a pivotal point for considerations for possible application of these strains. Screening for the presence of genes related to the production of biogenic amines is also considered a key test in the evaluation of LAB intended to be applied as a probiotic or starter cultures. The ingestion of excessive amounts of BA, especially the combinations of formed biogenic amines, can pose a serious health consequence for consumers [33]. In the detection assay conducted in this study for the detection of the crucial enzyme for BA production, only *hdc*1 was detected in *Lb. gasseri* ST16HK.

The molecular-based evaluation for the presence of virulence factors is one of the predictive approaches in the safety evaluation of potential beneficial bacteria [61]. However, the presence of specific genes needs to be discussed in the context of the presence of functional operons related to the effective expression of these virulence factors. It has previously been shown that some virulence genes can still be present, even though they may not be expressed. Thus, the expression of a specific factor should be further examined by determining whether a functional operon is present or not [62]. Possible horizontal gene transfer may occur between bacterial species and strains carrying the targeted virulence genes. It is likely that potential transfer occurred, involving only parts of the operon. Moreover, as suggested by Perin et al. [62], the expression of virulence genes (and other genes as well) can be related to environmental conditions, including temperature, pH, or specificity of the particular inductors. It must also be considered that these assessments are only carried out in vitro and in DNA-level screenings; thus, further evaluation of expression of the functionality of the virulence genes and the conditions required for their effective expression needs to be considered in future safety analysis.

Additional Beneficial Properties of the Selected Strains

β-*Galactosidase Production and Hydrophobicity*

Production of β-galactosidase is essential for bacterial growth when the available carbohydrate is limited to lactose [63]. From a probiotic point of view, the expression of β-galactosidase can be associated with the role of probiotics in the reduction of lactose intolerance in individuals [64]. However, the production of β-galactosidase is only considered an additional beneficial property of probiotics promoting oral health, since lactose digestion typically occurs in the small intestine, with little to no significance in the oral cavity. In this study, we have evaluated β-galactosidase production through ONPG disks. Among the evaluated strains, only *Str. salivarius* ST48HK showed a positive result by forming of a yellow color due to the action of β-galactosidase on ortho-nitrophenol.

The strains demonstrated a wide range of cell-surface hydrophobicity as indicated in Fig. 1. According to Krausova et al. [65], hydrophobicity above 40% can be considered as high. This characteristic has been considered a predictive tool for potential probiotic application because it determines the ability to adhere to the intestinal mucosa

Table 4 Antibiotic sensitivity of the potential probiotic strains in this study (*Streptococcus salivarius* ST48HK, ST59HK, ST61HK, and ST62HK; *Lactiplantibacillus plantarum* ST63HK and ST66HK; *Latilactobacillus sakei* ST69HK; and *Lactobacillus gasseri* ST16HK), shown as diameter (mm) inhibition zones

Antibiotics	ST16HK	ST48HK	ST59HK	ST61HK	ST62HK	ST63HK	ST66HK	ST69HK
Ampicillin	40 (S)	47 (S)	52 (S)	50 (S)	44 (S)	32 (S)	34 (S)	36 (S)
Daptomycin	21 (S)	27 (S)	50 (S)	30 (S)	36 (S)	34 (S)	36 (S)	41 (S)
Erythromycin	37 (S)	34 (S)	43 (S)	44 (S)	44 (S)	37 (S)	37 (S)	39 (S)
Gentamicin	10 (I)	13 (I)	20 (S)	14 (I)	14 (I)	13 (I)	13 (I)	16 (S)
Penicillin	38 (S)	22 (S)	47 (S)	21 (S)	22 (S)	26 (S)	26 (S)	26 (S)
Streptomycin	15 (S)	8 (R)	15 (S)	9 (I)	9 (I)	11 (I)	11 (i)	11 (I)
Tobramycin	0 (R)	9 (R)	14 (I)	9 (I)	9 (I)	9 (R)	9 (R)	9 (R)
Vancomycin	24 (S)	0 (R)	30 (S)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)

R resistant, diameter of inhibition zone < 10 mm; *I* intermediate, diameter of inhibition zone between 10 and 15 mm; *S* susceptible > 15 mm [81]

[30, 65]. However, this can only be regarded as a preliminary indication of mucus adhesion characteristics as the entire process is much more complex in vivo, and involves different factors such as expression of specific adherence proteins, distribution of carbohydrates on the cell surface, and electrical charges [66]. While a simple method, cell surface hydrophobicity to hydrocarbons serves as a presumptive assay for the adherence properties of the strains under investigation.

Detection of Adhesion and Beneficial Genes

The presence and expression of adhesion genes can be regarded as a desirable property for a strain to colonize the GIT and, therefore, interact with the host. Colonizing the digestive system, including the oral cavity, needs to be discussed as a functional characteristic in the context of the biological role and application of a specific probiotic strain. Adhesion may support prolonged "domestication" and can be considered as beneficial for long-term presence in the oral cavity and the GIT, thereby improving interaction with other bacterial species and promoting immune responses in the host [67]. In the scenario, where probiotics will interact with toxic metabolites (toxins, heavy metals, etc.) and be involved in their removal, quick transit passage may be considered as beneficial [68]. Results indicated that adhesion genes *map*, *mub*, and *eftu* were detected in *Str. salivarius* ST61HK and ST62HK. On the other hand, *eftu* gene was detected in both *Lb. gasseri* ST16HK and *Lb. sakei* ST69HK, while only *map* gene was present in *Lb. plantarum* ST63HK.

It is interesting to note that some of the evaluated strains were positive for *map*, *mub*, and *eftu* genes, which are typically present in *Lb. plantarum* strains [69]. However, the presence of these genes was determined even in representatives of *Enterococcus* and *Leuconostoc* [43], possibly making them more universally distributed. Moreover, the other evaluated genes (*EF*1249, *EF*2380, *EF*2662, and *prg*) were not detected in the strains being studied. The presence of a variety of adhesion proteins is strain-specific and may serve as functional support for possible application as novel probiotics.

Screening for the deconjugation-associated gene (*bsh*) showed its presence in *Str. salivarius* ST61HK and ST62HK, along with *Lb. sakei* ST69HK. This observation points to the possibility that these strains can be involved in various physiological processes including the metabolism of bile salts. However, based on the phenotypic assays, no evidence of degradation of the tested salts was demonstrated by the strains. The ability to deconjugate bile salts in vitro is considered a beneficial property of the strains since they can reduce cholesterol levels and the toxicity of bile salts [70], particularly those that are intended to be applied as probiotics. This characteristic was previously reported in *Lb. mucosae* CNPC006 and *Lb. mucosae* CNPC007 [41], with their ability to deconjugate GDC salts and TDC salts in a strain-specific manner. Additionally, some *Lb. reuteri* strains have also been reported to have the ability to degrade GDC salts [71]. Benefits associated with this characteristic lead to the reduction of the toxicity, formation of insoluble forms, and control of the presence of less absorbable bile acids in the intestinal lumen. Additionally, this may have an effect on the reduction of serum cholesterol levels [72].

The production of specific beneficial metabolites may also serve as an additional support for determining a newly isolated probiotic candidate's potential for its intended application. The presence of *gad* gene in all the strains may be considered as an additional beneficial characteristic. GABA, a known neurotransmitter, plays an important role in brain development. Aside from this, its other applications such as possible treatment for diabetes, positive influence and suppression inflammatory immune responses, and its ability to promote "regulatory" immune responses, especially concerning autoimmune diseases [73, 74], merit further evaluation, quantification, and exploitation.

Folate production is considered a beneficial property of LAB important in the formulation of functional fermented food products naturally enriched with vitamin B9. Laiño et al. [75] and Levit et al. [76] reported on different LAB strains with the ability to produce vitamin B9, and proposed the application of such strains in the formulation of dairy beverages for children to combat folate deficiency. In addition to previously identified beneficial genes, folate encoding genes were also screened. It was observed that *pab*B, *pab*C, *fol*KQ, and *fol*PE with the exception of *folPE* genes were detected in *Str. salivarius* ST48HK, but not in the other strains. In *Str. salivarius* ST61HK and ST62HK, the genes encoding for folate (*pab*B, *pab*C, *fol*KQ, and *fol*PE) and GABA (*gad*) production were detected in addition to all the adhesion genes. Only three genes (*pab*B, *pab*C, *fol*KQ) were found to be associated with *Str. salivarius* ST48HK, suggesting strain-specific diversity in this species, and also suggesting a potential for selecting of appropriate strains as oral probiotic candidates.

Antagonistic Properties of the Selected Strains

Production of antimicrobial peptides (bacteriocins) and cell-to-cell interactions were determined through activity against the test organisms listed in Table 2. *Lb. plantarum* ST63HK showed activity against most of the test organisms via cell-to-cell interaction, whereas a few positive results were observed for bacteriocin activity. *Lb. gasseri* ST16HK showed activity against *Lactobacillus paracasei*, *L. monocytogenes* ATCC 15313, *Staphylococcus delphini* KACC 13,58, and *Str. gordonii* KACC 13829. *Str. salivarius* ST48HK showed inhibitory activity against *L.*

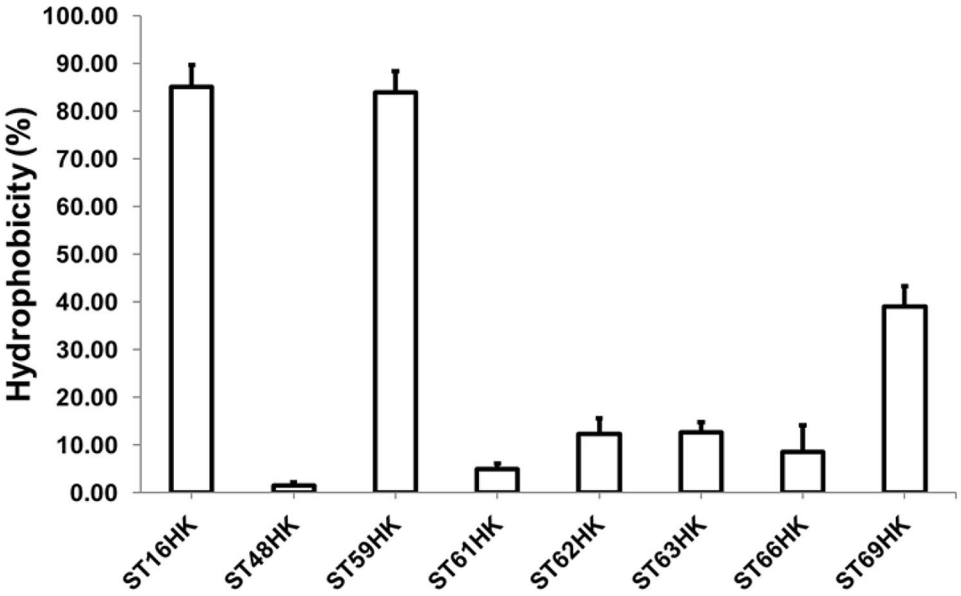

Fig. 1 Levels of the hydrophobicity, recorded for the evaluated in this study potential probiotic strains. SD were presented. *Lactobacillus gasseri* (ST16HK), *Streptococcus salivarius* (ST48HK ST59HK, ST61HK, and ST62HK), *Lactiplantibacillus plantarum* (ST63HK and ST66HK), and *Latilactobacillus sakei* (ST69HK)

monocytogenes ATCC 15,313, *St. delphini* KACC 13258, and *Str. gordonii* KACC 13829. The inhibition by *Lb. plantarum* ST63HK was detected against *E. coli*, *St. delphini* KACC 13258, and *Str. gordonii* KACC 13829, whereas *Lb. plantarum* ST66HK was active against *St. delphini* KACC 13258 (Table 2).

LAB can produce different antimicrobial metabolites as part of their defense mechanism. Organic acids, low molecular antimicrobials, diacetyl, H_2O_2, and antimicrobial peptides are just a few of the metabolites formed by LAB as a means to compete against other microorganisms present in the same ecological niche [77]. Moreover, Chikindas et al. [77] suggested that bacteriocins can be regarded as molecules of great complexity than just simple "killers," since they are actively involved in the regulatory cell processes and quorum sensing interactions. In addition to cell-to-cell inhibitory interactions, evidence for possible production of bacteriocins was sparse.

Growth in Media With Various Initial pH

The growth rate of the selected strains was determined in MRS broth with varying initial pH values. At pH values below 6.0, only slight growth was detected, most strains showed notable growth above pH 6.0, suggesting that they can grow effectively in the human oral cavity, emphasizing their potential as oral probiotics. In this study, bacterial growth on MRS broth adjusted to higher pH levels demonstrated slow growth of *Lb. gasseri* ST16HK compared to the control MRS medium (with pH 6.0). Optimal growth for *Lb. gasseri* ST16HK was recorded at pH 8.0, compared to other pH conditions. *Str. salivarius* ST48HK showed the lowest growth rate at pH 2.0 and 4.0, while other pH conditions showed no significant differences. *Str. salivarius* ST59HK showed the lowest growth rate at pH 2.0, pH 4.0, and pH 12.0 while continuous log phase was observed at pH 6.0. *Str. salivarius* ST61HK showed a significantly lower growth rate in pH 2.0 compared to the standard MRS medium. *Str. salivarius* ST62HK exhibited the lowest growth rate in pH 4.0 compared to the control and showed maximum growth rate at pH 6.0 and 8.0. For *Lb. plantarum* ST63HK, pH conditions showed no significant difference between pH 6.0, pH 8.0, pH 10.0, and pH 12.0 compared to the control with the lowest growth rate at pH 2.0 and pH 4.0. For *Lb. plantarum* ST66HK, growth was lowest in pH 2.0 and pH 4.0 compared to the control, with no significant differences at pH 6.0 and pH 8.0. Growth rate of *Lb. sakei* ST69HK was maximal at pH 8.0 and 10.0 compared to the other conditions. At pH values 2.0, pH 4.0, and pH 12.0, the lowest growth rate was detected for *Lb. sakei* ST69HK (Fig. 2).

Survival of Selected Strains in Commercial Drugs and Oral Hygiene Products

The efficacy of probiotics can be influenced by the interference of general drugs and hygienic products. Previous research [78–80] reported on the possible inhibitory effects of commercial non-antibiotic drugs on the viability of probiotic strains. In this study, 13 commercial drugs (Table 3) inhibited the growth of the strains. In order to estimate the potential effect, minimal inhibitory concentrations were calculated to evaluate the effective concentration against the selected strains. Nurofen, Doloran, Nurofurantiona, and oral hygienic products are inhibited all 8 strains. Buscopan inhibited the growth of *Str. salivarius* ST59HK, *Lb. plantarum* ST63HK, and *Lb. plantarum* ST66HK; Analgin inhibited the growth of *Lb. gasseri* ST16HK, *Lb. plantarum* ST63HK, and *Lb. plantarum* ST66HK. Empeace reduced the growth of *Str. salivarius* ST48HK, *Str. salivarius* ST61HK, and *Str. salivarius*

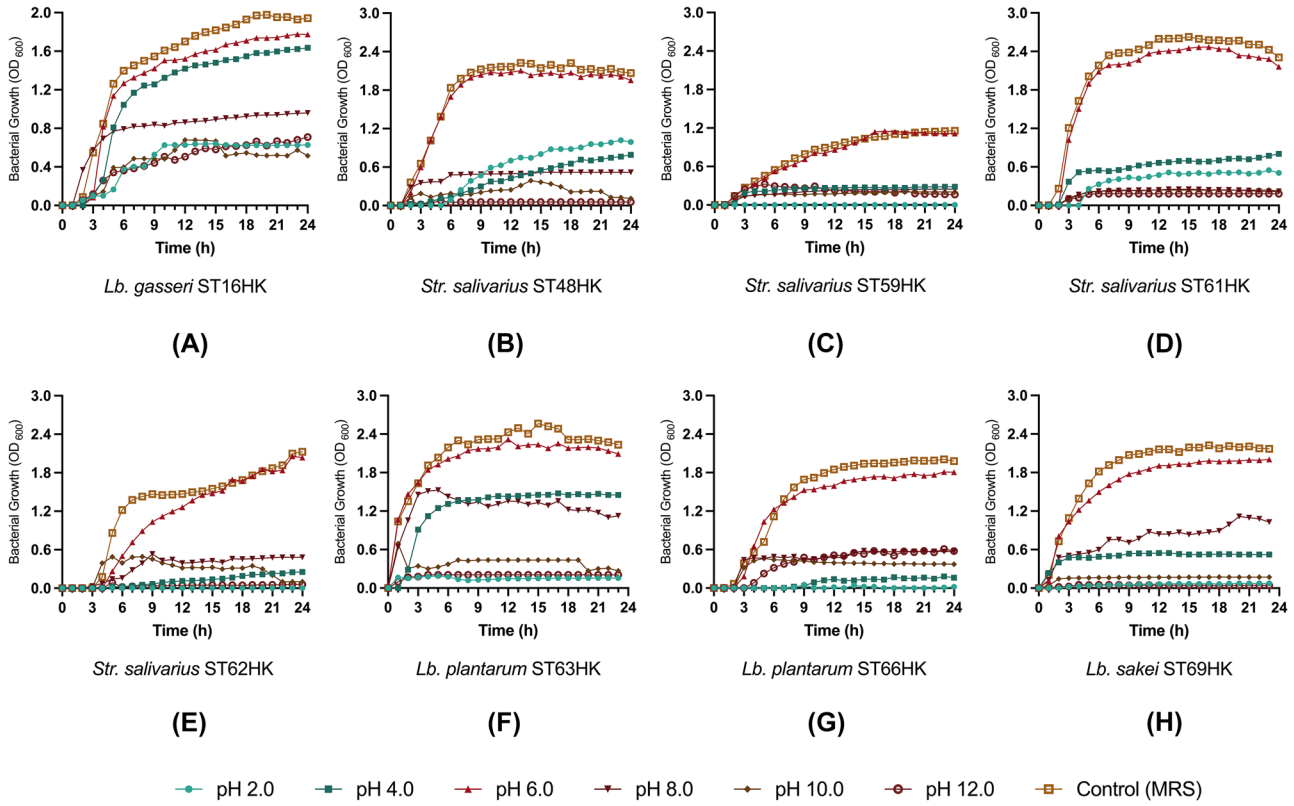

Fig. 2 Comparison of the growth of *Lactobacillus gasseri* ST16HK, *Streptococcus salivarius* ST48HK ST59HK, ST61HK, and ST62HK, *Lactiplantibacillus plantarum* ST63HK and ST66HK, and *Latilactobacillus sakei* ST69HK in MRS (Difco) at different initial pH levels. The experiment was performed in triplicate. SD were lower than 2%, and for simplicity, were not presented

ST62HK, while Mortin/Ibuprofen reduced the growth of all the strains except *Lb. plantarum* ST63HK. Treda did not influence the growth of *Lb. gasseri* ST16HK and *Lb. plantarum* ST66HK. Sandrin inhibitory effect was observed to the growth of *Lb. plantarum* ST66HK and *Str. salivarius* ST61HK. Different samples of Listerine inhibited the strains in a strain-specific manner (Table 3). The different ingredients of the two hygienic oral products did not show inhibitory effects against the strains. MIC levels were also calculated for the commercial drugs and hygienic products (Table 3), an information relevant to the long-term application of the drugs and/or hygienic products and potential negative effect on the probiotics. The generated inhibitory effects against the studied strains and the calculated concentrations are listed in Table 3.

Conclusions

Eight beneficial strains with potential as oral probiotics were isolated from the saliva of healthy individuals. Their differentiation and identification were based on repPCR and 16S rRNA partial genome sequencing. These strains were further evaluated by screening for the presence of different safety and beneficial properties. Results suggest that the strains *Lb. gasseri* ST16HK; *Str. salivarius* ST48HK, ST59HK, ST61HK, and ST62HK; *Lb. plantarum* ST63HK and ST66HK; and *Lb. sakei* ST69HK can be considered as promising oral probiotic candidates. In order to be recommended as probiotics, additional tests to confirm their efficacy in an appropriate animal model, as well as in vivo toxicological studies, have to be performed, in accordance to national and international regulations.

Author Contribution Concept: SDT; experimental work: HK, JIIF, SDT; data analysis: HK, JIIF, SDT; funds: WHH, SDT; writing of the manuscript: HK, JIIF, SDT; corrections and editing: WHH, SDT.

Data Availability All data generated or analyzed during this study are included in this published article and comply with research standards.

Declarations

Ethics Approval This article does not contain any studies with human or animal subjects.

Conflict of Interest The authors declare no competing interests.

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