ANTIMICROBIAL ACTIVITIES

Effect of probiotic bacteria on prevalence of yeasts in oropharyngeal biofilms on silicone rubber voice prostheses *in vitro*

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The proliferation of yeasts in the mixed bacterial and fungal biofilms colonising silicone rubber voice prostheses in laryngectomised patients is the main cause of malfunctioning of the valve mechanism on the oesophageal side of the prostheses. Indwelling voice prostheses usually have to be replaced every 3–4 months. The consumption of probiotic bacteria is largely motivated by health claims related to the urogenital and lower digestive tract, but not to the upper digestive tract. The present study examined the influence of probiotic bacteria on the prevalence of yeasts in oropharyngeal biofilms on silicone rubber voice prostheses, as formed in a modified Robbins device. Exposure of oropharyngeal biofilms on voice prostheses to suspensions of *Bifidobacterium infantis* 420 or *Enterococcus faecium* 603 did not significantly reduce the number of yeasts in the biofilm. However, suspensions of *Lactobacillus fermentum* B54, *L. rhamnosus* 744 or *L. lactis cremoris* SK11 led to a reduction in the number of yeasts harvested from the voice prostheses. Suspensions of *L. casei* Shirota and *Streptococcus thermophilus* B significantly reduced the number of yeasts in the biofilm to 39% and 33%, respectively. The reduction brought about in yeast prevalence in the mixed biofilm was greatest by exposure to a suspension of *L. lactis* 53, with yeast prevalence only 4% of the control. In conclusion, the study demonstrated that the prevalence of yeasts in oropharyngeal biofilms on silicone rubber voice prostheses might be controlled by consumption of probiotic bacteria.

Introduction

Indwelling silicone rubber voice prostheses in laryngectomised patients often fail within 3–4 months after insertion due to the formation of a biofilm on the oesophageal side of the prosthesis causing malfunctioning of the valve mechanism [1, 2]. Although the biofilm consists of various bacterial strains [3–5], including oral streptococci, skin staphylococci and enterococci, it is the yeasts isolated from malfunctioning prostheses which are generally held responsible for failure of the device, as their adhesion is most tenacious [6]. Scanning electron micrographs have demonstrated yeasts growing into the silicone rubber of explanted prostheses. *Candida albicans* is most frequently

isolated from voice prostheses, but *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. parapsilosis* have also been found. Modifications of the prosthesis surface, adjustment of dietary components or restoration of adequate salivary levels in the oral cavity are being explored to prolong the life-time of indwelling voice prostheses [7]. An alternative approach, hitherto rarely investigated, would be to challenge the colonising yeasts with competing probiotic bacteria.

Yeasts are seldom found in a biofilm without adhering bacteria and it has been suggested that their presence is essential in maintaining the integrity of mixed biofilms on surfaces [8]. Verran and Motteram [9] demonstrated that the presence of *Streptococcus sanguis* was a prerequisite for the adherence of *C. albicans* to denture acrylic. Yet, there are also certain bacterial strains that are known to compete effectively with yeasts in their colonisation of surfaces. Lactobacilli have a definitive role in the prevention of vaginosis [10], lactococci

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produce antimycotics [11] and thermophilic streptococci release biosurfactants that interfere with the adhesion of yeasts to silicone rubber [12].

The growth of biofilms on silicone rubber voice prostheses under laboratory conditions, showing similar features to those observed *in vivo*, including ingrowth into the silicone rubber, has only recently become possible [13]. A teleological approach [14] may be used in which a partly identified mixture of yeasts and bacteria, representing the total cultivable microflora from an explanted prosthesis, is employed as an inoculum. Voice prostheses have been inserted in a modified Robbins device and inoculated with the total cultivable microflora from an explanted prosthesis, complemented with yeasts. Cycling the device through periods of feast and famine permits growth of tenaciously adhering mixed biofilms on the prostheses with ingrowth features that cannot be distinguished from *in-vivo* biodeterioration seen on explanted prostheses. Interestingly, in a reductionistic approach with isolated single strains, biofilms resembling those found *in vivo* could not be grown in the modified Robbins device. For an investigation of isolated single factors influencing biofilm formation on voice prostheses, like the influence of probiotic bacteria, a laboratory model is indispensable, as it is not possible to control dietary factors, outside temperature and humidity over any prolonged period of time *in vivo*.

There is an increasing interest world-wide in alternative approaches [15] to control biofilms in the human body that are not based on antibiotics or antimycotics [16–18]. The aim of this study was to investigate whether the prevalence of yeasts in mixed oropharyngeal biofilms on silicone rubber voice prostheses could be reduced *in vitro* by exposing the prosthesis biofilm to suspensions of various probiotic bacteria.

Materials and methods

Voice prostheses and biofilm formation

'Low resistance' silicone rubber Groningen voice prostheses were provided by Medin Instruments and Supplies (Groningen, The Netherlands) and placed in three transparent modified Robbins devices, as shown schematically in Fig. 1. Each Robbins device was equipped with four Groningen voice prostheses. The total cultivable microflora from an explanted Groningen voice prosthesis, containing a variety of strains of yeast and bacteria, including *C. albicans*, *C. tropicalis*, streptococci and staphylococci, were cultured in a mixture containing Brain Heart Infusion Broth (Oxoid) 30% and defined yeast medium (/L: glucose 7.5 g, $(\text{NH}_4)_2\text{SO}_4$ 3.5 g, L-asparagine 1.5 g, L-histidine 10 mg, DL-methionine 20 mg, DL-tryptophane 20 mg, KH_2PO_4 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 500 mg, NaCl 500 mg, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 500 mg, yeast extract 100 mg, H_3BO_3

Fig. 1. Schematic presentation of the artificial throat, equipped with Groningen button voice prostheses.

500 µg, ZnSO₄·7H₂O 400 µg, Fe(III)Cl₃ 120 µg, Na₂MoO₄·2H₂O 200 µg, KI 100 µg, CuSO₄·5H₂O 40 µg) 70%. This mixed culture was used to inoculate the voice prostheses in the Robbins devices. Subsequently, a biofilm was allowed to grow on the voice prostheses over 3 days, in ambient air and at room temperature. On the fourth day, the throats were flushed with phosphate-buffered saline (PBS; 10 mM potassium phosphate, 150 mM NaCl, pH 7.0) to remove remnants of the growth medium.

One modified Robbins device served as a control and was perfused with 650 ml of PBS (35 ml/min) before being left in a moist environment. In the other Robbins devices, 650 ml of a bacterial suspension were perfused, after which the device was also left drained. To remove remnants of the bacterial suspension before the next perfusion, PBS was perfused through the device, followed by the bacterial suspension again. This perfusion scheme was repeated three times a day. At the end of the day, the modified Robbins devices were filled with growth medium for 30 min, while overnight the devices were left in the moist environment of the drained throat. Experiments were continued for 12 days at room temperature and the tracheal sides of the prostheses were left in ambient air, similar to the situation with a stoma.

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1, together with their growth medium and source. Test strains were inoculated from agar plates into the specified growth medium and incubated for 24 h at 37°C. The resultant culture was used to inoculate a second fresh medium that was similarly incubated for 16 h. All cultures were incubated aerobically at 37°C except for the strains of *Lactobacillus* spp. which were cultured in CO₂ 5% in air. Each bacterial suspension was centrifuged at 5000 g for 5 min and the pellet was washed twice with demineralised water. After counting in a Bürker-Türk counting chamber, bacterial pellets were resuspended in demineralised water at a concentration of 1×10^9 bacteria/ml. The pH of these suspensions is listed in Table 1.

Evaluation of biofilms

After removal of the voice prostheses from the modified Robbins devices, biofilm formation on the valve side of the prosthesis was assessed qualitatively by electron microscopy and quantitatively on the other three sides by plating of the biofilms on to agar plates.

For scanning electron microscopy (SEM), voice prostheses were flushed with sucrose 6.8% in 0.1 M cacodylate buffer (pH 7.4), fixed and stained in glutaraldehyde 2% and ruthenium red 0.2% in 0.1 M cacodylate buffer at 4°C and flushed again. Post-fixation and staining were performed in OsO₄ 1% and ruthenium red 0.2% in cacodylate buffer by gently shaking for 3 h at room temperature. Buffer washes and dehydration involved the following rinsing procedures: 20 min in sucrose 6.8% in 0.1 M cacodylate buffer; 3 × 10 min in bi-distilled water; 20 min in ethanol at respective concentrations of 30, 50 and 70% and finally 4 × 30 min in ethanol 100%. After critical-point drying with CO₂ for 4 h, the voice prostheses were sputter-coated with gold/palladium particles (15 nm). SEM observations were made with a JEOL 6301 electron microscope, with different magnifications at 15–25 kV.

The number of colonising yeasts (cfu) was determined after serial dilution in reduced transport fluid before inoculation on to plates of brain-heart infusion agar. The biofilms were removed by scraping the total biofilm from the voice prosthesis, which was suspended in reduced transport fluid and sonicated for 60 s. Agar plates were incubated at 37°C in aerobic conditions for 3 days. The number of fungal cfu/cm² was determined for all prostheses exposed to each probiotic strain. The discrimination between the resulting colony types for the yeasts and bacteria was done visually in combination with phase contrast microscopy. Enumeration of the total number of bacteria present in the biofilm was only done for the control, by plating on blood agar.

Results

Voice prostheses in the control Robbins device were consistently covered with a thick biofilm. Yeasts were recovered from the prostheses at a mean level of

Table 1. Probiotic strains and growth conditions used in this study

Strain	Growth medium	Source	pH
<i>Streptococcus thermophilus</i> B	M17 supplemented with 1% sucrose	NIZO, The Netherlands	4.0
<i>Lactococcus lactis</i> 53	BHI	Nutricia, The Netherlands	5.3
<i>Lactococcus lactis cremoris</i> SK11	BHI supplemented with 0.5% lactose	NIZO, The Netherlands	5.9
<i>Lactobacillus rhamnosus</i> 744 (ATCC7469)	MRS	Nutricia, The Netherlands	3.7
<i>Lactobacillus fermentum</i> B54	MRS	Dr G. Reid, Canada	6.3
<i>Lactobacillus casei</i> Shirota	MRS	Isolated from Yakult fermented-milk drink	4.3
<i>Enterococcus faecium</i> 603	BHI	Numico, The Netherlands	6.3
<i>Bifidobacterium infantis</i> 420	Defined yeast medium	Numico, The Netherlands	5.1

3×10^7 cfu/cm². Results were obtained from four experiments involving a total of 12 prostheses. The mean bacterial flora recovered from the prostheses was 1×10^8 cfu/cm².

The numbers of yeasts harvested from the prostheses after exposure of the oropharyngeal biofilms to various suspensions of probiotic bacteria are shown in Fig. 2. The number of yeasts isolated from prostheses in the control device was set to 100%. Exposure of the biofilms to suspensions of *B. infantis* 420 and *E. faecium* 603 did not significantly reduce ($p > 0.5$) the number of yeasts in the biofilm. Suspensions of *L. fermentum* B54, *L. rhamnosus* 744 and *L. lactis cremoris* SK11 produced a reduction ($p < 0.2$) in the number of yeasts harvested from the voice prostheses. Suspensions of *L. casei* Shirota and *S. thermophilus* B ($p < 0.1$) reduced the number of yeasts in the biofilm to 39% and 33%, respectively. The greatest reduction in yeast prevalence in the mixed biofilm (4% of the control) was produced by a suspension of *L. lactis* 53 ($p < 0.01$).

Scanning electron micrographs of the control biofilm and of biofilms after exposure to *S. thermophilus* B, *L. lactis* 53 and *L. rhamnosus* 744 suspensions are shown in Fig. 3. Ingrowth features of colonising yeasts together with bacteria can be clearly discerned for prostheses removed from the control device. Despite reduced yeast counts, fungal colonies on prostheses

exposed to a suspension of *S. thermophilus* B also showed similar ingrowth to the control, with a relatively greater involvement of bacteria when compared with the control. The *L. lactis* 53 and *L. rhamnosus* 744 suspensions fully inhibited the ingrowth of yeasts and bacteria, as shown in Fig. 3.

Discussion

The present study examined the effect of exposing a mixed oropharyngeal biofilm on silicone rubber voice prostheses to suspensions of probiotic bacteria. A reduction in the number of yeasts found in the biofilms may be beneficial, as proliferation of yeasts on the prosthesis surface is generally considered to be the main cause of device failure. Amongst the strains of lactobacilli studied, *L. casei* Shirota from a fermented milk product, *L. lactis* 53 and *S. thermophilus* B, isolated from heat exchanger plates in a pasteurising machine, caused the greatest reduction in the prevalence of yeast in the biofilms. These reductions appeared to be unrelated to the pH of the bacterial suspensions used. Previously, it has been demonstrated that mice with a normal, healthy oral microflora were highly resistant to colonisation by candida, whilst germ-free mice were extremely susceptible to candidal infection [19]. This susceptibility to candidal infection could be almost completely prevented by the restoration of a healthy microflora consisting of probiotic

Fig. 2. Recovery of yeasts from voice prostheses in the artificial throat after perfusion with different probiotic bacteria. All data are expressed as percentages of the control (100%), with a 30% mean difference over duplicate runs.

Fig. 3. Scanning electron micrographs of Groningen button voice prostheses removed from the artificial throats after perfusion three times daily with different probiotic bacteria or potassium phosphate buffer. Bar = 10 μ m. (a) Control, showing heavy biofilm formation; (b) after exposure to *S. thermophilus* B suspension; (c) after exposure to *L. lactis* 53 suspension; (d) after exposure to *L. rhamnosus* 744 suspension.

bacteria [20]. Disruption of the healthy oral microflora often occurs in laryngectomised patients due to reduced salivary flow following radiotherapy [21]. A similar situation occurs in patients with Sjögren's syndrome [22]. Furthermore, the wearing of dentures can lead to a candida-associated denture stomatitis, a condition caused by a disruption of the oral microflora due to the presence of a biomaterials prosthesis [23].

Although antimycotics have been used to reduce biofilm formation on voice prostheses, the success of this treatment is variable. Complete prevention of a biofilm is likely to be impossible and the ingrowth of the yeasts into the silicone rubber offers a protective environment against any antimicrobial therapy. A slow-

release tablet containing the antimycotic miconazole nitrate has been reported to eliminate candida from within the oropharynx of laryngectomised patients, but significant numbers of yeasts persisted in the biofilms on the valve side of the prostheses [2]. Similar results were found with the use of amphotericin B lozenges [3, 24] and rinsing the oral cavity twice a day with nystatin suspension [25, 26]. However, the long-term use of antimycotics is associated with an increased likelihood of inducing antimycotic resistance [15].

The use of probiotic bacteria to reduce yeast prevalence in biofilms on voice prostheses remains a worthwhile approach. Health claims associated with the use of probiotic bacteria are usually confined to the lower

digestive and urogenital tract [10, 27, 28]. For example, *L. acidophilus* has appeared to be effective in preventing candidal vaginitis in patients [29]. Lactobacilli are part of the healthy vaginal microflora [10] and can interfere with uropathogen colonisation of the urogenital tract by production of hydrogen peroxide and lactic acid, competitive adhesion and displacement and the release of anti-adhesive biosurfactants [10, 30, 31]. The release of anti-adhesive biosurfactants can be an especially powerful defence weapon against other colonising organisms, as the biosurfactant release of one adhering organism suffices to cover a substratum area 1000 times the geometrical area of the lactobacillus. *S. thermophilus* also releases anti-adhesive biosurfactants [32], that have been demonstrated to interfere with the initial adhesion of *C. albicans* and *C. tropicalis* to silicone rubber [12]. *L. lactis* 53 is not a biosurfactant producer but is known to release antimycotics [11].

In conclusion, the results of the present study indicate the potential health benefits of probiotic bacteria in the prevention of fungal infections occurring in the upper digestive tract. Specifically, such an approach could be helpful for patients who have undergone total laryngectomy with subsequent provision of an indwelling voice prosthesis.

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