RESEARCH ARTICLE

A concerted probiotic activity to inhibit periodontitis-associated bacteria

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Abstract

Periodontitis can result in tooth loss and the associated chronic inflammation can provoke several severe systemic health risks. Adjunctive to mechanical treatment of periodontitis and as alternatives to antibiotics, the use of probiotic bacteria was suggested. In this study, the inhibitory effect of the probiotic Streptococcus salivarius subsp. salivarius strains M18 and K12, Streptococcus oralis subsp. dentisani 7746, and Lactobacillus reuteri ATCC PTA 5289 on anaerobic periodontal bacteria and Aggregatibacter actinomycetemcomitans was tested. Rarely included in other studies, we also quantified the inverse effect of pathogens on probiotic growth. Probiotics and periodontal pathogens were co-incubated anaerobically in a mixture of autoclaved saliva and brain heart infusion broth. The resulting genome numbers of the pathogens and of the probiotics were measured by quantitative real-time PCR. Mixtures of the streptococcal probiotics were also used to determine their synergistic, additive, or antagonistic effects. The overall best inhibitor of the periodontal pathogens was L. reuteri ATCC PTA 5289, but the effect is coenzyme B12-, anaerobiosis-, as well as glycerol-dependent, and further modulated by L. reuteri strain DSM 17938. Notably, in absence of glycerol, the pathogen-inhibitory effect could even turn into a growth spurt. Among the streptococci tested, S. salivarius M18 had the most constant inhibitory potential against all pathogens, followed by K12 and S. dentisani 7746, with the latter still having significant inhibitory effects on P. intermedia and A. actinomycetemcomitans. Overall, mixtures of the streptococcal probiotics did inhibit the growth of the pathogens equally or—in the case of A. actinomycetemcomitans—better than the individual strains. P. gingivalis and F. nucleatum were best inhibited by pure cultures of S. salivarius K12 or S. salivarius M18, respectively. Testing inverse effects, the growth of S. salivarius M18 was enhanced when incubated with the periodontal pathogens minus/plus other probiotics. In contrast, S. oralis subsp. dentisani 7746 was not much influenced by the pathogens. Instead, it was significantly inhibited by the presence of other streptococcal probiotics. In conclusion, despite some natural limits such as persistence, the full potential for probiotic treatment is by far not utilized yet. Especially, further exploring concerted activity by combining synergistic strains, together with the application of oral prebiotics and essential supplements and conditions, is mandatory.
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Introduction

Periodontal diseases are one of the most prevalent diseases in the world and can result in chronic inflammation and tooth loss [1]. Beside these oral symptoms, prolonged chronic to severe cases are associated with several systemic diseases such as atherosclerosis and diabetes mellitus [2]. Periodontitis, as the irreversible and most prevalent form of periodontal diseases, is caused by a dysbiotic shift in the subgingival biofilm frequently provoking an immune response. This interplay is the driving force for the chronic inflammation. Several bacterial species such as Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedia, and fastidious anaerobes including Tannerella forsythia and Treponema denticola are implicated in these shifts [3].

Probiotic bacteria have been suggested as an alternative or supplement to the conventional treatment of periodontitis, which includes mechanical removal of biofilm and antibiotics in severe (high stage, high grade) cases [4]. Due to the dramatic increase of antimicrobial resistance, many efforts are made to reduce prescription of antibiotics and to change from therapy to prevention. Probiotics might help to prevent infectious diseases or microbial dysbiosis, with periodontal diseases as a popular target because of its high prevalence and risk of sequelae (Global Burden of Disease Study, [5]). Two groups of probiotics were subject of this study, three streptococcal strains releasing cell membrane directed antimicrobial peptides called bacteriocins, and Lactobacillus reuteri producing 3-hydroxypropanal (reuterin).

Streptococcus salivarius subspecies salivarius (abbreviated henceforward as S. salivarius) strains K12 and M18 (Mia) were already explored as anti-pharyngitis, anti-caries and anti-halitosis probiotics [6–8]. Furthermore, K12 and M18 both significantly inhibited the expression of the cytokines IL-6 and IL-8 of gingival fibroblasts, which both induce inflammation when co-incubated with P. gingivalis, F. nucleatum, and A. actinomycetemcomitans [9]. Two strains each of P. gingivalis and P. intermedia were inhibited by K12 and M18 using the simultaneous antagonism method [10]. However, no inhibition of P. gingivalis and P. intermedia was observed when using a deferred antagonism test with S. salivarius M18 [11]. S. salivarius K12 showed antibacterial effects on P. gingivalis, F. nucleatum, and A. actinomycetemcomitans in a liquid co-culture, albeit the concentration of the strain K12 had to be higher than the concentration of the periodontal pathogens [12]. Concentrated supernatants of Streptococcus oralis subspecies dentisani (abbreviated henceforward as S. dentisani) strain 7746, a probiotic primarily explored to inhibit cariogenic mutans streptococci, inhibited the growth of P. intermedia and F. nucleatum and changes in the cell wall of both species were observed [13]. Esteban-Fernández et al. observed a growth reduction rate of 35 to 38% of F. nucleatum and P. gingivalis by supernatants of S. dentisani 7746. They observed cell lysis of F. nucleatum and the formation of vesicles in P. gingivalis. S. dentisani 7746 also inhibited the adherence of F. nucleatum and P. gingivalis to gingival cells. It decreased the production of cytokines after exposure to F. nucleatum and P. gingivalis and, therefore, potentially lowered the inflammation in gingival tissue [14]. On the other hand, Conrads et al. did not observe any significant inhibition of P. gingivalis, P. intermedia and F. nucleatum by S. dentisani 7746 and only a weak inhibition of A. actinomycetemcomitans applying agar diffusion tests [15]. These discrepancies were explained with differences in the inhibitory potential of different (adapted, mutated) subcultures or clones of S. dentisani 7746, but also due to individual test-conditions.

Finally, Lactobacillus reuteri, a common colonizer of the human gastrointestinal tract [16], is used as a probiotic in humans and animals [17–19]. The antimicrobial effects of L. reuteri are mainly, but not exclusively, based on a released substance termed reuterin (3-hydroxypropanal, 3-HPA, synonym 3(β) hydroxypropionaldehyde) with a broad-spectrum antimicrobial activity including gram-positive and gram-negative bacteria as well as yeasts, fungi, and even
protozoa [20]. It is an intermediate in the metabolism of glycerol to 1,3-propanediol catalyzed by the coenzyme B12-dependent diol dehydrase (oxygen-sensitive and membrane-associated glycerol dehydratase). If not released, 3-HPA is reduced by the (NADH)+H+-dependent 1,3-propanediol-oxydoreductase regenerating NAD+ [21–23]. The production is rate-limited because, if overproduced, reuterin is toxic for the producer cell [21]. Schaefer et al. proposed that the antimicrobial effects of reuterin are based on exerting oxidative stress and its interactions with thiol groups [24]. L. reuteri has already been used as an oral probiotic in the treatment of periodontitis [25] and a commercial product is marketed (Sunstar GUM® PERIOBALANCE®, containing Prodentis® L. reuteri strains DSM 17938 and ATCC PTA 5289). In vivo studies were performed to assess the potential as a treatment for periodontal disease. Vivekananda et al. showed a significant reduction of several periodontitis indicators, such as periodontal pocket depth (PPD), gingival index (GI), plaque index (PI), gingival bleeding index (GBI), and clinical attachment level (CAL), after adjunct treatment with L. reuteri. The levels of P. intermedia, P. gingivalis, and A. actinomycetemcomitans were also significantly reduced after the treatment [26]. A study that determined the effects on gingivitis was done by Iniesta et al. with L. reuteri tablets [27]. The authors found no significant reduction of GI & PI compared to the placebo group but, however, they observed a significant reduction of P. intermedia in saliva and P. gingivalis in subgingival samples. In another study, Teughels et al. observed a reduction in pocket depth compared to mechanical treatment (scaling and root planning, SRP) alone. Also, lower numbers of P. gingivalis were detected after treatment with L. reuteri lozenges than with the placebo [25].

The aim of this study was to investigate single and combinatory inhibitory effects of streptococcal strains K12, M18, and 7746, as well as L. reuteri strains ATCC PTA 5289 and DSM 17938 on bacterial species associated with periodontal disease. The probiotics and pathogens were co-incubated in a simple model mimicking the conditions in a periodontal pocket. The resulting cell numbers of the periodontal pathogens and probiotics were determined by a quantitative real-time PCR (qRT-PCR). Genome numbers were analyzed to estimate the inhibitory effects of a specific probiotic strain or combination of probiotic strains on a particular periodontal pathogen. Also and rarely tested, the reverse effect of the pathogens on the growth of the probiotics was investigated. Our null hypothesis was that combinations of strains do not significantly increase the probiotic effect ($\alpha = 5\%$).

Materials and methods

Bacterial cultures

S. salivarius subsp. salivarius K12, Lactobacillus reuteri ATCC PTA 5289, and L. reuteri DSM 17938 were isolated from lozenges of the corresponding marketed products, namely BLIS K12 Throat Guard® (BLIS Technologies Limited, Wellington, New Zealand) and PERIOBALANCE® (Sunstar Europe SA, Switzerland). Based on GenBank genome sequence data available (GU564004.1 in case of ATCC PTA 5289, CP002844.1 in case of DSM 17938, the latter a plasmid-free progeny of strain SD2112) identity of both L. reuteri strains was confirmed by a multidrug ABC transporter gene directed PCR. Streptococcus salivarius subsp. salivarius M18 was kindly provided by R. Lütticken (Aachen, Germany) and S. oralis subsp. dentisani 7746 by A. Mira (Valencia, Spain). All strains (probiotic producers and periodontal test strains from our own collection) were grown on tryptic soy agar with sheep blood (TSASB, Oxoid Germany) for 48 hours at 37°C. Different atmospheric conditions were chosen. All probiotic strains were initially incubated in an atmosphere with 7–8% CO₂. The periodontopathogenic strains Porphyromonas gingivalis ATCC 33277, Prevotella intermedia ATCC 25611, and Fusobacterium nucleatum ATCC 25586 were grown anaerobically in a GasPak™ EZ anaerobe
pouch system, whereas *Aggregatibacter actinomycetemcomitans* ATCC 33384 was cultivated in a candle jar (MART Anaerobic jar). Stock suspensions of the probiotic strains with known concentrations of colony-forming units (cfu) were prepared and deep shock frozen (-73°C) in a mixture of 0.9% NaCl (850 μl) and glycerol (150 μl; Merck). The same aliquot used throughout all experiments. Fresh liquid cultures of the pathogenic strains were used to ensure optimal fitness. Colonies from the blood agar plates were used to inoculate 5 ml of a brain-heart-infusion (BHI) broth containing 50 μl of a vitamin K-hemin solution (Becton Dickinson) and incubated either anaerobically or microaerophilic (candle jar in case of *A. actinomycetemcomitans*) at 37°C for 48 hours.

**Growth inhibition of the periodontal pathogens by probiotic strains and vice versa**

A standard inhibition assay was developed to assess the inhibitory potential of probiotic strains against the periodontal pathogens. This was achieved by comparing the cell numbers (genome equivalents to be exact) of the pathogens after incubation with or without probiotic strains for 48 hours at 37°C anaerobically (in the case of anaerobes) or in a candle jar (in the case of *A. actinomycetemcomitans*). A 1:1 mixture of twofold concentrated BHI broth and autoclaved human saliva (donated and pooled 1:1 from two healthy probands) was used as standard growth medium (SGM) for the inhibition assay. Each well of a 96 well cell culture plate (Greiner Bio-One) contained 100 μl of the medium. To assess the effect of glycerol on inhibition, *L. reuteri* strains were cultivated with (1% w/v) or without glycerol. The stock suspensions of the probiotic strains (see above; except *L. reuteri* DSM 17938) were used to inoculate the microtiter wells generally reaching a start concentration of 10^4 cfu per 100 μl and well. Next, pathogenic strains were inoculated by adding 10 μl of the liquid pre-culture. The exact concentration of each inoculum was measured by quantitative real-time PCR. All tests were done in triplicates including the negative control and growth controls (without inhibiting probiotic strains). In order to find the most inhibiting probiotic formula, periodontal pathogens were co-incubated either with a singular probiotic strain or a mixture of two streptococcal or lactobacilli probiotics. However, mixtures of streptococci and lactobacilli were not tested so far to avoid genus-genus interactions and to ease interpretation. The overall concentration of all probiotic cells in a mixture was set to the maximum of 10^5 cfu per ml. After completing the inhibition assay, the cells were harvested by transferring culture from wells to 1.5 ml Eppendorf microliter tubes. Scraping the biofilm fraction was supported by the pipette tip, which was slightly cut back producing a sharper edge, and by pipetting up and down for dispersion. After centrifugation, the supernatant was discarded and the pellet was resuspended in 100 μl 0.9% NaCl. For washing, the steps centrifugation, discarding of the supernatant, and resuspension of pellet were repeated. Samples were then stored at -73°C until further use. After thawing, the samples were centrifuged and the supernatant discarded. The resulting pellet was treated with 20 μl of a lysozyme & mutanolysin solution (LM, 15 mg lysozyme and 500U mutanolysin in 1ml TE-buffer) for 30 minutes at 37°C lysing bacterial cell walls. DNA was extracted with the QIAmp DNA Mini Kit according to the manufacturer instructions and samples were stored at -20°C.

A qRT-PCR was performed to measure the genome number of both, the pathogenic and the therapeutic probiotic strains, before (inoculum, t = 0) and after (t = 48h) inhibition assays. The DNA of the stock suspensions was serially diluted in tenfold step with nuclease-free water to create a standard curve. The qRT-PCR was performed by the aid of a QuantStudio 3 and in 96 well plate block formats (Thermo Fisher Scientific, Dreieich, Germany). Except for the reciprocal inhibition experiments (limited to duplicates), every pathogen/probiotic
combination and controls were run in biological triplicates and the DNA extracted from each well (template) was measured in technical triplicates. The PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) was used to create a reaction mix. Each well contained 20 μl of the reaction mix with the following components: PowerUp™ SYBR™ Green Master Mix (10 μl), Forward Primer (0.1 μl), Reverse Primer (0.1 μl), nuclease-free water (8.8 μl), Template (1 μl). The concentrations of all primers (synthesized by TIB Molbiol, Berlin, Germany) were 100 μM. DNA of all pathogens and—for inverse experiments—two probiotics (M18, 7746) was amplified and quantified with strain specific primers (Table 1). As a negative control, nuclease-free water was added instead of the template.

The qRT-PCR was performed with the following temperature profile: initial denaturation at 95˚C (2 min); 40 cycles of: 95˚C (15 s), Ta (see Table 1, 15 sec), 72˚C (60 s); and final elongation at 72˚C (10 min).

Effect of glycerol on periodontal pathogens and on Lactobacillus reuteri
Since glycerol was added as substrate to L. reuteri cultures for reuterin (3-hydroxypropanal) production the sole influence of glycerol on the pathogen growth had to be investigated. The periodontal pathogens were grown in SGM with 1% w/v or without glycerol. The rest of the experiment was performed as described in the previous section and the cell density was measured by qRT-PCR.

L. reuteri ATCC PTA 5289 in combination with the L. reuteri strain DSM 17938 in commercially available lozenges (Sunstar GUM® PERIOBALANCE®) had been used for the treatment of periodontitis by re-establishing a healthy biofilm and combating dysbiosis [25]. Thus, the synergistic effects of these two lactobacilli strains on F. nucleatum were exemplarily investigated. F. nucleatum was chosen as its genome numbers varied the most in testing’s with or without glycerol. The SGM with 1% w/v or without glycerol was inoculated with 10⁴ cfu of F. nucleatum ATCC 25586. Except for the control, the different wells were inoculated with L. reuteri ATCC PTA 5289, L. reuteri DSM 17938 or a 1:1 mixture of both strains reaching a cell density of each probiotic of 10⁵ cfu/ml in a volume of 100 μl and the plates were incubated at 37°C in an anaerobic atmosphere for 48 hours. Anaerobiosis is important, as not only F. nucleatum, but also the reuterin-producing enzyme glycerol dehydratase, is oxygen-sensitive.

| Primer     | Sequence 5’-3’                      | Amplicon Size | Ta [˚C] | Reference   |
|------------|------------------------------------|---------------|---------|-------------|
| PF1-F      | AGAGTTTGATCTGGCTGAG                | 54–60 bp      |         | [28]        |
| Aa-R       | GGCAATGCTATTAACACAC                | 469 bp        | 54      |             |
| Fn-R (combined with PF1) | GTCATCGTGACACAGAATTGCTG       | 360 bp        | 60      |             |
| Pi-R (combined with PF1) | GATGCGTGACACTCAAGCTCCGCC     | 660 bp        | 56      |             |
| Pg-R (combined with PF1) | CAATAGCTGATACGGGTTTTATTC     | 478 bp        | 59      |             |
| SDent-16S-F | TGGAGGAGGGAGCTTGGTCTC             | 137 bp        | 59      |             |
| SDent-16S-R | CAAACAGTTATCATGCAATAACTG         | 137 bp        | 59      | [15]        |
| Ssal-M18-C4-F2 | GAGGTCGGTTTATGGTTGT        | 252 bp        | 54      | This study  |
| Ssal-M18-C4-R | CTATGCTGGGATGACGG              | 252 bp        | 54      | This study  |

Ta = annealing temperature of primer.

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DNA of cultures was isolated and the cell density of F. nucleatum again measured by qRT-PCR.

Statistical analysis

Data (bacterial cell or genome counts, respectively) were analyzed using GraphPad Prism (version 8.4.3; San Diego, CA). All data were not normally (not Gaussian) distributed. With non-pairing data, the non-parametric one-way ANOVA (Kruskal-Wallis) test was performed. Generally, the mean rank of columns (culture result) was compared to the mean rank of a control column (culture without probiotic). As the magnitude of genome counts varied over several logs, the uncorrected Dunn’s test was preferred. Correction of multiple comparisons was, however, also performed and differences were reported.

Results

Growth inhibition of the periodontal pathogens by probiotic strains

Without the addition of probiotics, the periodontal pathogens reached genome numbers between $1.14 \times 10^6$ (F. nucleatum) and $1.74 \times 10^5$ (P. gingivalis) after 48h of incubation (Fig 1A–1D). In principle, all probiotic strains inhibited the growth of periodontal pathogens, except L. reuteri in case that no glycerol was added to the medium. However, no probiotic strain or strain-combination—so far tested—was able to reduce the pathogen much below $10^5$ genomes. Glycerol for itself did only slightly, non-significantly, inhibit the growth of test strains (S1 Fig). The individual impact is reported below, firstly for single strains and secondly for strain combinations. The statistical results presented are based on an uncorrected Dunn’s Kruskall Wallis test, thus each comparison stands alone. Correction for multiple comparisons generally doubled the p-value and in case of p < .05 significance was lost.

The P. intermedia ATCC 25611 growth control reached an average genome number of $1.66 \times 10^5$, calculated from three measurements of three independent biological replicates (Fig 1A). The co-incubation with the streptococcal probiotics S. salivarius M18, S. salivarius K12, or S. dentisani 7746 resulted in a lowered growth of P. intermedia (M18 = $4.87 \times 10^5$ genomes; K12 = $2.20 \times 10^5$ genomes; 7746 = $1.41 \times 10^5$ genomes), with the latter reaching significance (p < .05). The inhibitory potential of L. reuteri ATCC PTA 5289 was dependent on the presence of glycerol in the growth medium. The overall strongest inhibition of P. intermedia was observed in co-incubation with strain PTA 5289 when glycerol was added to the medium ($2.02 \times 10^5$ genomes, p < .001). In contrast, in the absence of glycerol, P. intermedia was not inhibited at all by PTA 5289 reaching $1.63 \times 10^5$ genomes per well. The effect of glycerol supplementation was highly significant (p < .001).

The mean P. gingivalis ATCC 33277 genome number was $1.74 \times 10^5$ in the growth control (Fig 1B). Both S. salivarius strains, M18 and K12, did inhibit the growth of P. gingivalis but only K12 significantly ($M18 = 8.43 \times 10^5$ genomes; $K12 = 4.73 \times 10^5$ genomes, p < .05). In contrast S. dentisani 7746 had no inhibitory effect on P. gingivalis, still reaching $1.06 \times 10^6$ genomes. Compared to the control, the genome numbers of P. gingivalis were even higher when grown in the presence of L. reuteri ATCC PTA 5289 without glycerol ($2.48 \times 10^8$ genomes). However, the addition of glycerol resulted in a significant reduction of P. gingivalis if co-incubated with the PTA 5289 strain ($1.55 \times 10^7$ genomes, p < .01). Again, the effect of glycerol supplementation was highly significant (p < .001).

The mean F. nucleatum ATCC 25586 genome number in the growth control was $1.14 \times 10^6$ after 48h (Fig 1C). The co-incubation with S. dentisani 7746 resulted in a mean cell count of $8.49 \times 10^5$ genomes, slightly below the control. S. salivarius M18 inhibited the growth of F. nucleatum ($3.72 \times 10^5$ genomes), but not reaching significance. The co-incubation with S. salivarius K12 had no inhibitory effect ($1.19 \times 10^6$ genomes). Co-incubation with L. reuteri ATCC
PTA 5289 in a medium with glycerol reduced *F. nucleatum* significantly ($2.29 \times 10^5$ genomes, p < .05) but without glycerol supplementation the pathogen genome number increased more than 5-fold ($6.42 \times 10^6$ genomes, p < .001).

Finally, the mean number of *A. actinomycetemcomitans* ATCC 33384, measured after 48 hours of microaerophilic incubation in the growth control, was $8.17 \times 10^7$ genomes (Fig 1D).

The highest inhibition by streptococci was observed when co-incubated with *S. dentisani* 7746 (drop to $3.82 \times 10^6$ genomes, p < .01). Here and in contrast to the obligate anaerobic test strains, *L. reuteri* ATCC PTA 5289 resulted in a non-significant reduction of *A. actinomycetemcomitans*, even after glycerol supplementation, possibly because of the oxygen-sensitive nature of the glycerol dehydratase.
Next, the effect of mixtures of the oral streptococcal probiotics \textit{S. salivarius} M18, \textit{S. salivarius} K12, and \textit{S. dentisani} 7746 on the growth of the periodontal pathogens was investigated to assess synergistic, additive, or dilutive (antagonistic) inhibitory effects compared to addition of single probiotic strains (Fig 1A–1D, last three columns).

A strong inhibition of \textit{P. intermedia} was observed when co-incubated with a mixture of \textit{S. salivarius} K12 and \textit{S. dentisani} 7746 (9.90 \times 10^4 genomes, \textit{p} < .01). In comparison to the inhibitory effects of the two single probiotics (2.20 \times 10^5 in case of K12, \textit{p} = .08 and 1.41 \times 10^5 in the case of 7746, \textit{p} < .05), the significance was higher indicating a synergistic effect. A mixture of both \textit{S. salivarius} strains (K12 and M18) or of \textit{S. salivarius} M18 and \textit{S. dentisani} 7746 also inhibited the growth of \textit{P. intermedia} but with no significant additive or synergistic effect.

All mixtures of streptococcal probiotics lowered the genome numbers of \textit{P. gingivalis} compared to the growth control after 48h (Fig 1B). However, mixing the best streptococcal probiotic, namely K12, with either M18 or 7746, showed an antagonistic effect. As a result, no mixture reached the inhibitory and significant power of K12 applied as a single probiotic.

Only two of the three mixtures inhibited the growth of \textit{F. nucleatum} (Fig 1C). The best inhibitor was the mixture of both \textit{S. salivarius} strains M18 and K12 reducing \textit{F. nucleatum} down to 4.91 \times 10^5 genomes, followed by the mixture of M18 and \textit{S. dentisani} 7746 reducing to 4.83 \times 10^5 genomes. Both co-inhibitors, however, diluted the probiotic effect of sole M18 which was 3.72 \times 10^5 genomes for comparison. Interestingly, the mixture of K12 & 7746 slightly enhanced the growth of \textit{F. nucleatum} in comparison to the control (1.24 \times 10^6 genomes versus 1.14 \times 10^6 genomes), but this growth stimulation was not significant.

Finally, every mixture of the streptococcal probiotics inhibited the growth of \textit{A. actinomycetemcomitans} significantly (Fig 1D). The overall lowest number was observed when co-incubated with a mixture of \textit{S. salivarius} M18 & \textit{S. dentisani} 7746 (3.01 \times 10^5 genomes), indicating a significant synergistic effect (\textit{p} < .001). The increase of inhibition between applying individual versus mixed probiotics was also significant (\textit{p} < .01). The mixture of both \textit{S. salivarius} strains, in an additive manner, significantly (\textit{p} < .05) reduced \textit{A. actinomycetemcomitans} down to a mean of 5.90 \times 10^6 genomes. Worth mentioning, M18 compensated or even over-compensated the somewhat weak inhibitory effect of K12 here. In contrast, the mixture K12 / 7746 resulted in 4.44 \times 10^6 \textit{A. actinomycetemcomitans} genomes, indicating that the 7746-inhibitory effect was slightly diluted by K12.

All inhibitions were also calculated as percentages (Table 2) and graphically visualized in S2 Fig. Clearly, \textit{L. reuteri} ATCC PTA 5289 plus glycerol, as a single probiotic strain, had the best anti-pathogen effect. However, with depletion of glycerol, as essential for reuterin production, the inhibition can turn into a growth spurt (negative inhibition marked in bold in Table 2 and under the 0-line in S2 Fig).

### Table 2. Inhibition (in %) of four different periodontopathogenic species by four oral probiotic strains and single or in different combinations.

|                     | Ss M18 | Ss K12 | Sd 7746 | Lr PTA plus gly | Lr PTA minus gly | M18 & K12 | M18 & 7746 | K12 & 7746 |
|---------------------|--------|--------|---------|----------------|-----------------|-----------|------------|-----------|
| \textit{Prevotella intermedia} | 70.7   | 87.4   | 91.7    | 98.8           | 4.0             | 88.0      | 81.3       | 93.9      |
| \textit{Porphyromonas gingivalis} | 55.2   | 73.3   | 36.3    | 92.1           | -33.6           | 60.5      | 48.5       | 65.6      |
| \textit{Fusobacterium nucleatum} | 64.1   | -9.04  | 22.8    | 79.6           | 533             | 57.6      | 57.2       | -20.1     |
| \textit{Aggregatibacter actinomycetemcomitans} | 91.7   | 59.9   | 95.1    | 80.7           | 27.8            | 93.7      | 99.4       | 94.9      |
| Mean                | 70.44  | 52.90  | 61.47   | 87.82          | -133.9          | 74.94     | 71.61      | 58.55     |
| SD                  | 13.47  | 37.06  | 32.32   | 8.01           | 231.84          | 16.05     | 20.06      | 46.89     |

*As the results of four different species are integrated the standard deviation (SD) is naturally high. Abbreviations: M18 (\textit{S. salivarius} subsp. \textit{salivarius} M18), K12 (\textit{S. salivarius} subsp. \textit{salivarius} K12), PTA (\textit{Lactobacillus reuteri} ATCC PTA 5289), 7746 (\textit{Streptococcus oralis} subsp. \textit{dentisani} 7746), gly (glycerol).

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Growth inhibition of streptococcal probiotics by periodontal pathogens

Since the interaction between probiotic bacteria and periodontal pathogens is not one-sided, the effects of pathogens on the growth of *S. salivarius* M18 & *S. dentisani* 7746 were exemplarily assessed again by qRT-PCR. The resulting streptococcal genome numbers were compared to the probiotic growth control without pathogen (Fig 2A and 2B).

The co-incubation with every periodontopathogenic strain used in this study enhanced the growth of *S. salivarius* M18 (Fig 2A), but only significantly in the case of *P. gingivalis* (*p < .05*). In contrast, without any gram-negative target organism, the mean genome number was 3.56 \( \times 10^5 \). If co-incubated with *P. gingivalis* it reached 1.78 \( \times 10^6 \) genomes or with *P. intermedia* it reached 1.54 \( \times 10^6 \) genomes. The lowest growth-stimulation was detected when incubated with *A. actinomycetemcomitans* (9.05 \( \times 10^5 \) genomes).

The addition of another streptococcal probiotic (either *S. salivarius* K12 or *S. dentisani* 7746) to *S. salivarius* M18 plus any periodontal pathogen tested, generally (in 7 out of 8 cases) resulted in a further growth spurt of *S. salivarius* M18. The exception here was the mixture of *S. salivarius* M18 & K12 in the case of *P. intermedia*, which resulted in a lower genome number (decrease from 1.54 \( \times 10^6 \) to 5.19 \( \times 10^5 \) genomes) compared to the reference culture.

The genome numbers of *S. dentisani* 7746 were again higher compared to the growth control (6.25 \( \times 10^6 \)) when co-incubated with either *P. gingivalis* (1.40 \( \times 10^7 \)) or *A. actinomycetemcomitans* (8.34 \( \times 10^6 \)) (Fig 2B). The co-incubation with *F. nucleatum* or *P. intermedia* slightly lowered the genome number of *S. dentisani* 7746 (4.86 \( \times 10^6 \) and 4.54 \( \times 10^6 \)). Overall, the periodontal pathogens used in this study had little, non-significant effects on the growth of *S.*
dentisani 7746. With other words, the gram-negative targets did neither inhibit nor stimulate the growth of this probiotic. Interestingly, the overall genome numbers of 7746 were significantly (p < .05-.01) or by trend (p ≤ .17) lowered when additionally co-incubated with either S. salivarius M18 or K12, probably demonstrating a risk for antagonistic effects of other streptococcal strains.

Effect of glycerol on periodontal pathogens and on Lactobacillus reuteri
As outlined above, glycerol had an essential influence on the inhibitory effect of L. reuteri ATCC PTA 5289. For one, the inhibition of the three obligate anaerobic periodontal pathogens was highly significantly (p < .001) stronger when the medium was supplemented with 1% w/v glycerol. As an adverse effect, the growth of P. gingivalis (by 33.6%) and F. nucleatum (by 533%) was even stimulated when incubated with strain PTA 5289 in a glycerol-free culture (Table 2). Therefore, the sole effects of glycerol on the growth of periodontal pathogens had to be identified (S1 Fig). In principle, F. nucleatum, P. intermedia, P. gingivalis and A. actinomycetemcomitans did grow without (1.49 × 10^5; 1.14 × 10^5; 1.89 × 10^5; and 7.44 × 10^4 respectively) or with (1.25 × 10^5; 1.45 × 10^5; 1.04 × 10^5; and 6.08 × 10^5 respectively) 1% w/v glycerol. Although the numbers of periodontal pathogens were reduced by glycerol, the difference did not reach significance (S1 Fig). Taken all results together, the chemical effect of the essential glycerol has to be subtracted from the probiotic effect of Lactobacillus reuteri in glycerol-rich cultures.

Since strain L. reuteri ATCC PTA 5289 was isolated from lozenges (Sunstar GUM® PERI-OBALANCE®), that also contained another strain, L. reuteri DSM 17938, the synergistic effect of both strains was examined, again with and without the addition of glycerol (Fig 3). These tests were exemplarily performed with F. nucleatum ATCC 25586, as it showed the most pronounced influence of glycerol. Every well was inoculated with 6.2 × 10^5 F. nucleatum cells. After 48 hours, the mean genome-numbers of the growth control without glycerol (6.05 × 10^6) were higher than with glycerol (4.90 × 10^6), which was correspondent to the results of the aforementioned experiment. The co-incubation with strain PTA 5289 did significantly (p < .05) lower the number of F. nucleatum down to 9.16 × 10^5 genomes when the medium contained glycerol. However, after subtracting the sole inhibitory effect of glycerol, significance was just missed (p = .0578). Without glycerol, strain PTA 5289 enhanced F. nucleatum growth reaching 8.60 × 10^6 genomes. A reason for the upregulation in absence of glycerol could be catabolism gene-upregulation in F. nucleatum after coaggregation, as recently found in a co-culture with Streptococcus gordonii [32]. Interestingly, the sole incubation with strain DSM 17938 reduced F. nucleatum with and without glycerol (1.53 × 10^6 and 1.78 × 10^6 genomes, respectively), pointing on an inhibitory mechanism independent of the glycerol-depending reuterin-effect. Finally, a combination of both L. reuteri strains ATCC PTA 5289 and DSM 17938 inhibited F. nucleatum, reducing the effect of glycerol to a non-significant level (3.69 × 10^6 versus 1.04 × 10^6 genomes, with p = 0.1722). Inhibition of the probiotic strain mixture, however, reached the same magnitude as strain PTA 5289 alone. Thus, strain DSM 17938 seems to act as a helper strain being especially supportive in case of glycerol-shortage or absence.

Discussion
With a rising skepticism to use antibiotics in the treatment of chronic inflammatory diseases, such as periodontal diseases, alternatives including phage-therapy or probiotic approaches are gaining more attention. Several probiotics to re-establish a healthy, eubiotic oral microbiome have been isolated and explored. Among them are Firmicutes, including streptococci and lactobacilli, the most promising candidates. Recently, we have published about the in vitro anticariogenic potential of Streptococcus oralis subspecies dentisani 7746 in comparison to other
Fig 3. Inhibition experiments demonstrating the in vitro probiotic potential of *Lactobacillus reuteri* ATCC PTA 5289, DSM 17938, and a combination of both (minus/plus glycerol) on the growth of *Fusobacterium nucleatum* ATCC 25586. Level of significance *p* < .05. Abbreviations: Fn (*Fusobacterium nucleatum* PTA), PTA (*Lactobacillus reuteri* ATCC PTA 5289), DSM (*Lactobacillus reuteri* DSM 17938), gly (glycerol).

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probiotic strains or combinations of strains [15]. Here, we explored the in vitro anti-periodontitis potential of this species in comparison to *Streptococcus salivarius* subsp. *salivarius* M18 and K12, as well as *Lactobacillus reuteri* strains ATCC PTA 5289 and DSM 17938. The inhibitory effect was tested on four of the most prominent and cultivable periodontal pathogens, namely *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Prevotella intermedia*, and the key pathogen *Porphyromonas gingivalis*. As isolated from different research groups, so far their probiotic activity was tested only individually and applying different antagonist-tests, making the direct comparison of probiotic power difficult. Here for the first time, strains 7746, M18, K12 (separately and in combination), and PTA 5289 (plus/minus DSM 17938) were tested in parallel and under exactly the same conditions (BHI: saliva 1:1 medium, same atmospheric conditions, duration of 48h). Some combinations, such as mixtures of streptococcal and lactobacilli strains or mixtures of more than two strains, were excluded, keeping the culture conditions more controlled and easing the interpretation. In future experiments, more interactions based on the results here, should be tested.

Regarding the mostly bacteriocin-driven inhibitory potential of streptococcal strain mixtures in comparison to individual strains, it can be concluded from our results that additive or dilutive (antagonistic) inhibitory effects are more likely to occur than synergistic effects. Our null hypothesis, that combinations of strains do not significantly increase their probiotic effects, was approved. However, there were at least two exceptions found. A strong inhibitory
effect of *P. intermedia* was observed when co-incubated with a mixture of *S. salivarius* K12 and *S. dentisani* 7746. In comparison to the inhibitory effects of the two single probiotics, the significance was higher, indicating a synergistic effect. Furthermore, every mixture of the streptococcal probiotics improved *A. actinomycetemcomitans* growth-inhibition significantly. The overall lowest number was observed when co-incubated with a mixture of *S. salivarius* M18 and *S. dentisani* 7746, again indicating a concerted, synergistic effect. The increase of inhibition between applying individual versus mixed probiotics was also significant (*p* < .01). In contrast, for *P. gingivalis*, no mixture reached the inhibitory and significant power of K12 and for *F. nucleatum* both co-inhibitors diluted the probiotic effect of sole M18. In conclusion here, a concerted probiotic effect of otherwise individually explored streptococcal strains is possible, at least to target some pathogens. It is known that *S. dentisani* 7746 produces about ten different bacteriocins [15]. Combined with the plasmid-encoded salivaricin-variants A2, B, 9, and MPS produced by K12 and M18 [33], as well as bacteriocin immunity factors, a concerted activity directed against the pathogens is needed avoiding a friendly fire of against the co-producer.

As the interaction pathogen-probiotic is bidirectional, we tested the influence of pathogens on the growth of *S. salivarius* M18 & *S. dentisani* 7746 exemplarily. The co-incubation with every periodontopathogenic strain stimulated the growth of *S. salivarius* M18, significantly in the case of *P. gingivalis*. This was observed for the first time here. A possible explanation could be that a dying and disintegrating target provides more nutrients and supplements for the producer. In contrast, the co-incubation with pathogens had only non-significant effects on the growth of *S. dentisani* 7746.

Regarding the inhibitory potential of *L. reuteri* strains or strain mixtures on *F. nucleatum*, clearly pure *L. reuteri* ATCC PTA 5289 had the best anti-pathogen effect. By way of exception, *A. actinomycetemcomitans* was more reuterin-resistant and M18-susceptible, which had been reported before for culture supernatants of these probiotics [34]. Critically, with depletion of the essential glycerol the inhibition can turn into a growth spurt. The combination with *L. reuteri* DSM 17938, a putative immunomodulatory strain [35, 36], seems also to reduce the glycerol-dependency, an additional synergistic effect for the Sunstar GUM® PERIOBALANCE® Prodentis® mixture, overlooked so far. There is much to discuss about the reuterin (3-HPA) production of these strains. Many of the essential parameters are not taken into account when testing or applying *L. reuteri*, especially in clinical studies. It must be emphasized that 3-HPA production by the enzyme glycerol dehydratase is at least dependent on i) presence of substrate glycerol, ii) anaerobiosis as the enzyme is oxygen-sensitive, and iii) presence of traces of B12 [21–24, 37]. If one of these three conditions is not fulfilled, the probiotic activity will remain weak. On the other hand, if all conditions are perfectly concerted, the pathogen inhibition can be enhanced. In our experiments, we fulfilled these conditions in all wells (measurements) by adding 1% glycerol (versus control) and most plates were incubated anaerobically in a Brain-Heart-Infusion-broth with natural saliva containing B12-traces. However, the reduced reuterin-activity after microaerophilic incubation with *A. actinomycetemcomitans* could already be an indicator for glycerol dehydratase inhibition by traces of oxygen. Fortunately, B12 is heat-stable and the activity is not reduced much by autoclavation [38]. However, the exact B12-concentration was not determined in our experiment and we did not test whether addition of B12 would have further stimulated the reuterin-effect.

Underrating these conditions could be one reason why clinical studies on the anti-periodontitis activity revealed very different results, supporting [25, 39–43], intermediate [44, 45], or not-supporting [46–48] its application, and recently leading to rejection of a health claim [10]. This could further implicate that reuterin production needs a deep, anaerobic pocket and might thus not be helpful in mild cases. However, as many highly oxygen-sensitive, obligate
anaerobic pathogens are even present in shallowed periodontal pockets or in the healthy sulcus, it seems that—wherever anaerobic pathogens are able to multiply—reuterin will be produced as oxygen-concentration is locally low. As reported in a process engineering study, for the efficient reuterin production, the presence of a certain glycerol concentration is critical, as enough substance should be produced but too much antimicrobial reuterin is toxic for the producer [22]. Translated for clinical studies and applications, an optimal glycerol concentration should be ensured during probiotic therapy, at least initially or—after the strain is established in the mouth—in phases of inflammation. To our knowledge this condition was never considered enough in any clinical study. The probiotic lozenges were given as produced and marketed. And the same might be true for B12-supplementation, a condition easily to fulfill as this vitamin is cheap and stable. So far, PERIOBALANCE® does contain—besides bacteria and peppermint flavor—an isomalt cryoprotectant, emulsifier, sweetener, and a few percentages of hydrogenated palm oil. As at least palm oil is a source of triacylglycerole which can be degraded by bacteria releasing glycerol. Other glycerol-sources are blood and tissue in the inflamed gingiva. However, it might be helpful to measure glycerol in situ and—if suboptimal for reuterin production—to further supplement during probiotic therapy, ideally combined with B12.

Besides the direct antimicrobial activity subjected in our study, there are three additional central questions to answer when applying (concerted) probiotics.

Firstly, could lactic acid bacteria be cariogenic? The probiotics discussed here are acido- and aciduric and often found to be associated with caries. And while it can be effective in clearing periodontal bacteria in vitro or even in vivo, other impacts on the overall oral ecology and caries-etiology are important to consider [15, 49].

Secondly, is a reduction by 2 or 3 log-steps, usually measured by in vitro studies (including the present) enough to keep a chronic inflammatory reaction under control in vivo? This can only be answered in non-commercially biased, randomized, double blinded, placebo-controlled, and prognostic clinical studies with many subjects. At least for the application of L. reuteri such studies do exist but show, as outlined above, ambivalent results. A reason for hope here is, comparable to experience with antibiotics, that a reduction by only a few log-steps still gives the immune system the chance to take over, turning a vicious circle into an upwards spiral. The addition of immunostimulating strains might further improve the probiotic concert.

Thirdly, how long will the probiotic strain or a combination persist in situ and thus act at a particular oral side? For S. salivarius K12, in a former study, we found persistence on different mucosae for as long as three weeks, but with steadily decreasing numbers after day eight [7]. Other studies addressing this issue are very rare, as usually only the abundance before and shortly after end of treatment—but not the persistence—is measured. For instance, persistence of S. dentisani 7746 at mucosal membranes was never addressed before. In a study by Burton et al. the oral cavity “persistence” of S. salivarius M18 was investigated in 75 subjects receiving four different doses for 28 days [10]. The last testing for M18 was done as usual after the last administration, challenging the measurability of true persistence. The authors conclude that the percentage of subjects having the M18 strain detected in their saliva first increased with the dose, but after day seven slowly dropped down [10]. For gut flora, the clearance of probiotics is better studied. In rats it was shown that, from five different probiotic strains (Lactobacillus acidophilus LA742, Lactobacillus rhamnosus L2H, Bifidobacterium lactis HN019 and the oral probiotic S. salivarius K12) B. lactis and L. rhamnosus persisted seven days, but the oral K12 was already non-detectable at day three [50]. The latter result could be due to the “wrong” intestinal niche. Vice versa, intestinal L. reuteri might have reduced persistence in the oral cavity. Romani Vestman et al. conducted a randomized, double-blinded, placebo-controlled study with a 6-week PERIOBALANCE® intervention period and 3- and 6-month follow-up, investigating the effects on regrowth of mutans streptococci after full-mouth disinfection. L. reuteri
was frequently detected by culture during the intervention period but in only three test group subjects (10%) at follow-ups and in low numbers [51]. Indeed, the persistence of intestinal L. reuteri in the oral niche seems to be weak. As a matter of fact, clearance of niche-foreign bacteria is a basic defense principle in natural microbial ecosystems and this is why we call such intruders “transient”. A concerted probiotic activity should therefore, besides pathogen inhibition, also consider immunostimulation for pathogen clearance (e.g. by addition of L. reuteri DSM 17938) and niche-persistence (e.g. by addition of true residential strains linking others).

In conclusion, investigating the beneficial effects of probiotic bacteria to maintain or re-establish oral health is a very important topic. The full potential for probiotic treatment is by far not utilized yet. Especially, further exploring the concerted force on three levels, pathogen-inhibition, immunostimulation, and niche-persistence, together with the application of oral prebiotics and essential supplements and conditions, is desirable.

Supporting information

S1 Fig. Influence of 1% (w/v) glycerol on the growth of four periodontopathogenic bacteria. Abbreviations: Pi (Prevotella intermedia), Pg (Porphyromonas gingivalis), Fn (Fusobacterium nucleatum), Aa (Aggregatibacter actinomycetemcomitans), gly (glycerol).

S2 Fig. Overall in vitro percent inhibition of periodontal pathogens by probiotic strains S. salivarius M18 and K12, S. dentisani 7746, and L. reuteri ATCC PTA 5289. In a culture without glycerol, L. reuteri caused a growth spurt (negative inhibition) of some pathogens. For data see Table 2. Abbreviations: M18 (S. salivarius subsp. salivarius M18), K12 (S. salivarius subsp. salivarius K12), PTA (Lactobacillus reuteri ATCC PTA 5289), 7746 (Streptococcus oralis subsp. dentisani 7746), gly (glycerol).

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