Adhesion to pharyngeal epithelium and modulation of immune response: *Lactobacillus salivarius* AR809, a potential probiotic strain isolated from the human oral cavity

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ABSTRACT

Microbiome modulators such as probiotics are known to modulate oral diseases. Very few probiotics are commercially available for use in the oral cavity. In this context, we selected human-origin *Lactobacillus salivarius* AR809 as a promising oropharyngeal probiotic and characterized its functional and immunomodulatory properties. Results demonstrated that AR809 could efficiently adhere to pharyngeal epithelial FaDu cells, antagonize *Staphylococcus aureus*, adapt to the oral environment, and modulate host innate immunity by inducing potentially protective effects. Particularly, AR809 diminished proinflammatory activity by enhancing the production of *IL10* and inhibiting the expression of tumor necrosis factor- α , *IL1B*, inducible nitric oxide synthase, and *RELA*. Finally, we observed that AR809 grew efficiently when cultured in milk, suggesting that the preparation of a fermented milk product containing AR809 could be a practical way to administer this probiotic to humans. In conclusion, AR809 has high potential to adhere to the pharyngeal mucosa and could be applied in novel milk-based probiotic fermented food products.

Key words: *Lactobacillus salivarius*, pharyngeal epithelium, functional property, milk

INTRODUCTION

The oral cavity harbors hundreds of microbial species (Keijser et al., 2018); most are normal or commensal microbes and some are beneficial bacteria with oral mucosal barrier functions, conferring resistance to pathogen colonization and modulating the immune system of the host (López-López et al., 2017). However,

the oropharyngeal microbiota is a diverse and dynamic system (Lamont et al., 2018). Various uncertain risk factors (i.e., host susceptibility, poor oral hygiene, or dietary habits) can modify the oral microbiota, altering the natural balance between commensal and pathogenic microorganisms (Esteban-Fernández et al., 2018; Fan et al., 2018). These aberrations result in predominance of opportunistic pathogens in the oral cavity, causing bacterial infections such as pharyngitis, halitosis, caries, periodontitis, gingivitis, and other oral diseases (Amer et al., 2017; Gao et al., 2018). Hence, approaches to restore or replenish the composition and function of the normal oral microbiota are very important. The same benefit can be achieved using probiotics (Singh et al., 2018).

Probiotics are nonpathogenic microorganisms, which when consumed in adequate amounts can confer a positive health benefit on the host (FAO/WHO, 2006). They exert their key functions through immune-regulatory actions, thereby modifying host's immune system, by restoring gut barrier function, by lowering intestinal pH, by decreasing colonization and invasion by pathogenic organisms, and by the production of favorable metabolic products through modulation of the gut microbiota (Son et al., 2019). Recently, studies have indicated that probiotics can modify the oral microbiota, and many are under investigation to prevent or treat diseases of the oral cavity, such as dental caries and periodontal diseases (Krzyściak et al., 2017; Invernici et al., 2018; Lin et al., 2018). Such diseases are associated with a shift in the microbial composition and activity of biofilm and the resulting reaction of the host (Al-laker and Stephen, 2017). Several probiotics have been designed for the oral cavity (Ai et al., 2017; Rungsri et al., 2017). The use of probiotics in the oropharyngeal tract is particularly promising. Oral pathogens such as *Staphylococcus aureus*, group A streptococci, and *Porphyromonas gingivalis* often cause microbial dysbiosis and result in oropharyngeal tract dysfunctions (Schenck et al., 2016; Blasco-Baque et al., 2017). Therefore, the

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oropharyngeal tract is a potential target for developing new, specifically designed probiotic products.

Wescombe et al. (2006) isolated *Streptococcus salivarius* strain K12 from the oral cavity; this strain showed high adhesive ability and inhibitory activities against oral pathogens. The strain was used to create probiotic food products (powder and chewing gum), commercialized under the name of BLIS K12 (BLIS Technologies, Dunedin, New Zealand), specifically for prevention or treatment of dysfunction such as tooth decay, periodontitis, bad breath, and pharyngitis (Zupancic et al., 2017). The United States Food and Drug Administration has assigned the oral probiotic product BLIS K12 “generally recognized as safe” (GRAS) status, which allows the probiotic to be used as a food additive (Taverniti et al., 2012). To our knowledge, no other probiotics for the oral cavity are commercially available except *Streptococcus salivarius* K12 and *Streptococcus salivarius* M18. Isolation and characterization of new strains are needed.

The objectives of this study were to (1) select the oral isolate *Lactobacillus salivarius* AR809 as a new probiotic candidate for the pharyngeal mucosa and analyze its potential probiotic properties in vitro; (2) assess the responses induced by AR809 in RAW264.7 macrophage cell model; and (3) explore the potential application of AR809 in a fermented food product. The information obtained in this work should be of interest to the dairy industry, particularly with respect to producing a novel milk-based fermented food product for use as a probiotic for the oropharyngeal mucosa.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Sixty-three *Lactobacillus* strains isolated from the oral cavity and pickles were stored at Shanghai Engineering Research Center of Food Microbiology. Isolate AR809 was from the oral cavity of a healthy individual and identified as *Lactobacillus salivarius* (GenBank accession number MK680189). *Staphylococcus aureus* was isolated from the oral cavity of a patient with pharyngitis. *Streptococcus mutans* ATCC 25135 was generously provided by Tongji Hospital (Shanghai, China).

The *Lactobacillus* strains were initially cultured on de Man, Rogosa, and Sharpe (MRS) medium (BD Biosciences, Heidelberg, Germany) in an anaerobic incubator (85% N₂, 10% H₂, and 5% CO₂; Thermo Fisher Scientific, Waltham, MA) at 37°C for 24 h. The *Staph. aureus* strains were plated onto brain heart infusion (BHI) medium (BD Biosciences) and incubated overnight at 37°C under aerobic conditions. *Streptococcus*

mutans was cultured in BHI broth at 37°C for 24 h in a 5% CO₂ atmosphere.

Cell Culture

For cell assays, FaDu cells and RAW264.7 cells were cultured in Dulbecco's modified Eagle medium (DMEM; HyClone, Logan, UT) containing 10% (vol/vol) fetal bovine serum (FBS; HyClone) and 100 U/mL penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO) at 37°C under 5% CO₂. The medium was renewed every second day. Cells were subcultured once a week with a split ratio of 1:3 using 0.25% trypsin-EDTA solution (Sigma-Aldrich).

Growth Inhibition Assay

Agar-well diffusion assays (Abushelaibi et al., 2017) were used to evaluate inhibition of *Staph. aureus* by *Lactobacillus* strains. The *Staph. aureus* cultures (100 µL, 1×10^6 cfu/mL) were plated onto the surface of a plate containing 20 mL of BHI agar. Six-mm-diameter wells were made in the BHI agar and filled with 100 µL of cell-free culture supernatant from the 24-h *Lactobacillus* liquid culture (centrifuged at $8,000 \times g$ for 10 min at 4°C) that had been filtered using a 0.22-µm filter. Sterilized MRS broth (pH 7.0) was used as a negative control. The plates were incubated at 37°C for 24 h under aerobic conditions, and zones of inhibition were measured in millimeters.

An equal volume of cell-free supernatant was added into the BHI broth containing *Staph. aureus* cells (1×10^6 cfu/mL) and incubated at 37°C for 24 h. Cells were washed 3 times with PBS (pH 7.2) and then stained with 50 µg/mL propidium iodide (Molecular Probes Inc., Eugene, OR) in PBS for 30 min in dark at room temperature. Cells were washed again, mounted on slides using mounting medium, and photographed with a fluorescence microscope (Leica, München, Germany). The dead *Staph. aureus* strains fluoresced red.

Bacterial Adherence Assay

The adhesion assay was determined according to the method of Wang et al. (2014), with minor modifications. A human epithelial cell line (FaDu) was obtained from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were seeded in 6-well tissue culture plates (Gibco, New York, NY) at 37°C under 5% CO₂. The medium in the wells was replaced with fresh medium until a monolayer formed with no more visible differentiation. Before adherence assays, the FaDu monolayer was

washed twice with sterilized Dulbecco's PBS (DPBS). *Lactobacillus* strains were grown in MRS broth under anaerobic conditions at 37°C for 24 h and then labeled with fluorescein isothiocyanate (FITC; Sigma-Aldrich). Subsequently, 1 mL of FITC-labeled *Lactobacillus* suspension (1×10^6 cfu/mL in DMEM) and 1 mL of antibiotic-free DMEM were added to each well containing FaDu cells and incubated at 37°C in a 5% CO₂ atmosphere. After 2 h of incubation, cells were washed twice with DPBS, and examined using a multi-function microplate reader (Molecular Devices, Silicon Valley, CA). Lasers were set at 485 nm for excitation and 530 nm for emission. The adhesion percentage was calculated as follows: Adhesion index (%) = $(B_1/B_0) \times 100$, where B_0 and B_1 are the initial and final fluorescence intensities of bacteria.

Anti-Adherence Assays

Adherence Exclusion. The inhibition of *Staph. aureus* adhesion by *Lactobacillus* strains was performed using the method of Esteban-Fernández et al. (2018) with modifications. The FaDu cells were seeded into 6-well tissue culture plates and cultured until a monolayer of cells was formed in each well. The monolayer was washed twice with DPBS before the inhibition assay. One milliliter per well of *Lactobacillus* (1×10^6 cfu/mL) was first added and incubated for 2 h. Unbound bacteria were removed, 1 mL per well of FITC-labeled *Staph. aureus* suspension (1×10^6 cfu/mL) was added, and the mixture was incubated again for 4 h. Cells plus bound bacteria were then detached with trypsin-EDTA solution for 4 min. The number of *Staph. aureus* adhered to FaDu cells was then determined as follows: Relative adherence of *Staph. aureus* (%) = $(S_1/S_2) \times 100$, where S_1 and S_2 were the fluorescence intensities of *Staph. aureus* adhered in the presence and absence of *Lactobacillus*, respectively. Assays were performed in triplicate in 3 independent experiments.

Adherence Displacement. The displacement exclusion assay was explored as described above for the adherence exclusion assay except that the *Staph. aureus* suspension (1×10^6 cfu/mL) was added before the *Lactobacillus* suspension (1×10^6 cfu/mL). The displacement rate of pathogen was expressed as percentage adhesion (%) of *Staph. aureus* in the presence and absence of *Lactobacillus*, as described for the exclusion assay.

Adherence Competition. The similar experimental protocol to the exclusion assay was used to analyze competitiveness between *Lactobacillus* and *Staph. aureus*. For competition assays, 1 mL of *Lactobacillus* strain suspension (1×10^6 cfu/mL) and 1 mL of *Staph. aureus* suspension (1×10^6 cfu/mL) were added simultaneously to each well and incubated for 4 h. Com-

petitiveness was calculated as the percentage adhesion (%) of *Staph. aureus* in combination with *Lactobacillus* relative to *Staph. aureus* adhesion in the absence of *Lactobacillus* (control).

Potential Probiotic Properties of *L. salivarius* AR809 in an Oral Cavity Model

Acidic pH and Bile Tolerance. Overnight cultures of AR809 (1×10^6 cfu/mL) were inoculated at 3% (vol/vol) into MRS broth with pH value adjusted to 2.0, 3.0, 4.0, and 5.0 with 1.0 M HCl solution, or into MRS broth containing 0.5% and 1% (wt/vol) bile (Sangon Biotech, Shanghai, China), and incubated at 37°C for 24 h. Inoculated MRS broth (pH 6.5 and without bile) was used as control. Every 30 min, the optical density value at 600 nm (OD_{600nm}) was measured using an automatic growth curve (Bioscreen C, Helsinki, Finland) analyzer after shaking the culture for 10 s.

Resistance to Lysozyme and H₂O₂. Overnight cultures of AR809 (1×10^6 cfu/mL) were inoculated at 3% (vol/vol) into MRS broth containing lysozyme (100 and 200 µg/mL) or hydrogen peroxide (H₂O₂, 0.08 and 0.8 mM; Sangon Biotech), and incubated at 37°C for 24 h. Inoculated MRS broth without lysozyme and H₂O₂ was used as control. Every 30 min, the OD_{600nm} value was measured using the automatic growth curve analyzer after shaking for 10 s.

Tooth Degradation. A tooth degradation assay was conducted according to the method of Nikawa et al. (2004). Ten-milligram hydroxyapatite samples (20 nm; Sigma-Aldrich) were placed in a 50-mL centrifuge tube, which contained 10 mL of BHI broth. The hydroxyapatite-BHI broth was filtered with a 0.22-µm filter. The selected strain AR809 (1×10^6 cfu/mL) was inoculated (3%, vol/vol) into each tube followed by incubation for 0, 3, 6, 12, 24, and 48 h at 37°C; *Strep. mutans* was used as a control. After each period of incubation, the amount of calcium released was measured by using a commercial kit (CA590; Leadman, Beijing, China) according to the manufacturer's instructions.

Antibiotic Susceptibility Testing. Antibiotic susceptibility of AR809 was determined using the disk diffusion method (Dolatabadi et al., 2018). First, MRS agar plates were overlaid with 50 µL of overnight cultures of AR809 (1×10^6 cfu/mL). Commercial antibiotic disks (ampicillin, kanamycin, norfloxacin, amoxicillin, chloramphenicol, nalidixic acid, erythromycin, minocycline, sulfamethoxazole, vancomycin, tetracycline, and clindamycin; Hangzhou Binhe Microorganism Reagent Co. Ltd., Hangzhou, China) were placed on the plates and incubated. After 24 h incubation at 37°C, the diameter of inhibition zone was measured and expressed as susceptible (S), intermediate (I), or resistant (R) fol-

lowing the instructions given by the manufacturer. All analyses were done in triplicate.

In Vitro Functional Properties of L. salivarius AR809 in RAW264.7 Macrophage Cell Model

Cytotoxicity Assay. The cytotoxicity of *L. salivarius* AR809 on murine macrophage RAW264.7 cells was assessed using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Esteban-Fernández et al., 2018). RAW264.7 cells were seeded in 96-well plates (Corning Inc., New York, NY) and then AR809 cells were added at different multiplicities of infection (MOI, bacteria:macrophages) of 0.2:1, 2:1, and 20:1 before co-incubation for 24 h at 5% CO₂. Subsequently, 20 µL of MTT reagent (Sangon Biotech) diluted with sterilized DPBS (5 mg/mL) was added to each well and incubated for 4 h under the same conditions. After removal of the culture medium, 150 µL of dimethyl sulfoxide was added to each well. The whole plate was oscillated at room temperature until formazan crystals were dissolved completely. Absorbance of each well at 570 nm was measured using a multi-function microplate reader. Cell viability was calculated using the following equation: Cell viability (%) = (A_{sample}/A_{control}) × 100, where A_{sample} is the absorbance of RAW264.7 cells co-incubated with *Lactobacillus* cells; and A_{control} is the absorbance of RAW264.7 cells alone (Huang et al., 2017).

Phagocytosis Assay. The phagocytic ability of RAW264.7 cells was investigated by neutral red uptake (Motlagh et al., 2015). Cells were seeded onto 96-well plates, treated without or with AR809 cells at an MOI of 2:1, and incubated at 37°C for 12 h. Subsequently, 200 µL of 0.075% neutral red solution (wt/vol) was added to the cell cultures. After incubation for 1 h, 200 µL of cell lysate (ethanol and 0.01% acetic acid at the ratio of 1:1, vol/vol) was added to each well. Next, the mixtures were incubated at 4°C for 4 h. The optical density was measured at 570 nm (OD_{570nm}) in a multi-function microplate reader. RAW264.7 cells treated with LPS (1 µg/mL; Sangon Biotech) and *Staph. aureus* (MOI 2) acted as positive controls. The phagocytic ability of cells pretreated with AR809 followed by LPS stimulation for 12 h was also assessed.

Anti-Inflammatory Activity in Macrophage Cells RAW264.7. To determine the anti-inflammatory activity, RAW264.7 cells were treated with *Staph. aureus* (MOI 5) for 4 h. After washing, the cells were incubated with *Lactobacillus* cells (MOI 2) for 24 h at 5% CO₂. The production of nitric oxide (NO) was measured using the Griess reaction (Oh et al., 2018). The mRNA expression of cytokine genes *TNFA*, *IL1B*, *IL10*, nuclear factor-kappa B p65 (*RELA*), and induc-

Table 1. Primers used for reverse transcription PCR¹

Gene symbol	Primer sequence (5'-3')
<i>TNFA</i>	CACCCTTATTCTCGCTCAC CCCGCTTACAGTTCTCT
<i>IL1B</i>	CTCGTGCTGTCGGACCCAT CAGGCTTGTGCTCTGCTTGTA
<i>IL10</i>	TGGCCTTGTAGACACCTTGCTT CAGAGAAGCATGGCCAGAAATCA
<i>RELA</i>	GTATTGCTGTGCCTACCCGAAAC GTTTGAGATCTGCCCTGATGGTAA
<i>NOS2</i>	GAGCGAGTTGTGGATTGTC CCAGGAAGTAGGTGAGGG
<i>ACTB</i>	CTCTGCTCCTCCCTGTCC CAATCTCCACTTTGCCACT

¹Top = forward; bottom = reverse.

ible nitric oxide synthase (*NOS2*) were determined with real-time quantitative PCR (**RT-qPCR**). Total mRNA was isolated from RAW264.7 cells using TRI Reagent RNA isolation Reagent (Sigma-Aldrich) according to the manufacturer's instructions and cDNA was prepared using the iScript cDNA synthesis kit (BioRad, Hercules, CA). The RT-qPCR reaction was performed on a LightCycler Nano thermal cycler (Roche Applied Science, Penzberg, Germany), using SYBR Green Fast-Start kit (Roche, Basel, Switzerland). The PCR cycles were as follows: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers were used as shown in Table 1. Relative expression was calculated using the 2^{-ΔΔCt} method, where Ct is the cycle threshold.

Skim Milk Fermentation with AR809

Effect of Sugars on AR809 Growth. Strain AR809 was cultivated at 37°C in MRS broth. The growth curves of AR809 were determined with 96-well microtiter plates using the automatic growth curve analyzer to measure growth in MRS broth supplemented with 2% (wt/vol) of the following sugars (Sigma-Aldrich): glucose, fructooligosaccharides, lactose, sucrose, galactose, xylooligosaccharide, or oligomeric isomaltose.

Acidification Curves and Apparent Viscosity of Fermented Skim Milk. An overnight culture of AR809 was inoculated (2.5%, vol/vol) into skim milk supplemented with 2% (wt/vol) fructooligosaccharides or sucrose. After 24 h of fermentation at 42°C, acidification curves were determined using a pH meter recorder (Mettler-Toledo, Zurich, Switzerland), and apparent viscosity (expressed in mPa·s) was measured using a Discovery Hybrid Rheometer-3 (TA Instruments, New Castle, DE; 25°C, shear rate profile of 100 s⁻¹).

Inhibition of *Staph. aureus* Growth by Fermented Skim Milk. Antimicrobial effects of the supernatant of fermented skim milk were tested against

Staph. aureus using the agar well diffusion method. First, 100 μ L of *Staph. aureus* culture (1×10^6 cfu/mL) was plated onto the surface of a plate containing 20 mL of BHI agar. Then, 6-mm-diameter wells were uniformly bored in the BHI agar, and 100 μ L of fermented skim milk supernatant ($6,000 \times g$ for 15 min) was dispensed into each well. Plates were incubated at 37°C for 24 h. After incubation, inhibition zone diameters surrounding each agar well were measured. Inhibition was considered positive when the zone diameter was >6 mm.

Statistical Analyses

All experiments were performed at least 3 times. The data shown are representative results of triplicate experiments and are presented as means $\pm$ standard deviations. Data analysis was carried out with SPSS 22.0 software (SPSS Inc./IBM Corp., Armonk, NY), and Duncan's test was used to detect differences ($P < 0.05$).

RESULTS

Inhibition of *Staph. aureus* Growth by *Lactobacillus* Strains

Lactobacillus plantarum AR113, *L. plantarum* AR195, and *L. salivarius* AR809 showed different levels of inhibition against *Staph. aureus* by the agar well diffusion method (Figure 1, inset figures a–d). Strain AR809 exhibited higher inhibition of *Staph. aureus*, with inhibition zone diameters of 24.6 mm (Figure 1b). Fluorescence microscopy showed that most of the *Staph. aureus* in the culture medium (control group) were alive (Figure 1A) and results were significantly different ($P < 0.05$) from those in the experiment group (Figure 1B–D). Dead bacteria dominated in the experiment groups. Thus, the selected *Lactobacillus* strains inhibited the growth of *Staph. aureus*.

Adherence of *Lactobacillus* Strains to FaDu Cells

The adherence indices of *Lactobacillus* strains to FaDu epithelial cell layer demonstrated that the strains did not have similar adhesion abilities. Strains AR113 and AR195 manifested poor adhesion, from 4.44 to 8.28%, whereas AR809 exhibited higher adherence to FaDu cells, with an adhesion index of 31.1%.

Anti-Adhesive Effects on Adherence of *Staph. aureus* to FaDu Cells

The ability of *Lactobacillus* strains to prevent *Staph. aureus* from binding to FaDu cell monolayers was eval-

uated using exclusion, competition, and displacement assays. The adhesion of *Staph. aureus* to FaDu without interference by *Lactobacillus* was assigned as 100%. As shown in Figure 2, *Staph. aureus* adhesion was reduced significantly in the presence of *Lactobacillus* strains AR809 and AR195 in all assays. In the exclusion assay, AR809 exhibited higher inhibition against adherence of *Staph. aureus* in contrast to strains AR113 and AR195, resulting in a notable reduction (47%) in *Staph. aureus* adherence. In competition and displacement assays, AR809 also showed marked antagonistic effects, with the adhesion rate of *Staph. aureus* to FaDu cells reduced by 22.63% and 16.91%, respectively. The 3 assays demonstrated that AR809 reduced the adherence of *Staph. aureus* more significantly than did competition and displacement assays. Therefore, AR809 was selected as the potential probiotic for further trials.

Probiotic Properties of AR809 in an Oral Cavity Model

Resistance to Acidic pH, Bile, Lysozyme, and H_2O_2 . To maintain activity and viability through the oropharyngeal tract, a potential probiotic should be resistant to the extreme conditions of the oropharyngeal tract. Figure 3A shows that AR809 could survive at low pH and under a high concentration of bile salts. Moreover, AR809 could tolerate a wide range of acidic pH (3–5) but growth declined sharply at pH 2. Strain AR809 showed comparatively better tolerance when exposed to 0.5% bile than at 1% bile. In addition, AR809 exhibited good tolerance to 200 μ g/mL lysozyme and 0.8 mM H_2O_2 (Figure 3B). Thus, AR809 could overcome the hostile conditions in the oropharyngeal tract and subsequently colonize the oropharyngeal tract.

Tooth Degradation. Figure 4 shows that the effect of *Strep. mutans* on calcium release from hydroxyapatite was negligible within the first 12 h of incubation but then increased strongly during the rest of incubation. After 48 h of incubation, calcium released from hydroxyapatite caused by *Strep. mutans* was 2.0 μ g/mL. In contrast, calcium release by AR809 was almost negligible (0.06 μ g/mL) after 48 h of incubation, suggesting good potential protection against tooth degradation.

Antibiotic Susceptibility. The susceptibilities of AR809 toward 12 antibiotics are presented in Table 2. Strain AR809 was resistant to kanamycin, norfloxacin, chloramphenicol, nalidixic acid, vancomycin, and tetracycline and had intermediate resistance to ampicillin, sulfamethoxazole, and clindamycin. In addition, AR809 was susceptible to amoxicillin, erythromycin, and minocycline, antibiotics that interrupt either protein biosynthesis or cell wall biosynthesis in the bacteria.

***In Vitro* Functional Properties of AR809 in RAW264.7 Macrophage Cell Model**

Cytotoxicity Assay. To understand the possible cytotoxic effect of AR809 on RAW264.7 macrophages, we evaluated the viability of RAW264.7 macrophage cells in the presence of viable AR809 at certain MOI (0.2, 2, and 20). As shown in Figure 5A, no significant changes in cell viability were observed with AR809 at different MOI after 12 h of incubation. However, AR809 showed more than 120% cell viability after 24 h of incubation at an MOI of 0.2. In contrast, AR809 had no cytotoxic effect on RAW264.7 cells at an MOI of 2, similar to that of untreated macrophage cells, and

a low cytotoxic effect was shown in AR809 at an MOI of 20 (~11% dead cells).

Phagocytosis Assay. As shown in Figure 5B, the phagocytic activity of RAW264.7 cells induced by AR809 was significantly higher than that without AR809 or with *Staph. aureus*. This suggested that AR809 had a greater ability to activate the macrophages than *Staph. aureus*. The phagocytic activity of the macrophages triggered by AR809 might be effective in activating immune cells. Stimulation of RAW264.7 cells with LPS for 12 h led to a substantial increase in phagocytosis ($P < 0.05$). The percentage of macrophages to phagocytose neutral red increased from 12.58 to 68.05% following LPS stimulation. However,

Figure 1. Effects of *Lactobacillus* strains on the growth of *Staphylococcus aureus*. The antimicrobial activities of the *Lactobacillus* strains were tested using fluorescence microscopy (panels A–D) and the agar well diffusion method (inset figures a–d). Dead *Staph. aureus* fluoresced red. A, a = control group (culture medium only); B, b = *Lactobacillus salivarius* AR809; C, c = *Lactobacillus plantarum* AR113; and D, d = *L. plantarum* AR195.

phagocytosis was reduced to 39.73% in LPS-stimulated RAW264.7 cells pretreated with AR809.

Anti-Inflammatory Activity. To compare the anti-inflammatory activity of AR809 in *Staph. aureus*-stimulated RAW264.7 cells, we measured the amount of the key inflammatory mediators NO, cytokines (*TNFA*, *IL1B*, and *IL10*), *RELA*, and *NOS2* produced by treatment with AR809. As demonstrated in Figure 5C, the production of NO in RAW 264.7 cell culture (control) was 5.19 μ M, whereas that stimulated with AR809 was 11.9 μ M. After treatment with *Staph. aureus* for 4 h, the NO concentration in the supernatant increased compared with controls ($P < 0.05$). Strain AR809 decreased the NO level released from *Staph. aureus*-stimulated RAW264.7 and exhibited the strongest inhibition.

Figure 5D further demonstrated the effects of AR809 on gene expression of some upstream mediators (*TNFA*, *IL1B*, *IL10*, *RELA*, and *NOS2*) by RT-qPCR. As shown in Figure 5D, *TNFA*, *IL1B*, and *NOS2* mRNA levels were all sharply increased by *Staph. aureus* treatment for 4 h but decreased by treatment with AR809 for 24 h. The changes in gene expression confirmed the inhibition of *Staph. aureus*-induced inflammation by AR809 at the transcriptional level.

Figure 2. Effects of *Lactobacillus* strains (*L. salivarius* AR809, *L. plantarum* AR113, and *L. plantarum* AR195) on the adhesion behavior of *Staphylococcus aureus* to FaDu cells. Results are expressed as means $\pm$ SD ($n = 3$). Means with different letters (a–d) differ significantly ($P < 0.05$).

Fermentation of *L. salivarius* AR809 in Skim Milk

The growth curves of AR809 with 7 types of carbohydrates in MRS medium are shown in Figure 6A. Strain AR809 grew best in the presence of glucose, sucrose, and fructooligosaccharides, followed by oligomeric isomaltose, xylooligosaccharide, galactose, and lactose. The pH of skim milk supplemented with 2% (wt/vol) sucrose or fructooligosaccharides with a 2.5% (vol/vol) inoculum of AR809 decreased to 4.07 and 4.36, respectively (Figure 6B). Strain AR809 reached a viable cell count of 5.4×10^7 cfu/mL after 24 h of fermentation, indicating that it contributed to the acidification of skim milk. The apparent viscosity of skim milk with

Figure 3. Growth curves of *Lactobacillus salivarius* AR809 versus incubation time under different conditions: (A) acidic pH and 0.5 or 1.0% bile; and (B) 100 or 200 μ g/mL lysozyme and 0.08 or 0.8 mM hydrogen peroxide (H_2O_2). OD = optical density.

Figure 4. Effect of *Lactobacillus salivarius* AR809 on demineralization (calcium release from hydroxyapatite) of tooth enamel compared with *Streptococcus mutans*. Results are expressed as means $\pm$ SD ($n = 3$). After 48 h of incubation, calcium released from hydroxyapatite by *Strep. mutans* and AR809 were 2.0 and 0.06 $\mu\text{g/mL}$, respectively, indicating that AR809 has a good potential for protection against tooth degradation and could be a helpful aid to reduce risk of caries.

AR809 was increased (5.85 mPa·s) compared with that of the control (1.18 mPa·s; skim milk alone), and addition of 2% (wt/vol) fructooligosaccharides to the skim milk with AR809 further significantly increased viscosity, to 88.41 mPa·s ($P < 0.05$; Figure 6C). Moreover, AR809-fermented skim milk inhibited the growth of *Staph. aureus* in vitro, with inhibition zone diameters ranging from 8.7 to 12.6 mm (data not shown).

Table 2. Antibiotic susceptibility of *Lactobacillus salivarius* AR809¹

Antibiotic	Dose ($\mu\text{g/disk}$)	Sensitivity ²
Ampicillin	10	I
Kanamycin	30	R
Norfloxacin	10	R
Amoxicillin	10	S
Chloramphenicol	30	R
Nalidixic acid	30	R
Erythromycin	15	S
Minocycline	30	S
Sulfamethoxazole	25	I
Vancomycin	30	R
Tetracycline	30	R
Clindamycin	2	I

¹Dose values (dose of antibiotics on a standard antibiotic disk) are the mean of 3 experiments each with duplicates. Inhibition zone values (data not shown) were measured as means and then assigned to a sensitivity category (R, I, or S).

²S = susceptible; I = intermediate; R = resistant.

DISCUSSION

The aim of the initial part of this study was to screen antibacterial *Lactobacillus* strains with potential ability of inhibit adhesion of *Staph. aureus* to human oral epithelial cells. Of 63 *Lactobacillus* strains tested, 3 (AR809, AR113, and AR195) demonstrated high antimicrobial activity against *Staph. aureus*. A total of 6 *Lactobacillus* strains isolated from traditional pickles showed different antagonistic activity against *Staph. aureus* (data not shown). Compared with *Lactobacillus* strains isolated from traditional pickles, only 1 strain (*L. salivarius* AR809) isolated from a healthy oral cavity showed antagonistic activity against *Staph. aureus*. This finding indicates that it is not easy to screen antibacterial *Lactobacillus* strains isolated from a healthy oral cavity and that the antibacterial activity of *Lactobacillus* strains is closely related to their original source.

Adhesion to oral epithelium is among the main criteria for the selection of oral probiotic strains, because adequate host interaction is required for successful colonization of mucosal surfaces by probiotic bacteria and the resulting health benefits, including pathogen exclusion and stimulation of the mucosal immune system (Pérez Montoro et al., 2018). Because of the difficulties in studying bacterial adhesion in vivo, we used the FaDu cell model system in vitro to assess the adhesion rate of the selected *Lactobacillus* strains. Strain AR809 showed excellent adherence to FaDu cells (31.1%), higher than previously published data in which adhesion ranged from 0.9 to 20% (García-Ruiz et al., 2014). Bacterial adhesion is initially based on nonspecific physical interactions between 2 surfaces, which then enable specific interactions between adhesions (usually proteins) and complementary receptors (Kiňová Sepová et al., 2018). Cell-wall proteomic approaches have provided insight into the molecular mechanisms involved in the adhesion process. Some of the cytoplasmic housekeeping proteins detected in the extracellular proteome have been defined as “moonlighting” proteins, including proteins involved in glycolysis, protein folding and stress response, as well as transcription and translation. These proteins can be found on the surface of *Lactobacillus* spp., where they act as adhesion-promoting factors (Pérez Montoro et al., 2018).

Adhesion to and invasion of host tissues are crucial steps in the pathogenesis of many pathogenic bacteria and viruses (Kakisu et al., 2013; Tsai et al., 2018). Several reports have confirmed that *Lactobacillus* effectively inhibits cell association and invasion by pathogens (Wang et al., 2014; Angmo et al., 2016). The competitive exclusion of adhesion of pathogens to host cells by adherent *Lactobacillus* cells is thought to be an important factor enabling *Lactobacillus* to inhibit

Figure 5. Effect of *Lactobacillus salivarius* AR809 on the function of RAW264.7 macrophage cells. (A) Effect of AR809 on proliferation of RAW264.7 cells at multiplicities of infection (MOI) of 0.2, 2, and 20; (B) effect of AR809, LPS, and *Staphylococcus aureus* on RAW264.7 phagocytosis; (C) production of NO in RAW264.7 macrophages treated with AR809 and *Staph. aureus*; and (D) gene expression of cytokines and inflammatory mediators in RAW264.7 macrophage cells. Results are expressed as means $\pm$ SD ($n = 3$). Means with different letters (a–e) differ significantly ($P < 0.05$). OD = optical density.

pathogen infection (Wang et al., 2014). In this work, the colonization resistance provided by AR809 against *Staph. aureus* was demonstrated in competition, exclusion, and displacement assays. These results suggested that AR809 protected the host epithelium by forming a barrier through self-aggregation and adherence mechanisms, and possibly against potential pathogen binding to host cell receptors and by co-aggregation with potential pathogens.

An ideal probiotic strain intended for any human use must be (a) of human origin, (b) devoid of potential virulence genes, (c) sensitive to common antibiotics,

and (d) tolerant to oral, gastric, and intestinal physicochemical conditions; it should also be (e) catalase-negative, (f) able to adhere to epithelial membrane, and (g) able to compete with other microbes (Nagpal et al., 2018). Our thorough screening for these characteristics allowed us to select a strain (AR809) possessing these desirable traits. This also explained why we selected AR809 isolated from the oral cavity with potential ability of adhesion and inhibition of adhesion of *Staph. aureus* to the pharyngeal epithelium as good candidate probiotic strain. In addition, because AR809 was isolated from the oral cavity, which often contains

Figure 6. Optimized conditions for fermentation of skim milk by *Lactobacillus salivarius* AR809. (A) Growth curves of AR809 in de Man, Rogosa, and Sharpe medium supplemented with different carbon sources at 2% (wt/vol); (B) pH change during fermentation by AR809 at 42°C in skim milk supplemented with 2% sucrose or fructooligosaccharides; and (C) apparent viscosities of AR809-fermented skim milks (shear rate = 100 s⁻¹, temperature = 25°C). Control = skim milk, group 1 = AR809-fermented skim milk, and group 2: AR809-fermented skim milk supplemented with 2% (wt/vol) fructooligosaccharides. Results are expressed as means ± SD (n = 3). Means with different letters (a–c) differ significantly (*P* < 0.05). OD = optical density.

complex bacteria and may have been exposed to long-term antibiotic treatments, it was likely to have resistance to some antibacterial drugs. A combination of AR809 and antibiotics may improve oral and intestinal health.

Lactobacillus has a strong ability to produce lactic acid or be resistant to acid. One study noted that a possible side effect of applying *Lactobacillus* to improve oral health is the increased risk of caries due to the production of lactic acid (Devine and Marsh, 2009). Therefore, we determined the ability of *L. salivarius* AR809 to degrade hydroxyapatite. The result showed that AR809 had only a slight ability to degrade hydroxyapatite after 48 h of culture. Nikawa et al. (2004) noted that *Lactobacillus reuteri* had low ability to degrade hydroxyapatite, and side effects were not reported when *L. reuteri* was administered to treat oral diseases. Based on the results of the clinical studies of Nikawa et al. (2004) and the current study, because of strain differences in acid production ability, AR809 had a higher ability to degrade hydroxyapatite than *L. reuteri* but much lower ability than *Strep. mutans*. Compared with *Strep. mutans*, AR809 has negligible ability to degrade hydroxyapatite and thus would not increase the risk of caries.

Strain AR809 induced proliferation of RAW264.7 macrophage cells and triggered their phagocytic activity. A primary function of macrophages is to phagocytize and degrade invading pathogens. However, weakened phagocytosis occurred due to the lack of a key phagocytic component to increase the susceptibility to bacterial infection. An important step for immunomodulation by probiotic *Lactobacillus* strain is enhanced phagocytic activity in the RAW264.7 cells (Jaffar et al., 2018). Activated macrophages might function to kill microbes (classical activation), produce extracellular matrix components for the promotion of wound healing (alternative activation), or secrete anti-inflammatory cytokines to terminate inflammation (Jaffar et al., 2018; Kaji et al., 2018). In this study, we demonstrated an effect of AR809 in phagocytic activity of macrophage cells to combat LPS and *Staph. aureus* cells. Strain AR809 not only had antimicrobial activity but also immunomodulation capacity and displayed significant inflammation suppression by enhancing production of IL-10 and inhibiting the expression of TNF-α and IL-1β. Additionally, AR809 attenuated iNOS and repressed the nuclear transcription factor signaling pathway by impeding the nuclear translocation of p65.

The emerging demand for products having health benefits beyond nutrition has provided many opportunities to develop functional probiotic foods. In light of the in vitro probiotic and functional properties of

AR809, we examined the possibility of co-cultivating the bacteria in bovine milk and potentially using AR809 in dairy products. We found that AR809 showed optimal growth in the presence of glucose, sucrose, and fructooligosaccharides. It survived in skim milk supplemented with 2% (wt/vol) fructooligosaccharides, resulted in acidification, and significantly increased the apparent viscosity of milk. These results indicate that AR809 could be used as a starter culture for fermented milk. Moreover, AR809-fermented skim milk could be used to inhibit the growth of *Staph. aureus* in vitro. Taken together, these data supported the potential of using AR809 to ferment milk for the production of a novel food product.

CONCLUSIONS

We isolated antibacterial strain *L. salivarius* AR809 from the oral cavity and demonstrated that this strain has potential ability to adhere to the pharyngeal epithelium and modulate the immune response; thus, it is a good candidate probiotic strain for improving oral health. Because of its unique probiotic and functional properties, *L. salivarius* AR809 has the potential to enhance the immunity of the human oropharyngeal tract against invading pathogenic microbes for the prevention and treatment of pharyngeal infections. Moreover, AR809 has potential as a probiotic in health-promoting foods. It can be widely used in various commercial food products such as fermented milk. Using such foods as the delivery vehicle for probiotics leads to increased demand for these traditional products, indirectly improving the local and national economies. Before commercial application, further investigations may be warranted to elucidate the potential beneficial effects of fermented milk containing AR809 both in vitro and in vivo.

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