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Effects of probiotic *Lactobacillus salivarius* W24 on the compositional stability of oral microbial communities

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ABSTRACT

Probiotics are microorganisms beneficial to gastrointestinal health. Although some strains are also known to possess positive effects on oral health, the effects of most intestinal probiotics on the oral microflora remain unknown. We assessed the ability of the intestinal probiotic *Lactobacillus salivarius* W24 to incorporate into and to affect the compositional stability and cariogenicity of oral microbial communities. Microtiter plates with hydroxyapatite discs were incubated with W24 (''+W24'') or without W24 (''−W24'') and saliva from four individuals in plain (''−sucrose'') or sucrose-supplemented (''+sucrose'') medium. Biofilms were subjected to community profiling by 16S rRNA gene-based Denaturing Gradient Gel Electrophoresis (DGGE) after 72 h growth. Diversity (Shannon–Weaver index) and similarities (Pearson correlation) between biofilm communities were calculated.

Microcosms ''+sucrose'' were less diverse and more acidic than ''−sucrose'' microcosms ($p < 0.001$). The effects of W24 on the community profiles were pH dependent: at pH 4 (''+sucrose''), the respective ''+W24'' and ''−W24'' microcosms differed significantly more from each other than if the pH was ~7 (''−sucrose''). The pH of ''+W24/+sucrose'' microcosms was lower ($p < 0.05$) than the pH of the microcosms supplemented with sucrose alone (''−W24/+sucrose'').

Although not able to form a monospecies biofilm, *L. salivarius* W24 established itself into the oral community if inoculated simultaneously with the microcosm. In the presence of sucrose and low pH, W24 further lowered the pH and changed the community profiles of these microcosms. Screening of probiotics for their effects on oral microbial communities allows selecting strains without a potential for oral health hazards.

1. Introduction

Probiotics are defined as live microorganisms which when administered in adequate amount confer a health benefit on the host.[1] The effective use of probiotics has been reported in treatment of intestinal diseases such as inflammatory bowel disease, antibiotics-associated diarrhoea, and irritable bowel syndrome,[2–4] as well as non-gastrointestinal diseases, such as

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atopy, respiratory infections, vaginitis and hypercholesterolaemia.[5] Probiotic supplements are generally regarded as safe because the microorganisms they contain are identical to those found in the human gastrointestinal and vaginal microflora. Although probiotics are administered orally by ingestion, so far the studies on these microorganisms with respect to oral health are scarce[6] and their effects on oral microbial ecology remain unknown.

The most abundantly used probiotic strains are of the genus *Lactobacillus*. Lactobacilli are commensal lactic acid producing bacteria with high aciduric potential. A probiotic lactobacillus strain, *Lactobacillus salivarius LS1952R* was found to be highly cariogenic in rats,[7] while the oral administration of probiotics containing seven *Lactobacillus* species significantly increased the salivary counts of lactobacilli in healthy adults and had no effect on *Streptococcus mutans*.[8] In contrary to these results, there are clinical studies on probiotics and oral health suggesting that probiotic bacteria may have beneficial effects on dental health.[6] Children that were exposed to milk containing the probiotic *Lactobacillus rhamnosus* GG for 7 months, showed less dental caries and lower mutans streptococci counts than children in the control group.[9] In addition, a study on an adult population also found reduced salivary mutans streptococci, in this case after 3-week ingestion of *Lactobacillus reuteri* (ATCC 55730).[10]

Among the various intestinal probiotic lactobacillus strains tested, *L. salivarius* W24 was superior in inhibiting coagulase negative *Staphylococcus* and *Staphylococcus aureus*, as well as other clinical pathogens such as *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Escherichia coli*.[11,12] Moreover, W24 inhibited pro-inflammatory cytokine production in unstimulated peripheral blood mononuclear cells and had no negative selection criteria such as antibiotic resistance.[11] This strain is included in commercially available probiotic products (Winclove Bio Industries BV, Amsterdam, The Netherlands) used to restore the gastrointestinal microbial balance, *e.g.* after antibiotic-associated diarrhoea or traveler's diarrhea.[11,13] *L. salivarius* belongs to obligatory homolactic lactobacilli that produce only lactic acid during glucose fermentation. We hypothesised that addition of *L. salivarius* to oral microbial community may increase cariogenicity of dental plaque biofilm.

Our aim was to test the ability of intestinal probiotic *L. salivarius* (strain W24) to establish itself into the saliva-derived microbial communities. Furthermore, we aimed to assess the effects of W24 establishment on the compositional stability and cariogenicity of the microbial communities derived from individual salivas.

2. Materials and methods

2.1. The inoculum for microcosms

The use of human saliva was approved by institutional review board. Stimulated saliva was collected during parafilm chewing from four healthy adults with caries experience in the past and no use of antibiotics within last 3 months. The donors were asked not to brush their teeth for 24 h and to abstain from any food or drink intake for at least 2 h before donating saliva. During collection, saliva was kept on ice. After that, the saliva was filtered through sterilised glass-wool and diluted in glycerol (final concentration 30%). The mixture of saliva and glycerol was aliquoted in 2-mL sterile tubes, and stored at −80 °C. One of the frozen aliquots was processed in advance of the experiment to quantify the bacteria by colony counting on blood agar plates after anaerobic incubation (80% N_2, 10% CO_2 and 10% H_2) at 37 °C for 48 h. An inoculum of 10^6 colony forming units (cfu)/mL was subsequently used in microcosm experiments.

2.2. Biofilm growth conditions and harvesting

The growth medium comprised artificial saliva medium described by McBain et al.,[14] and contained mucin (type II, porcine, gastric), 2.5 g/L; bacteriological peptone, 2.0 g/L; tryptone, 2.0 g/L; yeast extract, 1.0 g/L; NaCl, 0.35 g/L, KCl, 0.2 g/L; $CaCl_2$, 0.2 g/L; cysteine hydrochloride, 0.1 g/L; haemin, 0.001 g/L; vitamin K1, 0.0002 g/L, pH 7. Sterilised hydroxyapatite (HA) discs (∅10.6 mm) were put into the wells of polystyrene, 24-well flat-bottomed microtiter plates. Each well was filled with 2 mL of growth medium either with or without 0.2% (v/v) sucrose supplement. As inoculum the saliva-glycerol stock was added (10^6 cfu/mL) and the plates were incubated anaerobically at 37 °C for 72 h. The medium was refreshed every 24 h.

After growth the HA discs were removed from the wells, put into tubes with 1 mL of cysteine peptone water (CPW) and vortexed at maximum speed for 2 min. The biofilm samples were then centrifuged at 16,100 × *g* for 1 min. The samples were processed for Denaturing Gradient Gel Electrophoresis (DGGE). The pH of the spent medium was measured by pH electrode (PHM 220 Lab pH Meter, Meterlab®, Radiometer Analytical SAS, France).

2.3. Ability of L. salivarius strain W24 to establish into microcosms

A freezer stock (overnight culture + 30% glycerol) of *L. salivarius* W24 was streaked onto a MRS agar plate and grown at 37 °C anaerobically for 48 h. One colony of W24 from the agar plate was used to inoculate 10 mL of artificial saliva medium with 0.2% sucrose and grown anaerobically at 37 °C for 16 h.

The optimal concentration of W24 to inoculate the microcosm was determined by inoculating a series of concentrations of W24 (from 10^2 to 10^8 cfu/mL) with 10^6 cfu/mL of saliva in sucrose-supplemented artificial saliva medium and incubating the microcosms for 72 h as described above. A concentration of 10^4 cfu/mL W24 was found to give the DGGE profiles consisting of multiple bands including a distinct W24 band, while higher concentrations yielded a single dominant W24 band on the DGGE gel, and lower concentrations showed no W24 band at all (data not shown). The concentration of 10^4 cfu/mL of W24 was chosen as the optimal concentration to inoculate the microcosms described below.

Saliva-derived microcosms were grown anaerobically, in the medium supplemented with 0.2% sucrose, on the HA discs at six different conditions: the microcosm alone (a control), the probiotic strain W24 introduced once (at 0, 24 or 48 h), twice (at 24 and 48 h) or thrice (at 0, 24 and 48 h) into the