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ORIGINAL SCIENTIFIC ARTICLE

In vitro inhibitory effect of two commercial probiotics on chromogenic actinomycetes

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Abstract

Purpose Black extrinsic discoloration is a common clinical and aesthetic problem. This study aims to evaluate the potential in vitro antagonistic activity of two commercial probiotics, *Streptococcus salivarius* M18 and *Lactobacillus reuteri* ProDentis, against microorganisms associated with black stains.

Methods *Streptococcus salivarius* M18 and *Lactobacillus reuteri* were tested against *Aggregatibacter actinomycetemcomitans* and *Actinomyces naeslundii*using their cell-free fermentative broth in a planktonic growth inhibition test.

Results Both probiotic cell-free supernatants showed the ability to reduce the pathogenic bacteria growth in a dose-dependent way. *Streptococcus salivarius* M18 showed a stronger antimicrobial activity than *Lactobacillus reuteri* ProDentis against the two indicator strains used. *A. naeslundi* was less susceptible to the probiotic activity of both *S. salivarius* and *L. reuteri* compared to *A. actinomycetemcomitans*.

Conclusions The obtained results demonstrate a potent antagonistic ability of probiotics to reduce the growth of microorganisms associated with black tooth stains. Therefore, these strains could be evaluated for a therapeutic use against dental pigmentations.

Keywords *Aggregatibacter actinomycetemcomitans* · *Actinomyces naeslundii* · *Streptococcus salivarius* M18 · *Lactobacillus reuteri* · Black stain

Introduction

Black extrinsic discoloration is a common clinical and aesthetic problem in childhood. Both primary and permanent teeth can be affected, with a reported prevalence of 1–20% (Ronay and Attin 2011). Black stain (BS) is considered a special form of dental plaque unique for its insoluble iron salts and high calcium and phosphate contents (Hattab et al. 1999; Reid and Beeley 1976). Previous culture-based studies have indicated an association between black tooth stain and chromogenic bacteria. While at first periodontal bacteria, such as *Porphyromonas gingivalis*, *Prevotella intermedia* and *Prevotella melaninogenica* were reported to be closely related to BS (Soukos et al. 2005), traditional and molecular examinations have proposed Actinomycetes as an etiological factor in the production of black pigment. Specifically, *Aggregatibacter actinomycetemcomitans* and *Actinomyces naeslundii* are reported as significantly predominant cultivable microorganisms found in patients with BSs (Saba et al. 2006; Li et al. 2015; Heinrich-Weltzien et al. 2014).

Current treatments focus on mechanical removal of the stains, which are hard to be removed by daily tooth brushing and are prone to resurface after professional scaling (Hattab et al. 1999). Therefore, further research on prevention and therapy of BSs is needed.

Probiotic bacteria are live microbial food supplements that confer a health benefit on humans (de Vrese and Schrezenmeir 2008). Their use to effect an improvement in

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oral health is a relatively undeveloped strategy and it has been investigated for prevention and treatment of dental caries, periodontal disease and for halitosis (Meurman 2005; Twetman and Stecksén-Blicks 2008; Allaker and Stephen 2017; Teughels et al. 2011).

To the best of our knowledge, no report on the introduction of beneficial bacterial population to help counter the proliferation of chromogenic species can be found in the published literature so far. Generally, probiotic activity is considered to influence the balance among the many species of commensal flora in the oral cavity. It is our view that probiotics may have beneficial applications in the reduction of BSs by inhibiting the proliferation of the BS associated bacteria, as demonstrated in vivo in a clinical trial (Bardellini et al. 2020), still the reasons why a probiotic intake inhibits the formation of BS requires further studies.

Among probiotics, *Streptococcus salivarius* possesses excellent credentials as an oral probiotic (Tagg and Dierksen 2003; Burton et al. 2011). It is a pioneer colonizer of the human oral cavity and produces bacteriocins, ribosomally synthesized proteinaceous antibiotics typically encoded by megaplasmid-borne loci (Wescombe et al. 2006). More specifically, the strain *S. salivarius* M18 has been shown to colonize and persist in the human oral cavity (Burton, Wescombe et al. 2013b), to reduce plaque formation (Burton and Drummond et al. 2013a) and to reduce gingivitis and periodontitis (Litty et al. 2015) Another probiotic is *Lactobacillus reuteri,* an obligate heterofermentative resident in the gastrointestinal tract in humans (Itsaranuwat et al. 2003) which is reported to produce antimicrobial substances with a broad-spectrum activity (Talarico et al. 1988; Ganzle et al. 2000), and it is effective against *Streptococcus mutans* (Caglar et al. 2008), as well as against periodontal pathogens (Vivekananda et al. 2010; Vicario et al. 2013).

To be able to display a probiotic effect against BSs a bacterium must compete with chromogenic bacteria, thus reducing the level of their colonization. The purpose of this study was to evaluate the inhibitory effects of two commercially available probiotics *Streptococcus salivarius* M18 and *Lactobacillus reuteri* Prodentis on growth inhibition of chromogenic bacteria, specifically *A. actinomycetemcomitans* and *A. naeslundii,* using their cell-free fermentative broth in a planktonic growth inhibition test.

Materials and methods

Bacteria and bacterial culture

Streptococcus salivarius M18 (IDA classification: DSM 14,865), also named by the manufacturer as BLIS M18 (BLIS Technologies, Dunedin, New Zealand), distributed as Carioblis® by Omeopiacenza (Pontenure, Italy), and *Lactobacillus reuteri* (1×10^8 CFU) strains DSM17938 and ATCC PTA5289 (Prodentis; BioGaia, Lund, Sweden) were used as probiotic strains. Two types of oral bacteria were used to test the antibacterial effect of these probiotic strains: *Aggregatibacter actinomycetemcomitans* DSM-13386 (DSMZ, Braunschweig, Germany) and *Actinomyces naeslundii* DSM-11123 (DSMZ, Braunschweig, Germany). They were cultured using Schaedler agar with vitamin K and 5% Sheep Blood (BD bioscience, Sparks, MD, USA) in anaerobic conditions.

Antimicrobial activity of *S. salivarius* and *L. reuteri* against oral bacteria

Bacterial strains were stored in tryptic soy broth (TSB) medium with 20% of glycerol at − 80 °C and used as required. Bacterial isolates (*Streptococcus salivarius* and *Lactobacillus reuteri*) from frozen stock were grown overnight and 500 µl was then subcultured in brain heart infusion (BHI) medium (BD bioscience, Sparks, MD, USA) for 48 h at 37 °C in anaerobic conditions. The bacterial suspensions were centrifuged at 4 °C for 10 min at 12.000*xg* and sterilized by filtration trough a 0.22 mm Millipore filter (Merck Millipore, Milano, Italy).

A test of the antibacterial properties of this medium was performed in accordance with recommendations from the Clinical and Laboratory Standards Institute (CLSI) (2012, 2016). A serial dilution was performed starting with 180 µl of cell-free supernatants (CFS) of *S. salivarius* and *L. reuteri* in BHI medium to reach a proportion range of 10–90%.

A. actinomycetemcomitans and *A. naeslundii* culture suspensions were diluted using fresh sterile BHI medium, so that the turbidity was equal to that of a McFarland 0.5 standard. Afterwards, 20 µl of each bacterial suspension was inoculated in each well of a 96-well plate up to a final volume of 200 µl. BHI medium without inoculum and BHI medium with only the indicator bacteria were used as negative and positive control, respectively. The plate was then incubated for 24 h at 37 °C in an anaerobic chamber. Bacterial growth was measured at a wavelength of 600 nm using a microplate spectrometer. The entire experiment was performed three times with three independent repetitions.

Statistical analysis

The primary outcome of this study was the bacterial growth mean of *A. actinomycetemcomitans* and *A. naeslundii* culture suspensions evaluated when grown separately in BHI (controls) and in BHI added with different dilutions of CFS of *S. salivarius* or *L. reuteri*. Two hierarchical models were built, one for each culture, to estimate bacterial growth

(measured 3 times per dilution, from 10 to 90%) in relation to dilution (from 10 to 90%) and "CASES" (control, CFS of *S. salivarius,* CFS of *L. reuteri*). Because normality assumptions were rejected, logarithm transformation was performed and estimates were than back-transformed to the original unit of measurement. Results are reported as estimates and 95% confidence intervals. All analyses were performed assuming a significance level of 5% using the statistical software R (version 3.4.2).

Results

Antibacterial activity of *Streptococcus salivarius* M18 and *Lactobacillus reuteri*

The effect of the two probiotics on the chromogenic actinomycetes' growth was explored by adding increasing concentration of single CFS of the 48-h-old probiotic cultures to the culture medium of the actinomycetes.

Anti-*A. actinomycetemcomitans* activity of *S. salivarius* M18 occurred drastically at CFS concentrations of 40% and higher (Fig. 1a) with a significant reduction of the *A. actinomycetemcomitans'* growth (p value = 0.001) and a complete growth inhibition at 90%. Differently, anti-*A. actinomycetemcomitans* activity of *L. reuteri* occurred at CFS concentrations higher than 60% (p value = 0.001) (Fig. 1a).

Anti-*A. naeslundii* activity of *S. salivarius* M18 occurred at concentrations higher than 40% with a complete growth inhibition at 90% (Fig. 1b), while anti-*A. naeslundii* activity of *L. reuteri* occurred at a concentration of 80% without reaching a complete growth inhibition at 90% (Fig. 1b). Both *S. salivarius* M18 and *L. reuteri* significantly reduced the *A. naeslundii* growth ranging between the concentrations of 40% (p value < 0.040) and of 70% (p value < 0.0001), respectively.

Discussion

BSs are characteristic pigmented deposits that may occur at any age but seem to peak in childhood with a decrease in prevalence during pubescence and adulthood. Even if not

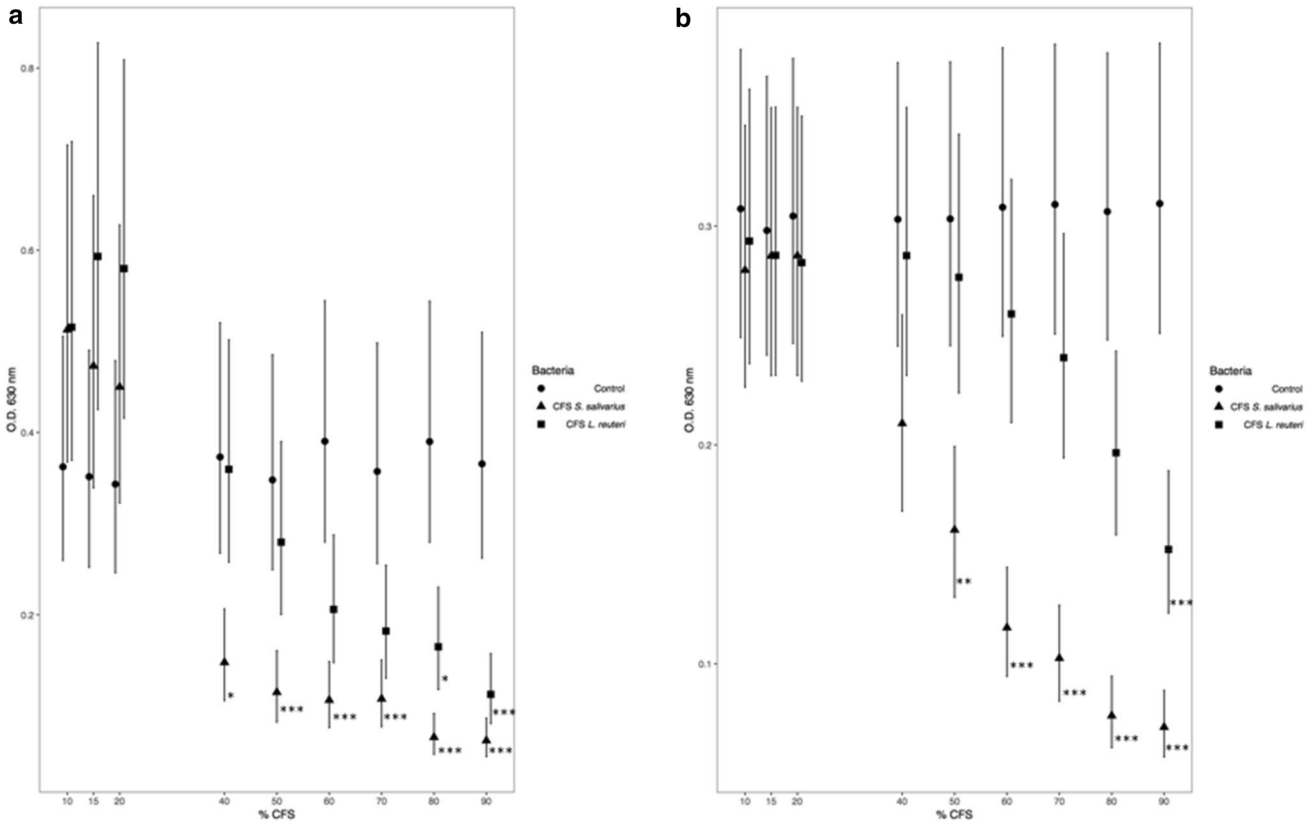

Fig. 1 Antibacterial activity of *Streptococcus salivarius* M18 and *Lactobacillus reuteri* ProDentis against *Aggregatibacter actinomycetemcomitans* (**a**) and *Actinomyces naeslundii* (**b**). CFS, cell-free supernatant. Each point represents the mean of three determinations ($n = 9$, average + 95% confidence interval). Asterisks are used to denote relevant comparisons and statistical significance as follows: *$p = 0.001$, **$p = 0.0002$, ***$p \leq 0.0001$

correlated with caries, BS represents an aesthetic problem, especially in the parents' eyes.

Probiotics are generally defined as microorganisms that confer a health benefit on humans by establishing a balance between beneficial and pathogenic bacteria (Koll et al. 2008; Li et al. 2015; Krasse et al. 2006). Their use is a relatively undeveloped strategy for prevention and treatment of caries and periodontitis (de Vrese and Schrezenmeir 2008).

BSs too are associated with a segregation of microbial communities in the oral cavity (Krasse et al. 2006), thus this approach has been considered as potentially beneficial in the prevention and treatment of BSs and applied for the first time in a clinical trial (Bardellini et al. 2020).

This short note is a preliminary contribution to unravel the underlying mechanisms. The study was undertaken to compare the in vitro efficiencies of two commercial probiotic products, including that used in the above mentioned clinical trial, against planktonic oral actinomycetes which are commonly reported as associated to BSs.

Both probiotics show antagonistic capacity, as their fermentative broth was able to significantly reduce the growth of the tested bacterial strains. Notably, in this study *Streptococcus salivarius* M18 showed a stronger antimicrobial activity than *Lactobacillus reuteri* ProDentis against the two indicator strains used. Furthermore, this study shows that *A. naeslundi* is less susceptible to the probiotic activity of both *S. salivarius* and *L. reuteri* compared to *A. actinomycetemcomitans*, whose growth is completely inhibited at high cell-free fermentative broth concentrations. This study also indicates a dose-dependent inhibiting activity of the two probiotics as higher the concentrations of their fermentative broth used in the experiments, the higher was the reduction of the bacterial indicators growth.

The activities of *S. salivarius* and *L. reuteri* against these actinomycetes have already been reported, although strains and culture media may differ and generally the pathogenic bacteria were in association to periodontal diseases (Teughels et al. 2013; Kang et al. 2011; Twetman et al. 2009).

The nature of the antimicrobial activity exerted by the two probiotics in this work remains unclear. Because in this study, pH evaluation of CFS was not performed, the presented data do not support whether the inhibition is due to some secreted metabolite, present in the cell-free supernatants of *S. salivarius* and *L. reuteri* or due to organic acids production, which could contribute to reducing the number of indicator bacteria. However, some previous studies demonstrated that the acidic environment provided by *Lactobacillus* strains can directly affect their antibacterial activities against *S. mutans* (Lin et al. 2015; Keller et al. 2011). Therefore, the role of the acids produced by probiotic strains on bacterial growth is still unclear.

This is corroborated by many reports showing that Lactobacilli can produce organic acids, H_2O_2 and bacteriocins (Cintas et al. 2001) while *S. salivarius* M18 releases into saliva copious quantities of bacteriocins and enzymes as dextranase and urease (Heng et al. 2011). Additional research is required to verify the mechanism of antibacterial activity found here and if and which probiotic metabolites are eventually produced in vitro and in vivo during the interactions studied in this work and what is their role in the oral cavity for preventing BSs.

This study ascertained in vitro the potential antagonistic activity of two commercial products, *Streptococcus salivarius* M18 and *Lactobacillus reuteri* ProDentis: these strains are to be considered for a therapeutic use against dental pigmentations.

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Compliance with ethical standards

Conflict of interest The Authors declare that they have no conflict of interest.

Ethical approval This study did not involve any human participants or animals.

Informed consent This study did not need informed consent from the patients, being an in vitro study.

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