Microbial community interactions: effects of probiotics on oral microcosms
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Chapter II - Effects of probiotic *Lactobacillus salivarius* W24 on the compositional stability of oral microbial communities

This chapter has been published as:

ABSTRACT

Probiotics are microorganisms beneficial to gastro-intestinal 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. Microriters plates with hydroxylapatite 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 rDNA 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.

INTRODUCTION

Probiotics are defined as live microorganisms which when administered in adequate amount confer a health benefit on the host (FAO/WHO 2006). The effective use of probiotics has been reported in treatment of intestinal diseases such as inflammatory bowel disease, antibiotics-associated diarrhea, and irritable bowel...
syndrome (ARMUZZI et al. 2001; MACFARLANE and CUMMINGS 2002; VAN SANTVOORT et al. 2008), as well as non-gastrointestinal diseases, such as atopy, respiratory infections, vaginitis and hypercholesterolaemia (ZUCCOTTI et al. 2008). 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 (TWETMAN and STECKSEN-BLICKS 2008) 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 lactobacilli strain, *Lactobacillus salivarius* LS1952R was found to be highly cariogenic in rats (MATSUMOTO et al. 2005), 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* (MONTALTO et al. 2004). 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 (TWETMAN and STECKSEN-BLICKS 2008). Children that were exposed to milk containing the probiotic *Lactobacillus rhamnosus* GG for seven months, showed less dental caries and lower mutans streptococci counts than children in the control group (NASE et al. 2001). 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 (CAGLAR et al. 2006).

Among the various intestinal probiotic lactobacilli strains tested, *Lactobacillus 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* (TIMMERMAN et al. 2004; TIMMERMAN et al. 2007). Moreover, W24 inhibited pro-inflammatory cytokine production in unstimulated peripheral blood mononuclear cells and had no negative selection criteria such as antibiotic resistance (TIMMERMAN et al. 2007). 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 diarrhea or traveler’s diarrhea (KONING et al. 2008; TIMMERMAN et al. 2007). L. salivarius belongs to obligatory homolactic lactobacilli that produce only lactic acid during glucose fermentation. We hypothesized 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.

MATERIALS AND METHODS

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 that no use of antibiotics within last three 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 sterilized 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% N₂, 10% CO₂ and 10% H₂) at 37 °C for 48 h. An inoculum of 10⁶ colony forming units (cfu)/mL was subsequently used in microcosm experiments.

Biofilm growth conditions and harvesting

The growth medium comprised artificial saliva medium described by McBain et al. (McBAIN et al. 2005), and contained mucin (type II, porcine, gastric), 2.5 g/L; bacteriological peptone, 2.0 g/L; trypptone, 2.0 g/L; yeast extract, 1.0 g/L; NaCl, 0.35 g/L; KCl, 0.2 g/L; CaCl₂, 0.2 g/L; cysteine hydrochloride, 0.1 g/L; haemin, 0.001 g/L; vitamin K₁, 0.0002 g/L; pH 7. Sterilized hydroxylapatite (HA) discs (Ø: 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 supplementation. 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 two minutes. The biofilm samples were then centrifuged at 16,100 x g for one minute. 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).

**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 - 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 10^6 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 10^6 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 h, 24 h or 48 h), twice (at 24 h and 48 h) or thrice (at 0 h, 24 h and 48 h) into the microcosm. The microcosms were harvested after 72 h and were processed for DGGE.
To control for pH-induced effects resulting from the metabolism of sucrose, the same conditions as above were tested in a medium supplemented with PIPES buffer (Sigma-Aldrich, USA).

**The effects of sucrose and the probiotic *L. salivarius* strain W24 on microcosms derived from four individual salivas**

Saliva from four healthy adults was used to inoculate microcosms. Two independent experiments per saliva were performed with triplicate samples per experimental condition. The microcosms were grown at four experimental conditions: 1) in a plain medium without W24; 2) in a plain medium with W24 added at 0 h; 3) in a sucrose-supplemented medium without W24; 4) in a sucrose-supplemented medium with W24 added at 0 h. The microcosms were harvested after 72 h and processed for DGGE. The pH of the spent medium was determined after 24 h, 48 h (data not shown) and 72 h.

**Denaturing gradient gel electrophoresis (DGGE)**

DNA was extracted with the DNeasy blood and tissue kit following the instructions of the manufacturer (Qiagen, Germany). PCR was carried out in a 25 μL (total volume) mixture containing 0.4 μM Muyzer primer F357-GC, 0.4 μM primer R518 (Table 1) (MUYZER et al. 1993), each deoxynucleoside triphosphate at a concentration of 0.4 mM, 10 μg of bovine serum albumin (Biolabs, 10 mg/mL), 1 x Taq buffer, 0.25 U of Taq enzyme, and 1 μL of undiluted DNA template. Amplification was performed with a PCR machine (Tprofessional thermocycler, Biometra, Germany) as follows: 94 °C for four minutes, followed by 35 cycles of 94 °C for 0.5 minute, 54 °C for one minute, and 72 °C for one minute, and a final elongation at 72 °C for five minutes.

DGGE was performed with the Bio-rad DCode system. The PCR product was loaded onto 1-mm-thick 8% (wt/vol) polyacrylamide (ratio of acrylamide to bisacrylamide, 37.5:1) gels containing a 35% to 60% linear denaturing gradient; 100% denaturant was defined as 7 M urea and 40% (vol/vol) formamide. The outer two lanes of each gel were not used. The samples were distributed randomly among
different gels. To aid in the conversion and normalization of the gels, a marker consisting of 7 reference strains was added at both sides of each gel, as well as after every four samples. The marker consisting of a mixture of 7 reference strains (*Porphyromonas gingivalis*, *L. salivarius* W24, *Agregatibacter actinomycetemcomitans*, *Streptococcus mutans*, *Veillonella parvula*, *Peptostreptococcus assacharolyticus* and *L. reuteri*), for which the V3 region (*Escherichia coli* positions 357 to 518) was amplified.

The gels were electrophoresed in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM Na-EDTA; pH 8) at 200 V and 60 °C for 4 h. The gels were stained in 1 x TAE buffer containing 1 μg/mL of ethidium bromide and were recorded with a charge-coupled device camera system (The imager; Appligen, Illkirch, France).

Table 1. Primers used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primers (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC clamp</td>
<td>CGC CCG CCG CCG CCC GCG CCC GCG CCG CCG CCG CCC CCG CCC</td>
</tr>
<tr>
<td>F357*</td>
<td>GC CGC CCG CCG CCG GGC GGG CCT ACG GGA GGC AGC AG</td>
</tr>
<tr>
<td>R518*</td>
<td>ATT ACC GCG GCT GCT GG</td>
</tr>
</tbody>
</table>

* - Numbers 357 and 518 indicate nucleotide position in *E. coli*.

Analysis of the community profiles

Microbial diversity is the variation in microbial species in an ecosystem. To estimate microbial diversity, diversity indices are calculated statistically. A frequently used index is the Shannon Weaver index of general diversity (VERSEVELD and RÖLING 2004). Here, it takes into account the number of DGGE bands and the relative contribution of each band to the whole set of bands. Band positions were assigned manually. The individual bands and their intensities in the community profiles were determined using GelCompar. The Shannon Weaver index (H') was calculated.
Effects of *Lactobacillus salivarius* W24 as follows: $H' = - \sum p_i \log p_i$ where $p_i$ is the relative contribution of band $i$ to the whole set of bands in a track calculated as $p_i = n_i/N$; $n_i$ is the height of a peak in a densitometric curve and $N$ is the sum of all peak heights. A higher $H'$ indicates a higher diversity (VERSEVELD and RÖLING 2004).

Similarities between DGGE profiles were calculated using GelCompar software (version 4.0, Applied Maths, Sint-Martens-Latem, Belgium) by Pearson product-moment correlation coefficient and visualized using the Unweighted Pair Group Clustering Method with Arithmetic Averages (UPGMA) (VERSEVELD and RÖLING 2004). The average similarity values per growth condition from two independent experiments were calculated. UPGMA is a clustering method used to construct a tree from a matrix of pairwise distances between samples. The first step in UPGMA is identification of the pair of samples with the smallest distance between them. The branch point is estimated as half the distance between the two samples. The two samples are then defined as a cluster and the matrix is recalculated with the first two samples combined. This process is repeated with the number of entries in the matrix reduced by each time from the root by adding clusters defined in each of the matrices.

**Statistical analyses**

The Statistical Package for the Social Sciences (SPSS version 14) was used to perform statistical analyses. For all tests, triplicate samples from two independent experiments were averaged into a single value per individual saliva and growth condition. The normality of data was confirmed with Shapiro-Wilk normality test ($p>0.05$), the homogeneity of variance – with Levene’s test ($p>0.05$). The effects of the experimental conditions on the pH and the diversity values of the microcosms were calculated using one-way ANOVA and the Bonferroni post-hoc test. The correlation between the pH and the diversity data of 72-h old microcosms was calculated using Pearson’s correlation analysis. The significance level of all tests was 0.05.
RESULTS

Ability of *L. salivarius* strain W24 to establish into microcosms in the presence of sucrose

In preliminary experiments where the ability of W24 to grow in a biofilm was tested we found that this strain is not able to form a biofilm when incubated as a monoculture in the microtiter plate model.

The pH of spent medium of microcosms grown without PIPES supplement was pH 4.0 (SD 0.08), while with PIPES the pH was 6.8 (SD 0.02). W24 established itself into the biofilm community irrespective of the pH (Figure 1). However, W24 was able to establish itself into the microcosm only if W24 was added simultaneously with the inoculum (T = 0 h) (Figure 1). Microcosms that were inoculated with W24 once or twice at a later time point (T = 24 h or T = 48 h) did not show a distinct *L. salivarius* band on the DGGE gel.

The impact of W24 on the community profiles was pH-dependent. In the samples that were grown with W24 but without PIPES, some distinct DGGE bands either appeared (Figure 1, band “a”) or disappeared (Figure 1, band “b”) compared to the microcosms grown without W24, while no changes in the band profiles due to W24 were observed in microcosms supplemented with PIPES.
Figure 1. The effects of the inoculation time and frequency on the establishment of the *L. salivarius* W24 into the microcosms grown for 72 h, as revealed by DGGE profiling of amplified bacterial 16S rDNA gene fragments. Biofilms were grown in either medium supplemented with 0.2% sucrose alone (No PIPES) or in medium supplemented with 0.2% sucrose and PIPES (PIPES). Six conditions per growth medium were compared: 1) no W24, 2) W24 inoculated simultaneously with saliva into the microcosm at the start of the experiment (W24: at T = 0 h), 3) W24 inoculated into 24-h-old microcosms (W24: at T = 24 h), 4) W24 inoculated into 48-h-old microcosms (W24: at T = 48 h), 5) W24 inoculated twice – into 24- and 48-h-old microcosms (W24: at T = 24 h, 48 h), 6) W24 inoculated thrice – simultaneously with saliva at the start of the experiment and into 24- and 48-h-old microcosms (W24: at T = 0 h, 24 h, 48 h).

* - the band corresponding to the *L. salivarius* W24 position in the marker line.
a, b - examples of individual bands affected by the presence of W24.
Effects of sucrose and *L. salivarius* W24 on microbial communities derived from four individual salivas

The biofilm communities in microcosms with or without sucrose were unique for each individual (data not shown). However, W24 was able to establish itself into all microcosms irrespective of individual or the presence of sucrose. Figure 2 shows the average pH of the spent medium and the average diversity of the biofilm communities, derived from four individual saliva donors after 72-h growth. There was a significant correlation between the diversity of DGGE profiles and the pH of the spent medium (p<0.001; Pearson correlation coefficient 0.893). Sucrose had a significant effect on both pH and the diversity of the microcosms. The pH and the diversity of the sucrose-exposed microcosms were significantly lower (p<0.001) than the respective values of the microcosms grown in the plain medium. W24 lowered the pH of sucrose-exposed microcosms for additional 0.3 pH unit, which was significantly lower (p=0.046) than the pH of the microcosms exposed to sucrose alone.

Sucrose alone had a strong effect on the community profiles in all four individual microcosms: the sucrose-exposed samples of each individual clustered separately from the respective samples grown in plain medium (avg. similarity 23%, SD 12.2). The effects of W24 on community profiles of all four individual microcosms were dependent on the presence of sucrose (Figure 3). In the absence of sucrose, W24-supplemented microcosms remained rather similar to the microcosms without W24 added (avg. similarity 56%, SD 12.3). When W24 was added in the presence of sucrose, the microcosms showed significantly reduced similarity (avg. similarity 27%, SD 12.4) and a changed band pattern of the DGGE profiles (Figure 1).
Effects of *Lactobacillus salivarius* W24

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**Figure 2.** Shannon-Weaver Index of Diversity (H’) and the pH of the spent medium of the 72-h old microcosms grown in the medium with or without 0.2% sucrose and with (+W24) or without *L. salivarius* W24 (-W24) added to the inoculum. The medium was refreshed after 24 h and 48 h. The values are average from the duplicate experiments (N=3 per experiment) with four microcosms derived from individual saliva donors. Error bars indicate standard deviations.

**Figure 3.** The effects of sucrose on the similarity between the DGGE profiles of the 72-h microcosms with *L. salivarius* W24 and the microcosms without W24 added per individual saliva used to inoculate the microcosms. The similarity values were obtained from Pearson correlation after UPGMA analysis of the DGGE profiles. The values are average from two independent experiments with triplicate samples per growth condition. ‘No sucrose’ – the microcosms grown in the plain medium; ‘Sucrose’ – the medium was supplemented with 0.2% sucrose.
DISCUSSION

We studied the effects of two ecological perturbations – sucrose and a probiotic strain – and their mutual interaction on the stability of oral microcosms. Under specific growth conditions (low pH and concurrent inoculation with the microcosm), intestinal probiotic *Lactobacillus salivarius* W24 was able to affect the biofilm ecology of the microcosms derived from saliva of different individuals and appeared to have a cariogenic potential.

We employed sucrose as a well-known modulator of the ecology of oral communities: microbial shifts due to sucrose metabolism and resulting low pH have been demonstrated for defined microbial consortia (Bradshaw et al. 1989) and complex saliva-derived microcosms (Filoché et al. 2004; Filoché et al. 2007b). We also observed clear microbial shifts in our study, which provides further support to the ecological plaque hypothesis proposed by Marsh (Marsh 1994). In our model, the pH of the spent medium decreased from pH 7 to about pH 4 in the presence of sucrose, while it remained around pH 7 when sucrose was not added to the growth medium. The selective pressure of the low pH on microbial community was visualized in changed patterns of the DGGE fingerprints and summarized in decreased diversity of the profiles. Interestingly, a strong positive correlation between the pH and the diversity of the microcosms was found. More diverse microbial communities have been shown to be associated with caries-free individuals compared to caries-active subjects (Li et al. 2007; Li et al. 2005).

*L. salivarius* W24 is one of the probiotic strains available in multispecies commercial probiotic products aimed at the improvement of gastrointestinal microbial balance (Koning et al. 2008; Timmerman et al. 2004; Timmerman et al. 2007). Assessment of the potential of W24 to establish into the microcosms showed that this strain, although unable to form a biofilm while growing in a monoculture, was able to establish into saliva-derived communities irrespective of the pH of the medium. Interestingly, the prerequisite for successful establishment of W24 was the concurrent inoculation of the saliva microorganisms and W24. This suggests that, in *vivo* situation, this strain might be able to establish on dental surfaces within newly developing dental biofilm if exposed to the oral cavity right after tooth
brushing. This also proposes that besides the availability of surface for attachment, an interaction with other microorganisms or their products was mandatory for the biofilm formation of W24.

Furthermore, for W24 to have an impact on the ecology of the microbial community, a low pH was necessary. Only in the presence of un-buffered growth medium and sucrose, changes in the DGGE profiles of the W24-supplemented microcosms were observed. Two possible mechanisms might explain the observed effects of W24 on the biofilm communities. Firstly, the addition of W24 lowered pH even further than sucrose alone and thus might have selected the most aciduric members in the microcosms. Secondly, a common feature of probiotic strains is the release of bacterocins or bacterocin-like substances (CORR et al. 2007; JOERGER and KLAENHAMMER 1986; KLAENHAMMER 1993; VIGNOLO et al. 1993) that negatively affect other, potentially pathogenic microbial species. In our preliminary studies, W24 did not affect the biofilm formation of *S. mutans* in a dual species model (unpublished findings), while it does inhibit clinical isolates associated with infections elsewhere in the human body, such as *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Klebsiella pneumoniae* (TIMMERMAN et al. 2007).

It has been shown that individual saliva ‘begets unique microcosms’ (LEDDER et al. 2006) and each individual community may respond in its own manner to ecological perturbations (FILOCHE et al. 2007b). To increase the relevance of our findings, we assessed the effects of W24 on microcosms derived from saliva of four individuals and repeated each experiment twice. Even though each individual community and the microcosm derived from it is unique (LEDDER et al. 2006; RASIAH et al. 2005), sucrose together with W24 introduced the same phenomena – reduced diversity and lowered pH – in all individual microcosms in a reproducible manner. On the other hand, the appearance or disappearance of individual DGGE bands due to establishment of W24 in the presence of sucrose, was highly individual (data not shown) and is attributable to inter-individual differences in microbial community composition.

Our results showed that addition of an aciduric and obligatory homolactic microorganism, *L. salivarius* W24, to oral microcosms in the presence of sucrose
lowered the pH and thus might increase the cariogenic potential of the oral microbial community. The model described here is an oversimplification of the complex dynamic community interactions occurring in the oral cavity. Our approach could be used as a high throughput screening of existing and newly developed probiotics with regard to their effects on the oral microbial communities. Further clinical testing would be necessary to exclude the potential risks, e.g., increased acid formation rather than inhibiting cariogenicity, for the oral health of the commercially available intestinal probiotic products.

In conclusion, we showed that the intestinal probiotic *L. salivarius* W24 was not only able to establish into saliva-derived microcosms, but also affected the compositional stability and reduced the pH of these microcosms. Screening of intestinal probiotic strains for their effects on oral microbial communities could improve the selection of beneficial strains for the host as a whole.

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