Biologia Futura
https://doi.org/10.1007/s42977-021-00091-3

ORIGINAL PAPER

Interactions between probiotic and oral pathogenic strains

Noémi N. Gönczi[1] · Orsolya Strang[1] · Zoltán Bagi[1] · Gábor Rákhely[1,2] · Kornél L. Kovács[1,3]

Received: 19 August 2020 / Accepted: 19 May 2021

Abstract
More than 6 billion bacteria and other microorganisms live in the adult oral cavity. As a result of any deleterious effect on this community, some microorganisms will survive better than others, which may trigger pathogenic processes like caries, halitosis, gingivitis or periodontitis. Oral dysbiosis is among the most frequent human health hazards globally. Quality of life of patients deteriorates notably, while treatments are often unpleasant, expensive and irreversible, e.g. tooth loss. In the experiments reported here, we investigated the individual interactions between 8 pathogenic and 8 probiotic strains and a commercially available probiotic product. Almost all pathogens, namely *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus gordonii*, *Enterococcus faecalis* and *Prevotella buccae* are pathogens frequently occurring in the oral cavity. The used probiotic strains were *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Bifidobacterium thermophilum* and two *Streptococcus dentisani* isolates. Using a modified agar diffusion method, we investigated capability of the probiotic bacteria to prevent the growth of the pathogenic ones in order to identify candidates for future therapeutic treatments. The results indicated successful bacteriocin production, i.e. growth inhibition, against every pathogenic bacterium by at least 5 probiotic strains.

Keywords Oral diseases · Dysbiosis · Bacteriocin · Modified agar diffusion test

Introduction

Human oral cavity is a moist environment, having relatively stable temperature, ranging between 34 and 36 °C, and pH that is close to neutral (Marcotte and Lavoie 1998). Thanks to these favourable conditions, the microbiome of the mouth is very rich and diverse. In general, at least 6 billion bacteria live in the oral cavity of every human, consisting of more than 700 different species (Metwalli et al. 2013). Moreover, other microorganisms, i.e. fungi, mycoplasma, protozoa and viruses, also populate the main entrance of our body (How et al. 2016). The microorganisms tend to form biofilms to prevent microbial washout (Berger et al. 2018).

The primary colonizers of the dental biofilm are *Streptococcus* species (Dige et al. 2009; Brennan and Garrett 2019). The oral streptococci are Gram-positive, facultative anaerobes, which initiate the formation of dental plaque (Wang and Kuramitsu 2005). *Streptococcus mutans*, a member of the oral streptococci, was described in 1924 as the causative agent of the dental caries (Clarke 1924; How et al. 2016). *S. mutans* has multiple cariogenic effects. It can produce large amounts of extracellular polysaccharides (EPS) and organic

✉ Noémi N. Gönczi
nikolett.noemi.gonczi@gmail.com

Orsolya Strang
strang@bio.u-szeged.hu

Zoltán Bagi
bagi.zoltan@bio.u-szeged.hu

Gábor Rákhely
rakhely@brc.hu

Kornél L. Kovács
kovacs.kornel@bio.u-szeged.hu

[1] Department of Biotechnology, University of Szeged, Közép fasor 52., Szeged 6726, Hungary

[2] Institute of Biophysics, Biological Research Centre, Temesvári krt. 62., Szeged 6726, Hungary

[3] Department of Oral Biology and Experimental Dental Research, University of Szeged, Tisza Lajos körút 64-66., Szeged 6726, Hungary

Published online: 02 June 2021

acids and tolerates low pH (Lemos et al. 2019). The EPS-matrix provides protective environment for the other microorganisms. Elevated concentrations of acids demineralize the enamel and cause tooth decay (Hata and Mayanagi 2003; Lemos and Burne 2008; Forssten et al. 2010; Bowen 2016). The other two *Streptoccus* strains used in the experiments were *S. oralis* and *S. gordonii*. These bacteria do not cause tooth decay directly like *S. mutans* does. However, they produce EPS as well and play key role as primary colonization (Banas et al. 2007).

After primary colonizers attach to the surface successfully, a bridge bacterium, called *Fusobacterium nucleatum* binds to them and provides a link between the early, Gram-positive and the late, mostly Gram-negative bacteria (Park et al. 2016; Brennan and Garrett 2019). *F. nucleatum* is a Gram-negative (Han 2015), obligate anaerobic bacterium (How et al. 2016) with a tapered rod shape (Brennan and Garrett 2019). This bacterium expresses numerous adhesins, and the elongated shape helps to establish connection with many microorganisms (Brennan and Garrett 2019). This is the reason why *F. nucleatum* is one of the key players in dental plaque formation. Moreover, *F. nucleatum* was identified as a volatile sulphur compound (VSC) producer bacterium. The VSCs, such as methyl mercaptan and hydrogen sulphide, can cause halitosis, which affects 2.4% of the adult population in industrialized societies (Krespi et al. 2006).

Periodontal diseases affect 10–15% of the adult population worldwide (Petersen and Ogawa 2012). Although the latest models show that periodontal diseases are linked to a dysbiotic community rather than to a single bacterium, strong correlation was found between the periodontal diseases and *Porphyromonas gingivalis* (Hajishengallis and Lamont 2014; How et al. 2016; Ebbers et al. 2018). *P. gingivalis* is a Gram-negative, anaerobic bacterium (How et al. 2016), which requires heme or hemin and vitamin K for growth (Bostanci and Belibasakis 2012). The main habitat of *P. gingivalis* is the deep subgingival pocket where the level of sugars is low; therefore, this bacterium ferments amino acids as energy source (Bostanci and Belibasakis 2012). The main end-products of the fermentation are propionate, n-butyrate and acetate (Holt et al. 1999). The virulence factors of this bacterium could penetrate the gingival tissue, cause inflammation and inhibit the regeneration (Kato et al. 2014; How et al. 2016).

A special type of periodontitis is the localized aggressive periodontitis (LAP), which appears frequently in juvenile people and primarily affects the first molars. The main symptom is fast bone destruction (Miller et al. 2018). This disease is rare, affects only 1% of the population worldwide, whereas the pathogenic agent—namely *Aggregatibacter actinomycetemcomitans*—is present in at least one-third of the oral normal population (Zambon 1985). *A. actinomycetemcomitans* is a Gram-negative bacterium (Fine et al. 2015) and for a long time it was regarded as a late colonizer, but recent experiments showed that this bacterium colonizes the cleaned tooth surface in 6 h (Fine et al. 2010). As a slow-growing bacterium, *A. actinomycetemcomitans* has to choose an alternative survival method; hence, this bacterium uses lactate as carbon source (Brown and Whiteley 2007). During infection, *A. actinomycetemcomitans* first colonizes the supragingiva. In the next step, it integrates and survives in the biofilm's environment. Then, it migrates below the gum, where the bacterium suppresses the immune system of the host (Fine et al. 2019). The virulence factors of *A. actinomycetemcomitans* are able to modulate inflammatory response, cause tissue destruction and prevent from healing. It induces a quick course (Raja et al. 2014).

Prevotella buccae is a Gram-negative anaerobic bacterium (Cobo et al. 2017). Correlation has been found between this bacterium and periodontitis associated with advanced caries (Borsanelli et al. 2017; Praveen et al. 2018).

Most of the above-mentioned bacteria are normal members of the human oral cavity, dysbiosis occurs when their abundances increase in the community (Wilkins et al. 2003; Do et al. 2009; Raja et al. 2014; Dadon et al. 2017; Brennan and Garrett 2019). *Enterococcus faecalis*, on the contrary, can be found in the oral cavity, but it is not indigenous to the normal oral flora (Distel et al. 2002). This Gram-positive facultative anaerobic bacterium is well known as a nosocomial infective agent (Anderson et al. 2016). It is often detected in patients who have post-treatment apical or marginal periodontitis (Et and Mpa 2014), or primary or persistent endodontic infections (Karayasheva and Radeva 2017). There are three factors, which help *E. faecalis* to become a successful survivor. The antibiotic resistance level of this bacterium is very high (Distel et al. 2002); it has a strong biofilm forming potential and powerful capability to attach to the host cells (Anderson et al. 2016).

Metchnikoff was one of the first who defined dysbiosis and probiotics. He thought that there were toxin-producer bacteria in the gut, which cause diseases and shorten the lifetime, but when we supply our body with useful bacteria, then the number of harmful bacteria will decrease, and consequently, life expectancy and quality of life can improve (Anukam and Reid 2007; Andrade et al. 2012). Following Metchnikoff, we have a substantial amount of information on the probiotics in the gut. Pan et al. examined the probiotic effect of *L. casei* on rheumatoid arthritis in rat model and they found that probiotics ease the symptoms in sick animals (Pan et al. 2019). Kim et al. made a comprehensive research on probiotics affecting neuronal system disorders, like autism, Alzheimer's disease, Parkinson's disease, depression or stress (Kim et al. 2018). The results have been very impressive, but the precise mechanism is not yet disclosed. Firstly, probiotic bacteria compete for binding site and nutrients with other microorganisms. Secondly, they can

degrade toxins and produce antimicrobial substances, like short-chain fatty acids, hydrogen peroxide, nitric oxide and bacteriocins (Dobson et al. 2012). Finally, they are able to induce local or systemic immune modulation (Jakubovics and Palmer Jr. 2013). Of course, in most cases, one probiotic strain does not have all three effects; therefore, they are mostly combined for efficient treatment (Jakubovics and Palmer Jr. 2013).

Bacteriocins are small, bacterially produced, ribosomally synthesized peptides which are effective against other bacteria. There are numerous broad- and narrow-spectrum bacteriocins identified, which affect targeted pathogens without hurting the normal microflora. Bacteriocins have many benefits like their potency, low toxicity and the availability of both broad and narrow-spectrum peptides (Cotter et al. 2013). Lactic acid bacteria (LAB) such as lactobacilli, bifidobacteria, non-pathogenic *Escherichia coli* and bacilli are used to prepare probiotic products (Dobson et al. 2012). Several bacteriocins have been characterized from the probiotic strains employed in the present study (Barefoot and Klaenhammer 1984; Müller and Radler 1993; Simova et al. 2008; Martinez et al. 2013; da Silva Sabo et al. 2014; Jeong and Moon 2015; Ullah et al. 2017; Zhou and Zhang 2018; Gaspar et al. 2018). The main goal of our study was to test probiotic bacteria (such as lactobacilli, bifidobacteria and particularly *Streptococcus dentisani*, a recently recognized probiotic bacterium) against selected oral pathogenic strains.

Materials and methods

Strains and media

During the experiments, the following pathogenic strains were used: *Fusobacterium nucleatum, Porphyromonas gingivalis, Streptococcus mutans, Streptococcus gordonii, Streptococcus oralis, Aggregatibacter actinomycetemcomitans, Prevotella buccae, Enterococcus faecalis*. The applied probiotic strains were: *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus delbrueckii,* two *Streptococcus dentisani* strains (DSMZ 27088, 27089) and the mixed probiotic product, Florabalance Plus (Goodwill Pharma Ltd.) which is commercially available, including the following strains: *Bifidobacterium infantis, Bifidobacterium longum, Bifidobacterium bifidum, Lactobacillus rhamnosus, Lactobacillus bulgaricus, Lactobacillus plantarum, Lactobacillus helveticus* and *Lactobacillus lactis.* Strains were provided by University of Szeged, Institute of Clinical Microbiology, except *L. delbrueckii* and *L. casei*, which were from the microbial strain collection of University of Szeged, Department of Biotechnology. The two *S. dentisani* were purchased from DSMZ. Strains were stored at − 80 °C with 50 v/v% glycerine.

Two media were used for propagation of the strains—the ATCC 2722 (2722) and a modified DSMZ 58 (58). ATCC 2722 had the following composition: tryptic soy broth (see below) 30.00 g, yeast extract 5.00 g L-cysteine HCl 0.50 g, hemin stock (5%) (see below) 1.00 ml, vitamin K1 stock (1 mg/ml) 1.00 ml, in 1000.00 ml distilled water. The modified DSMZ 58 had the following composition: hemin stock (5%) (see below) 1.00 ml, tryptic soy broth 15.00 g, casein peptone (tryptic digest) 1.50 g, yeast extract 5.00 g, meat extract 5.00 g, glucose 8.75 g, K_2HPO_4 0.75 g, $MgSO_4 \times 7\ H_2O$ 0.20 g, $MnSO_4 \times H_2O$ 0.05 g, Tween 80 1.00 ml, NaCl 2.5 g, L-cysteine HCl 0.50 g, salt solution (see below) 40.00 ml, in 960.00 ml distilled water. The composition of the hemin stock (5%) solution was: hemin 0.25 g, distilled water 50.00 ml. The tryptic soy broth had the following components: casein peptone 15.00 g, soymeal peptone 3.00 g, glucose 2.50 g, NaCl 5.00 g, K_2HPO_4 2.50 g in 30 g. The salt solution used in modified DSMZ 58 had the following composition: $CaCl_2 \times 2\ H_2O$ 0.25 g, $MgSO_4 \times 7\ H_2O$ 0.50 g, K_2HPO_4 1.00 g, KH_2PO_4 1.00 g, $NaHCO_3$ 10.00 g, NaCl 2.00 g, in 1000.00 ml of distilled water.

Media were prepared for solution, agar plate and top agar. 20 ml solution was filled in hypovial bottles. Each bottle was sealed with butyl rubber stopper and an aluminium cap and was flushed with nitrogen gas for 10 min to establish anaerobic environment. For making the agar plates, 1.5% agar was added to the solution and for the top agar 2% agar was added to the solution.

Modified agar diffusion test

The basic agar disc-diffusion method has been developed in 1940 (Balouiri et al. 2016). In this, well-known procedure plates are inoculated with a single test microorganism in a lawn. Then, paper discs, containing the test compounds/microbes, are placed on the agar surface. During incubation, the test compound, a potential antimicrobial agent, diffuses into the agar plate and inhibits the growth of the test microorganism. In the end, the zones of inhibition are measured (Balouiri et al. 2016). In our case, the plates were inoculated with probiotic microbes, and the top agar with the pathogenic strain was spread onto it. During the incubation, the antimicrobial agent produced by the probiotic culture, diffuses into the top agar and in optimal case, interferes with the growth of the pathogen, which appears as an inhibition zone in the top agar. The experimental workflow consisted of 5 steps (Fig. 1): inoculation, preparation, top agar formation, incubation and evaluation.

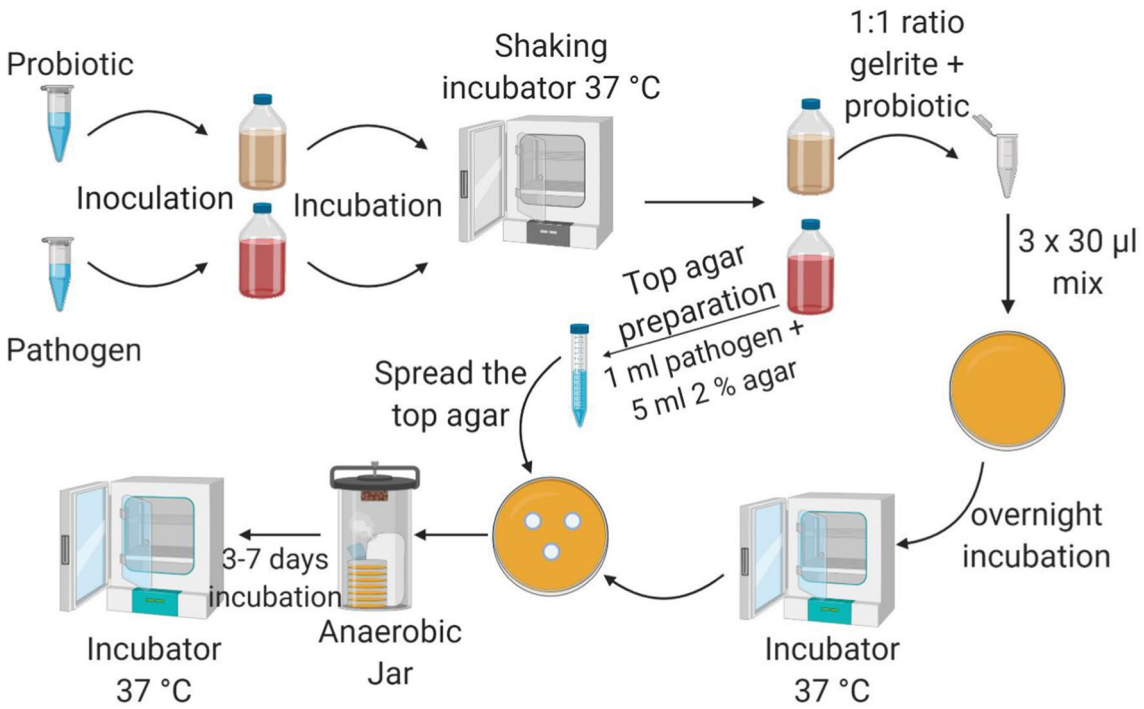

Fig. 1 The workflow of the experiments. The cultures were inoculated a day prior application (except *Pr. buccae*). First, the test probiotic strain (0.5–1 ml) was inoculated to the medium (20 ml) and was placed on a shaking incubator (37 °C, 160 rpm) overnight. Next day, the plates were prepared. The probiotic culture was mixed with gelrite in the ratio of 1:1, and then, 3 drops of mixture were placed on the plate. One drop had 30 µl volume (15 µl probiotic culture and 15 µl gelrite). Simultaneously with the preparation, CFU from the used probiotic culture was made to determine the living cell concentration of the culture (the figure does not show this step). The plates were incubated overnight (37 °C). Test pathogen was inoculated on the medium and incubated overnight on a shaking incubator (37 °C, 160 rpm). Next day, the top agar layer was poured on the prepared plates (plates with probiotic drops, after the overnight incubation). During the top agar preparation, 1 ml pathogenic culture and 5 ml 2% agar were used; it was stirred in a 15 ml sterile Falcon tube, and poured on the prepared plate. Finally, test plates were placed in an anaerobic jar and put into an incubator (37 °C) for 3–7 days. After incubation, plates were evaluated; the zones of inhibition were measured and averaged

Experimental set-up

Inoculation

Pure single colonies of test bacteria were inoculated into 20 ml of anaerobic medium (2722 or 58) one day before application, except *Pr. buccae*, which needs two days to grow up. The anaerobic, sealed bottles were placed on a shaking incubator (37 °C, 160 rpm) overnight.

Preparation

The fresh probiotic cultures (OD_{600} was between 0.5–0.7 / cm in case of medium #2722 and between 0.6 and 0.8 /cm in case of medium #58) were mixed with gelrite (Sigma-Aldrich) in the volumetric ratio of 1:1. Gelrite is a transparent biopolymer, which forms a gel in the presence of cations at room temperature (Shungu et al. 1983). The gelrite solute ion is made from 0.04 g of gelrite powder and 10.00 ml of triply distilled water and sterilized by passing through a 0.2 µm pore size syringe filter. Each drop had 30 µl volume; one drop contained 15 µl gelrite and 15 µl of fresh probiotic culture. Three drops were placed on each Petri dish. After the drops solidified on the plates, they were placed to 37 °C in an incubator overnight. The initial cell concentration (living cell/ml) of the used probiotic cultures during preparation was determined with CFU (colony forming unit).

Top agar layer

For the top agar, 5 ml media (2722 or 58) containing 2% agar were mixed with 1 ml of pathogenic culture (OD_{600} was between 0.5 and 1.0 /cm, which is about $1–4 \times 10^8$ cell/ml in case of medium #2722 and between 0.5 and 1.2 /cm, which is about $3–6 \times 10^8$ cell/ml in case of medium #58) a sterile centrifuge tube and were spread on the agar plates having the probiotic spots. Control plates were prepared without the probiotic strains, only with top agar containing the pathogenic bacteria.

Incubation

The plates were placed into anaerobic jars (Thermo Fisher Scientific) with an anaerobic atmosphere generation bag (Thermo Fisher Scientific), and they were put into an incubator at 37 °C. The incubation lasted from 3 to 7 days.

Evaluation

The zone of inhibition is the area where no growth is visible to the unaided eye (Fig. 2). The distance from the edge of the probiotic drop to the distant edge of the zone was measured. Three probiotic drops were placed on each plate; the size of zones was measured and averaged. Zones less than 1 mm were recorded as 0.5 mm; complete inhibition reached 15-mm-wide zones under these experimental conditions.

Statistical analysis

In the figures, we show the data's mean values, with their standard deviations. Statistical analyses were performed with SigmaPlot 14.0 software (Systat Software Inc., Erkeath, Germany) using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) at 5% significance level.

Results and discussion

Two experimental series were studied on two growth media: DSMZ 58 and ATCC 2722. The total number of the individual plate tests using medium 58 and 2722 was 63 and 70, respectively (Table 1). In all cases, we had at least three averaged readings.

Table 1 Effects of the media

Medium	Plates grown	Successful interaction	(%)	Average zone (mm)
2722	70	33	47.1	1.9 ± 1.6
58	63	53	84.1	8.8 ± 4.8

Two types of media were used, ATCC 2722 and DSMZ 58. The "plates grown" means the plates where pathogens were grown successfully. In regular case 72 types of experiments (8 pathogens and 9 probiotics mean 72 interactions (8*9)) were prepared on plate #2722 and on #58 as well. *P. gingivalis* could not grow in two cases on plate #2722, so it means only 70 interactions, *P. gingivalis* with *L. casei* and *P. gingivalis* with Florabalance Plus interactions were not investigated. In medium #58, *P. gingivalis* could not grow, which means 9 interactions (*P. gingivalis* and the 9 probiotics) could not be investigated. "Successful interaction" indicates plates, where both visible growth of the pathogenic strain and an inhibition zone were observed. "Average zone" was referred to the average size of all inhibition in mm

We can evaluate the results from two aspects, i.e. the sensitivity of the pathogens and the effectiveness of the probiotics.

The percentages of plates displaying inhibition zone over total number of plates indicate the sensitivity of pathogens (Table). In this context, a pathogen is considered sensitive if > 80% of the tested plates showed inhibition zone. The number of effective probiotic strains is proportional to the non-specific sensitivity of the pathogenic strain in question (Table 2).

According to the data, the most sensitive bacteria were *Pr. buccae, P. gingivalis* and *S. oralis*, while the most resistant was *E. faecalis*, since *E. faecalis* is a multidrug-resistant bacterium (Kouidhi et al. 2011). *F. nucleatum*, *A. actinomycetemcomitans*, *S. oralis* and *Pr. buccae* were inhibited by all probiotic strains, while *E. faecalis* was inhibited only by

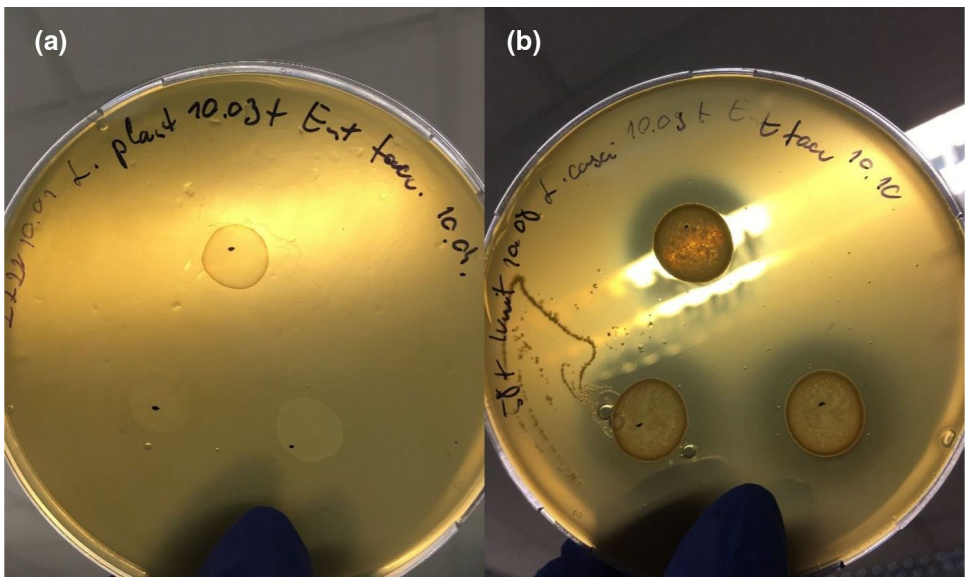

Fig. 2 **a** Pathogenic growth in the top agar layer without inhibition zone **b** Pathogenic growth in the top agar layer with inhibition zone

Table 2 Sensitivity of the pathogens

	F. n	P. g	A. a	S. m	S. o	S. g	E. f	Pr. b
Plates grown	18	7	18	18	18	18	18	18
Successful interaction	11	6	14	9	15	10	5	16
Sensitivity (%)	61.1	*85.7*	77.7	50	*83.3*	55.5	**27.2**	*88.8*

F. n.—F. nucleatum, *P. g.—P. gingivalis*, *A. a.—A. actinomycetemcomitans*, *S. m.—S. mutans*, *S. o.—S. oralis*, *S. g.—S. gordonii*, *E. f.—E. faecalis*, *Pr. b.—Pr. buccae*. In regular case, all pathogenic strain had 18 interactions (interactions with 9 probiotics on the plates #2722, and another 9 on plates #58). "Plates grown" means the interactions where the pathogens could grow in the top agar. *P. gingivalis* could not grow in medium #58 and was not able to grow in two cases in medium #2722. "Successful interaction" indicates, where both visible growth of the pathogenic strain and an inhibition zone were observed. "Sensitivity" is the percentage of "Plates grown" and "Successful interaction". *Pr. buccae*, *P. gingivalis* and *S. oralis* were the most sensitive, and *E. faecalis* was the most resistant in this the experimental arrangement. Outstanding sensitive strains are italicized, and insensitive is marked with bold in Table 2

L. rhamnosus, *L. plantarum*, *L. acidophilus*, *L. casei* and the Florabalance Plus.

During preparation, the initial cell number of the probiotic cultures was determined before using them with CFU. During the evaluation, we used these CFU data to compare the effectiveness of the different probiotic bacteria (not all data showed).

In Fig. 3, we present the results of plates #2722 (not all data showed). The maximum value was under 6 mm. *S. dentisani* (89) was the most effective. Camelo-Castilo et al. isolated two novel strains, which could inhibit the growth of *S. mutans* and named them *S. dentisani* (Camelo-Castillo et al. 2014). Similar results were reported in a separate study, where *S. dentisani* inhibited the growth of *S. mutans* and killed *F. nucleatum* (López-López et al. 2017). Others added *A. actinomycetemcomitans* to the list of oral pathogens inhibited by *S. dentisani* (Conrads et al. 2019). Our results corroborate these observations.

In Fig. 4, the results are presented in case of $1–2.5 \times 10^8$ living cell/ml initial concentration of the probiotic cultures on plates #58. Results of *L. plantarum* can be compared on Fig. 3 to Fig. 4. On plate #2722 and #58 *L. plantarum* caused inhibition in case of *Pr. buccae* and *S. oralis* as well and the initial probiotic concentration was similar ($1.5–5 \times 10^8$ cell/ml vs. $1–2.5 \times 10^8$ cell/ml). But the difference is well marked between the two media types. On #2722 *L. plantarum* caused only 1 mm zones in

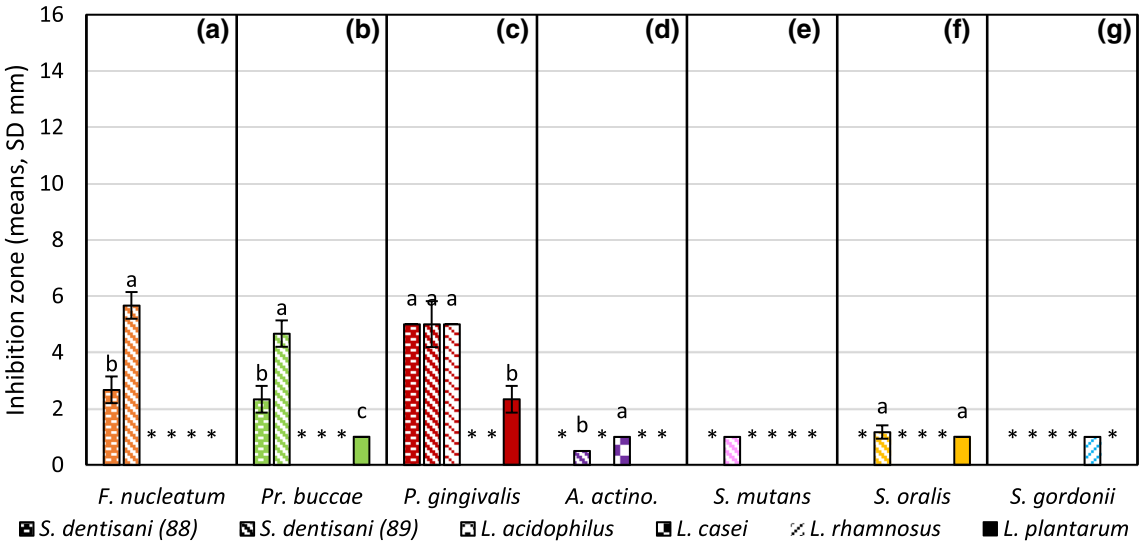

Fig. 3 Effectiveness of the probiotics on plate #2722 in case of $1.5–5 \times 10^8$ cell/ml initial probiotic concentration. Orange marks *F. nucleatum* **a**, light green is *Pr. buccae* **b**, dark red is *P. gingivalis* **c**, purple is *A. actinomycetemcomitans* **d**, lavender is *S. mutans* **e**, gold is *S. oralis* **f**, and light blue is *S. gordonii* **g**. Trellis pattern-filled column marks inhibition zones caused by *S. dentisani* (DSMZ 27088) strain, downward diagonal stripes pattern is *S. dentisani* (DSMZ 27089), zigzag pattern is *L. acidophilus*, checker board pattern is *L. casei*, upward diagonal stripes pattern is *L. rhamnosus*, and solid-filled columns marks inhibitions caused by *L. plantarum*. Whiskers on the columns mark the standard deviations. Different lower-case letters indicate statistical differences among treatments ($n = 3$, $p \leq 0.05$), *means data not shown. $F_{(A)}$ (1, 4) = 40,500; $F_{(B)}$(2, 6) = 46,500; $F_{(C)}$(3, 8) = 16,000; $F_{(D)}$(1, 4) = > 1e40; $F_{(F)}$(1, 4) = 1000

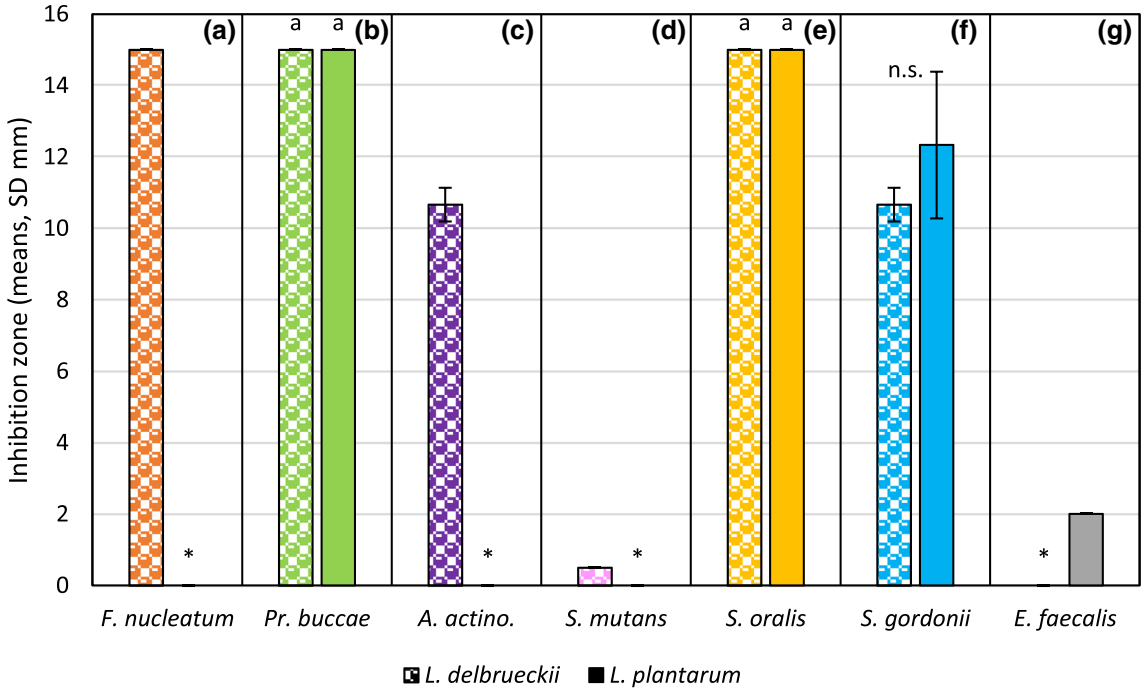

Fig. 4 Effectiveness of the probiotics on plate #58 in case of $1–2.5 \times 10^8$ cell/ml initial probiotic concentration. Orange marks *F. nucleatum* **a**, light green is *Pr. buccae* **b**, purple is *A. actinomycetemcomitans* **c**, lavender is *S. mutans* **d**, gold is *S. oralis* **e**, light blue is *S. gordonii* **f**, and grey is *E. faecalis* **g**. Sphere pattern-filled column marks inhibition zone caused by *L. delbrueckii* and solid-filled column marks the effect of *L. plantarum*. Whiskers on the columns mark the standard deviations. Different lower-case letters indicate statistical differences among treatments ($n = 3$, $p \leq 0.05$), *means data not shown, n.s. means not significant. $F_{(B)}(1, 4) = 1000$; $F_{(E)}(1, 4) = 1000$; $F_{(F)}(1, 4) = 1{,}250$

case of *Pr. buccae* and *S. oralis*. On the contrary, on #58 *L. plantarum* caused complete inhibition in both cases.

Figure 5 shows the result of plates #58 in case of $8.7 \times 10^8–3 \times 10^9$ cell/ml initial concentration of the used probiotic cultures. The effectiveness of few different probiotics can be compared, like *L. acidophilus, L. casei, L. rhamnosus* and the Florabalance Plus. *L. rhamnosus* was the most effective against *F. nucleatum* and the pathogenic *Streptococcus* strains. *L. casei* was the most effective against *A. actinomycetemcomitans* and *E. faecalis*. *Pr. buccae* was the most sensitive pathogenic bacterium, which was inhibited completely by *L. acidophilus, L. casei* and *L. rhamnosus*. *S. mutans* and *E. faecalis* were the most resistant as can be seen in Fig. 5. According to other studies, *L. casei* and *L. rhamnosus* could inhibit the growth of *S. mutans*, and *L. rhamnosus* and *L. plantarum* were effective against *A. actinomycetemcomitans* and *P. gingivalis* (Badet and Thebaud 2008). These findings are in line with our results. Florabalance Plus was quite effective against all of the test pathogens. Florabalance Plus could be a good basis for an effective probiotic mixture against oral pathogens. An improved Florabalance Plus could also be developed into a product family targeted against oral pathogens predominating specific dysbiotic microbiota causing various oral diseases.

We can speculate about the mechanism of the probiotic inhibition as follows. Three mechanisms could be considered: 1. competition for binding site and nutrients, 2. production of antimicrobial agents, bacteriocins and 3. alerting the response of the host immune system. The experiments were done in vitro, which precludes the last possibility. Under the employed experimental arrangements, the probiotic bacteria were fixed in gelrite and the pathogens were grown in the top agar. Consequently, there was no chance for competition between the two bacteria on the plate. Therefore, the most likely option to explain the observed inhibitory effect invokes the antimicrobial substances, which could freely diffuse in the agar surrounding the probiotic drop and reach the pathogens to inhibit the growth of the pathogens. The size of the inhibition zone should be proportional to the amount and biological activity of the excreted bacteriocins.

We noted substantial differences between the two growth media used to cultivate the bacteria. This points at the need for thoroughly defined, standardized experimental conditions to be maintained in such studies in order to allow a straightforward comparison of the data. In our case, medium #58 gave clearer and larger inhibition zones than medium #2722 did. The exceptions were the two *S. dentisani* strains where there was no pronounced

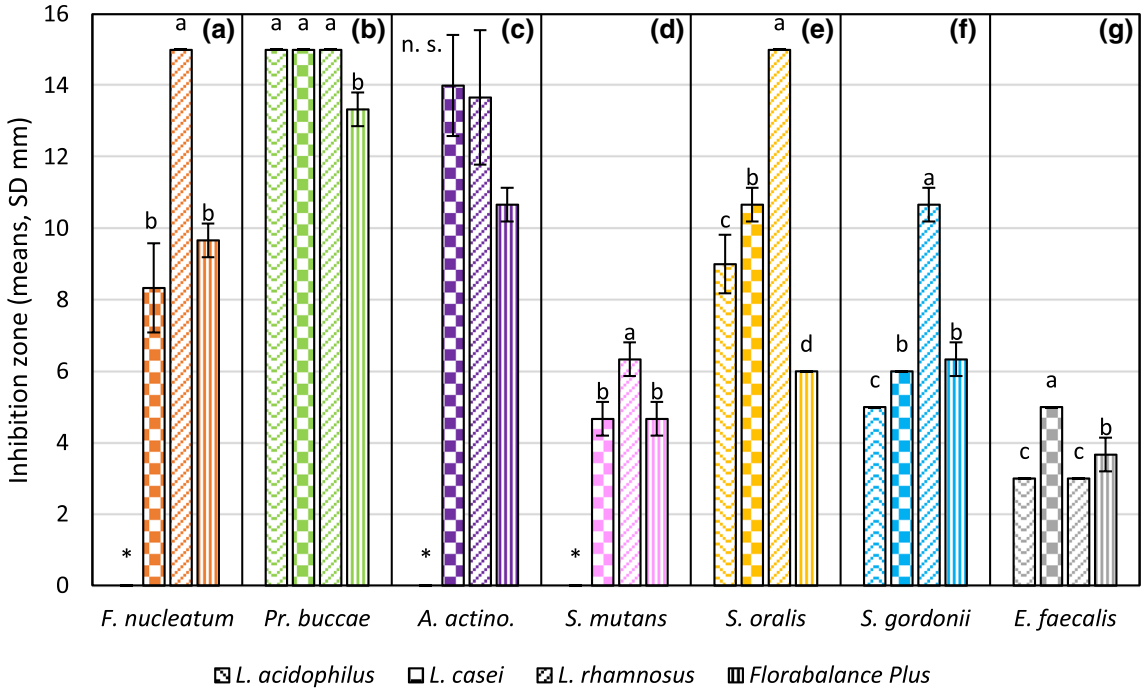

Fig. 5 Effectiveness of the probiotics on plate #58 in case of 8.7×10^{8}–3×10^{9} cell/ml initial probiotic concentration. Orange marks *F. nucleatum* **a**, light green is *Pr. buccae* **b**, purple is *A. actinomycetemcomitans* **c**, lavender is *S. mutans* **d**, gold is *S. oralis* **e**, light blue is *S. gordonii* **f**, and grey is *E. faecalis* **g**. Zigzag pattern-filled column marks inhibition caused by *L. acidophilus* strain checker board pattern is *L. casei*, upward diagonal stripes pattern is *L. rhamnosus* and vertical stripes pattern column marks the effect of the Florabalance Plus. Whiskers on the columns mark the standard deviations. Different lower-case letters indicate statistical differences among treatments ($n=3$, $p\leq0.05$), *means data not shown, n.s. means not significant. $F_{(A)}(2,\ 6)=42{,}000$; $F_{(B)}(3,\ 8)=25{,}000$; $F_{(C)}(2,\ 6)=3500$; $F_{(D)}(2,\ 6)=8{,}333$; $F_{(E)}(3,\ 8)=127{,}000$; $F_{(F)}(3,\ 8)=113{,}333$; $F_{(G)}(3,\ 8)=32{,}000$

difference in either medium, and apparently *S. dentisani* performed better medium #2722 than on #58.

A major difference between the two media is in the Tween-80, which is absent from medium 2722. Tween-80, also known as polyoxyethylene (20) sorbitan monooleate, is a non-ionic surfactant and emulsifier, which is often used in foods, cosmetics and pharmacology as additive. Tween-80 has been found to have beneficial effect on lactobacilli growth. Some *Lactobacillus* species need detergents like Tween-80 or Tween-20 which supports growth as fatty acid source (Reitermayer et al. 2018). It is relevant to recall that under adequate conditions Tween-80 elevated bacteriocin production of *L. cremoris* by about fourfold relative to the control medium (Huot and Petitdemange 1996). The detergent Tween-80 also promotes bacteriocin detachment from the probiotic cell wall (Md Sidek et al. 2018; Reitermayer et al. 2018).

The other important difference between the two media is in their glucose content, which is one fourth in medium #2722 compared to #58. The high sugar content could result in high acid production. Acids are antimicrobial agents themselves, which may supplement the bacteriocin effect. According to a new study, acids have other effects as well, more recently, a facilitated release of bacteriocins from the *L. plantarum* cells at lower pH (De Giani et al. 2019).

In the future studies, composite mixtures of probiotics should be tested to elicit comprehensive probiotic effect in the complex oral microbial community. Specific interactions among the various strains may alter the effective biological activity of probiotic mixture preparations (Jeong and Moon 2015; Conrads et al. 2019).

Conclusions for future biology

In the tests of individual probiotic candidates, at least 5 probiotic strains effectively inhibited the growth of each selected pathogenic strains. Successful inhibitions were also observed in case of the multidrug-resistant *E. faecalis* and the acid-tolerating *S. mutans*. It is noteworthy that substantial differences were observed between probiotic-pathogenic pairs depending on the composition of the growth medium. This emphasized the importance of using standardized conditions in these experiments. The findings corroborate that a rational management of oral pathogens by properly selected, well-defined, synthetic probiotic communities is feasible. In this study, we tested potential probiotic bacterial strains

for their ability of controlling the growth of oral pathogens and established an experimental set-up and collected useful information for further development of efficient probiotic composite microbial communities. However, we need more experiments in the future, including clinical experiments. Our results indicate that the probiotics can inhibit the growth of the studied pathogenic strains, so we could use them for future therapeutic treatments in odontology.

Acknowledgements We thank University of Szeged, Institute of Clinical Microbiology and Goodwill Pharma Ltd. for providing strains and the probiotic preparation for this study.

Author Contributions NG, with the help from OS, designed and performed the experiments and contributed to the evaluation of the data. KLK conceived the project and participated in its design. NG, OS, ZB and KLK drafted the manuscript. All the authors have read and approved the final manuscript.

Funding This study has been supported in part by the Hungarian National Research, Development and Innovation Fund projects GINOP-2.3.2-15-2016-00011, GINOP-2.2.1-15-2017-00081, GINOP-2.2.1-15-2017-00033 and EFOP-3.6.2-16-2017-00010. ZB received support from the Hungarian NKFIH fund FK123902.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Anderson AC, Jonas D, Huber I, Karygianni L, Wölber J, Hellwig E, Arweiler N, Vach K, Wittmer A, Al-Ahmad A (2016) *Enterococcus faecalis* from food, clinical specimens, and oral sites: Prevalence of virulence factors in association with biofilm formation. Front Microbiol. https://doi.org/10.3389/fmicb.2015.01534

Andrade E, dos Santos Pires AC, Soares M, Jan G, de Carvalho AF (2012) Probiotics in dairy fermented products. Probiotics. https://doi.org/10.5772/51939

Anukam KC, Reid G (2007) Probiotics: 100 years (1907-2007) after Elie Metchnikoff 's observation. Commun Curr Reserach Educ Top Trends Appl Microbiol 466–474

Badet C, Thebaud NB (2008) Ecology of lactobacilli in the oral cavity: a review of literature. Open Microbiol J 2:38–48. https://doi.org/10.2174/1874285800802010038

Balouiri M, Sadiki M, Ibnsouda SK (2016) Methods for in vitro evaluating antimicrobial activity: a review. J Pharm Anal 6:71–79

Banas JA, Fountain TL, Mazurkiewicz JE, Sun K, Margaret Vickerman M (2007) *Streptococcus mutans* glucan-binding protein-A affects *Streptococcus gordonii* biofilm architecture. FEMS Microbiol Lett 267:80–88. https://doi.org/10.1111/j.1574-6968.2006.00557.x

Barefoot SF, Klaenhammer TR (1984) Purification and characterization of the *Lactobacillus acidophilus* bacteriocin lactacin B. Antimicrob Agents Chemother 26:328–334. https://doi.org/10.1128/AAC.26.3.328

Berger D, Rakhamimova A, Pollack A, Loewy Z (2018) Oral biofilms: development, control, and analysis. High-Throughput 7:1–8. https://doi.org/10.3390/ht7030024

Borsanelli AC, Gaetti-Jardim E, Schweitzer CM, Viora L, Busin V, Riggio MP, Dutra IS (2017) Black-pigmented anaerobic bacteria associated with ovine periodontitis. Vet Microbiol 203:271–274. https://doi.org/10.1016/j.vetmic.2017.03.032

Bostanci N, Belibasakis GN (2012) *Porphyromonas gingivalis*: an invasive and evasive opportunistic oral pathogen. FEMS Microbiol Lett 333:1–9

Bowen WH (2016) Dental caries—not just holes in teeth! A perspective. Mol Oral Microbiol 31:228–233

Brennan CA, Garrett WS (2019) *Fusobacterium nucleatum*—symbiont, opportunist and oncobacterium. Nat Rev Microbiol 17:156–166

Brown SA, Whiteley M (2007) A novel exclusion mechanism for carbon resource partitioning in *Aggregatibacter actinomycetemcomitans*. J Bacteriol 189:6407–6414. https://doi.org/10.1128/JB.00554-07

Camelo-Castillo A, Benítez-Páez A, Belda-Ferre P, Cabrera-Rubio R, Mira A (2014) *Streptococcus dentisani sp*. nov., a novel member of the mitis group. Int J Syst Evol Microbiol 64:60–65. https://doi.org/10.1099/ijs.0.054098-0

Clarke JK (1924) On the bacterial factor in the etiology of dental caries. Br J Exp Pathol 5:141

Cobo F, Rodríguez-Granger J, Sampedro A, Navarro-Marí JM (2017) Infected breast cyst due to *Prevotella buccae* resistant to metronidazole. Anaerobe 48:177–178. https://doi.org/10.1016/j.anaerobe.2017.08.015

Conrads G, Westenberger J, Lürkens M, Abdelbary MMH (2019) Isolation and bacteriocin-related typing of *Streptococcus dentisani*. Front Cell Infect Microbiol 9:1–11. https://doi.org/10.3389/fcimb.2019.00110

Cotter PD, Ross RP, Hill C (2013) Bacteriocins-a viable alternative to antibiotics? Nat Rev Microbiol 11:95–105

da Silva-Sabo S, Vitolo M, González JMD, de Oliveira RPS (2014) Overview of *Lactobacillus plantarum* as a promising bacteriocin producer among lactic acid bacteria. Food Res Int 64:527–536

Dadon Z, Cohen A, Szterenlicht YM, Assous MV, Barzilay Y, Raveh-Brawer D, Yinnon AM, Munter G (2017) Spondylodiskitis and endocarditis due to *Streptococcus gordonii*. Ann Clin Microbiol Antimicrob 16:4–7. https://doi.org/10.1186/s12941-017-0243-8

De Giani A, Bovio F, Forcella M, Fusi P, Sello G, Di Gennaro P (2019) Identification of a bacteriocin-like compound from *Lactobacillus plantarum* with antimicrobial activity and effects on normal and cancerogenic human intestinal cells. AMB Express. https://doi.org/10.1186/s13568-019-0813-6

Dige I, Nyengaard JR, Kilian M, Nyvad B (2009) Application of stereological principles for quantification of bacteria in intact dental biofilms. Oral Microbiol Immunol 24:69–75. https://doi.org/10.1111/j.1399-302X.2008.00482.x

Distel JW, Hatton JF, Gillespie MJ (2002) Biofilm formation in medicated root canals. J Endod 28:689–693. https://doi.org/10.1097/00004770-200210000-00003

Do T, Jolley KA, Maiden CJ, Gilbert SC, Clark D, Wade WG, Beighton D (2009) Population structure of *Streptococcus oralis*. Microbiology 155:2593–2602. https://doi.org/10.1099/mic.0.027284-0

Dobson A, Cotter PD, Paul Ross R, Hill C (2012) Bacteriocin production: a probiotic trait? Appl Environ Microbiol 78:1–6

Ebbers M, Lübcke PM, Volzke J, Kriebel K, Hieke C, Engelmann R, Lang H, Kreikemeyer B, Müller-Hilke B (2018) Interplay between *P. gingivalis*, *F. nucleatum* and *A. actinomycetemcomitans* in murine alveolar bone loss, arthritis onset and progression. Sci Rep 8:1–10. https://doi.org/10.1038/s41598-018-33129-z

Et P, Mpa M (2014) JBR J Interdiscip *Enterococcus faecalis* Oral Infect 3:1–5

Fine DH, Markowitz K, Furgang D, Velliyagounder K (2010) *Aggregatibacter actinomycetemcomitans* as an early colonizer of oral tissues: *Epithelium* as a reservoir? J Clin Microbiol 48:4464–4473. https://doi.org/10.1128/JCM.00964-10

Fine DH, Patil AG, Velusamy SK (2019) *Aggregatibacter actinomycetemcomitans* (Aa) under the Radar: Myths and misunderstandings of AA and its role in aggressive periodontitis. Front Immunol 10

Fine HD, Karched M, Furgang D, Sampathkumar V, Velusamy S, Godboley D (2015) Colonization and presistence of labeled and foreign strains of Aa inoculated into the mouths of rhesus monkeys. J Oral Biol 136:554–561. https://doi.org/10.1016/j.ygyno.2014.12.035.Pharmacologic

Forssten SD, Björklund M, Ouwehand AC (2010) *Streptococcus mutans*, caries and simulation models. Nutrients 2:290–298

Gaspar C, Donders GG, Palmeira-de-Oliveira R, Queiroz JA, Tomaz C, Martinez-de-Oliveira J, Palmeira-de-Oliveira A (2018) Bacteriocin production of the probiotic *Lactobacillus acidophilus* KS400. AMB Express 8:153. https://doi.org/10.1186/s13568-018-0679-z

Hajishengallis G, Lamont RJ (2014) Breaking bad: manipulation of the host response by *Porphyromonas gingivalis*. Eur J Immunol 44:328–338. https://doi.org/10.1002/eji.201344202

Han YW (2015) *Fusobacterium nucleatum*: a commensal-turned pathogen. Curr Opin Microbiol 23:141–147

Hata S, Mayanagi H (2003) Acid diffusion through extracellular polysaccharides produced by various mutants of *Streptococcus mutans*. Arch Oral Biol 48:431–438. https://doi.org/10.1016/S0003-9969(03)00032-3

Holt S, Kesavalu L, Walker S, Genco CA (1999) Virulence factors of *P.gingivalis*. Periodontol 2000(20):168–238

How KY, Song KP, Chan KG (2016) *Porphyromonas gingivalis*: an overview of periodontopathic pathogen below the gum line. Front Microbiol 7:1–14

Huot E, Petitdemange H (1996) Tween 80 effect on bacteriocin synthesis by *Lactococcus*. Lett Appl Microbiol 22:307–310

Jakubovics NS, Palmer RJ Jr (2013) Oral Microbial ecology current research and new perspectives. Caister Academic Press, Norfolk, UK

Jeong YJ, Moon GS (2015) Antilisterial bacteriocin from *Lactobacillus rhamnosus* CJNU 0519 presenting a narrow antimicrobial spectrum. Korean J Food Sci Anim Resour 35:137–142. https://doi.org/10.5851/kosfa.2015.35.1.137

Karayasheva D, Radeva E (2017) Importance of enterococci (*Enterococcus faecalis*) for dental medicine ? Microbiological characterization, prevalence and resistance. Int J Sci Res 6:1970–1973. https://doi.org/10.21275/art20175821

Kato H, Taguchi Y, Tominaga K, Umeda M, Tanaka A (2014) *Porphyromonas gingivalis* LPS inhibits osteoblastic differentiation and promotes pro-inflammatory cytokine production in human periodontal ligament stem cells. Arch Oral Biol 59:167–175. https://doi.org/10.1016/j.archoralbio.2013.11.008

Kim N, Yun M, Oh YJ, Choi HJ (2018) Mind-altering with the gut: modulation of the gut-brain axis with probiotics. J Microbiol 56:172–182. https://doi.org/10.1007/s12275-018-8032-4

Kouidhi B, Zmantar T, Mahdouani K, Hentati H, Bakhrouf A (2011) Antibiotic resistance and adhesion properties of oral *Enterococci* associated to dental caries. BMC Microbiol. https://doi.org/10.1186/1471-2180-11-155

Krespi YP, Shrime MG, Kacker A (2006) The relationship between oral malodor and volatile sulfur compound-producing bacteria. Otolaryngol Head Neck Surg 135:671–676

Lemos JA, Burne RA (2008) A model of efficiency: stress tolerance by *Streptococcus mutans*. Microbiology 154:3247–3255

Lemos JA, Palmer SR, Zeng L, Wen ZT, Kajfasz JK, Freires IA, Abranches J, Brady LJ (2019) The biology of *Streptococcus mutans*. Microbiol Spectr 7:1–18. https://doi.org/10.1128/microbiolspec.gpp3-0051-2018

López-López A, Camelo-Castillo A, Ferrer MD, Simon-Soro Á, Mira A (2017) Health-associated niche inhabitants as oral probiotics: the case of *Streptococcus dentisani*. Front Microbiol 8:1–3. https://doi.org/10.3389/fmicb.2017.00379

Marcotte H, Lavoie MC (1998) Oral microbial ecology and the role of salivary immunoglobulin A. Microbiol Mol Biol Rev 62:71–109

Martinez FAC, Balciunas EM, Converti A, Cotter PD, de Souza Oliveira RP (2013) Bacteriocin production by *Bifidobacterium* spp. A Rev Biotechnol Adv 31:482–488. https://doi.org/10.1016/j.biotechadv.2013.01.010

Md Sidek NL, Halim M, Tan JS, Abbasiliasi S, Mustafa S, Ariff AB (2018) Stability of bacteriocin-like inhibitory substance (BLIS) produced by *Pediococcus acidilactici* kp10 at different extreme conditions. Biomed Res Int. https://doi.org/10.1155/2018/5973484

Metwalli KH, Khan SA, Krom BP, Jabra-Rizk MA (2013) *Streptococcus mutans*, *Candida albicans*, and the human mouth: a sticky situation. PLoS Pathog. https://doi.org/10.1371/journal.ppat.1003616

Miller K, Treloar T, Guelmann M, Rody WJ, Shaddox LM (2018) Clinical characteristics of localized aggressive periodontitis in primary dentition. J Clin Pediatr Dent 42:95–102. https://doi.org/10.17796/1053-4628-42.2.3

Müller E, Radler F (1993) Caseicin, a bacteriocin from *Lactobacillus casei*. Folia Microbiol (praha) 38:441–446. https://doi.org/10.1007/BF02814392

Pan H, Guo R, Ju Y, Wang Q, Zhu J, Xie Y, Zheng Y, Li T, Liu Z, Lu L, Li F, Tong B, Xiao L, Xu X, Leung EL-H, Li R, Yang H, Wang J, Zhou H, Jia H, Liu L (2019) A single bacterium restores the microbiome dysbiosis to protect bones from destruction in a rat model of rheumatoid arthritis. Microbiome 7:1–11. https://doi.org/10.1186/s40168-019-0719-1

Park J, Shokeen B, Haake SK, Lux R (2016) Characterization of *Fusobacterium nucleatum* ATCC 23726 adhesins involved in strain-specific attachment to *Porphyromonas gingivalis*. Int J Oral Sci 8:138–144. https://doi.org/10.1038/ijos.2016.27

Petersen PE, Ogawa H (2012) The global burden of periodontal disease: Towards integration with chronic disease prevention and control. Periodontology 2000 60(1):15–39. https://doi.org/10.1111/j.1600-0757.2011.00425.x

Praveen T, Kotrashetti VS, Pattanshetty S, Hosmani J V, Babji D, Ingalagi P (2018) Detection of different *Prevotella* species from deep dentinal caries of primary teeth—a culture and biochemical study. J Adv Clin Res Insights 5:65–68. https://doi.org/10.15713/ins.jcri.213

Raja M, Ummer F, Dhivakar CP (2014) *Aggregatibacter actinomycetemcomitans*—a tooth killer. J Clin Diagn Res 8:13–17

Reitermayer D, Kafka TA, Lenz CA, Vogel RF (2018) Interrelation between Tween and the membrane properties and high pressure tolerance of *Lactobacillus plantarum*. BMC Microbiol 18:1–14. https://doi.org/10.1186/s12866-018-1203-y

Shungu D, Valiant M, Tutlane V (1983) GELRITE as an agar substitute in bacteriological media. Appl Environ Microbiol 46:840–845

Simova ED, Beshkova DM, Angelov MP, Dimitrov ZP (2008) Bacteriocin production by strain *Lactobacillus delbrueckii ssp. bulgaricus* BB18 during continuous prefermentation of yogurt starter culture and subsequent batch coagulation of milk. J Ind Microbiol Biotechnol 35:559–567. https://doi.org/10.1007/s10295-008-0317-x

Ullah N, Wang X, Wu J, Guo Y, Ge H, Li T, Khan S, Li Z, Feng X (2017) Purification and primary characterization of a novel bacteriocin, LiN333, from *Lactobacillus casei*, an isolate from a Chinese fermented food. LWT Food Sci Technol 84:867–875. https://doi.org/10.1016/j.lwt.2017.04.056

Wang B, Kuramitsu HK (2005) Interactions between oral bacteria: Inhib Soc 71:354–362. https://doi.org/10.1128/AEM.71.1.354

Wilkins JC, Beighton D, Homer KA (2003) Effect of acidic pH on expression of surface-associated proteins of *Streptococcus oralis*. Appl Environ Microbiol 69:5290–5296. https://doi.org/10.1128/AEM.69.9.5290-5296.2003

Zambon JJ (1985) *Actinobacillus actinomycetemcomitans* in human periodontal disease

Zhou B, Zhang D (2018) Antibacterial effects of bacteriocins isolated from *Lactobacillus rhamnosus* (ATCC 53103) in a rabbit model of knee implant infection. Exp Ther Med 15:2985–2989. https://doi.org/10.3892/etm.2018.5790