Aus dem Institut für Zahn-, Mund- und Kieferheilkunde
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Effects of Selected Probiotic Strains *Lactobacillus reuteri* and *Streptococcus oligofermentans* on Composition and Caries Activity of Multispecies Biofilm *In Vitro*

Auswirkungen der probiotischen Stämme *Lactobacillus reuteri* und *Streptococcus oligofermentans* auf die Zusammensetzung und Kariesaktivität von Multispezies-Biofilmen *in vitro*

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Abstract (English)

Introduction: While an increased interest in the possible oral health benefits of probiotics has emerged, there is a lack of understanding of their anti-caries properties on complex dental biofilms. The present study aimed to investigate the effect of the probiotics *Lactobacillus reuteri* and *Streptococcus oligofermentans* on multispecies biofilm composition, architecture, and caries activity *in vitro*.

Methods: A continuous-cultured biofilm model containing multiple cariogenic species, *Streptococcus mutans*, *Lactobacillus rhamnosus* and *Actinomyces naeslundii*, was employed. Cariogenic biofilms were preliminarily cultured on bovine enamel specimens with identical surface areas (4.5 × 4.5 mm), which were randomly allocated in separate chambers (n = 21/group). The specimens were challenged with *L. reuteri* whole culture (LC) or its cell-free supernatant (LS), *S. oligofermentans* whole culture (SC) or its cell-free supernatant (SS), or fresh medium (negative control, NC) once per day, followed by five cycles of alternate rinses of modified Brain Heart Infusion (MBHI) broth and defined mucin medium (DMM). After 10-day cultivation, biomass amount and biofilm composition, e.g., total viable cells and strain-specific bacterial numbers, were evaluated via colony-forming units (CFU) counts and quantitative polymerase chain reaction (qPCR) (n = 5/group). The biofilm architecture was visualized via fluorescence *in situ* hybridization (FISH), with one specimen randomly selected from each group. Caries activity was determined by pH measurement and by assessing biofilm-induced lesions, e.g., mineral loss (ΔZ) and lesion depth (LD), via transverse microradiography (TMR, n = 15/group).

Result: The cariogenic bacterial amounts (*L. rhamnosus* and *S. mutans*) and the total living cell number were significantly decreased after the application of *L. reuteri* whole culture compared to NC ($p < 0.05$). Meanwhile, ΔZ (*vol* % × μm, mean ± SD) and LD (μm, mean ± SD) were significantly decreased in the LC group (1846.67 ± 317.89; 78.2 ± 13.13; $p < 0.05$). In the SC group, the pathogenic bacterial counts (*A. naeslundii* and *S. mutans*), total living bacterial count, ΔZ (3315.87 ± 617.30), and LD (101.35 ± 15.08) were significantly reduced ($p < 0.05$). No significant reductions in bacterial numbers or ΔZ were observed in the two supernatant groups ($p > 0.05$). Biofilm architecture was

the typical structure of supragingival plaque and not affected by types of probiotic interventions.

Conclusion: Viable cultures of *L. reuteri* and *S. oligofermentans* could significantly reduce the caries activity of the multispecies biofilm *in vitro* and display anti-caries potential, while no significant effects from their cell-free supernatants were observed.

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Abstract (Deutsch)

Einleitung: Während Probiotika ihr Antikaries-Potential in Monospeziesbiofilmen demonstriert haben, fehlt es an Verständnis über ihre Auswirkungen auf komplexe dentale Biofilme. Wir verglichen daher *in vitro* die Wirkung der Probiotika *Lactobacillus reuteri* und *Streptococcus oligofermentans* auf die Zusammensetzung, Architektur und Kariesaktivität von komplexen Biofilmen aus verschiedener Bakterienspezies.

Methoden: Es wurde ein kontinuierlich kultivierbares, künstliches Biofilmmodell verwendet, das aus drei kariogenen Spezies, *Streptococcus mutans*, *Lactobacillus rhamnosus* und *Actinomyces naeslundii*, zusammengesetzt war. Diese kariogenen Biofilme wurden auf Rinderschmelzproben mit der identischen Fläche (4,5 × 4,5 mm) kultiviert, die nach dem Zufallsprinzip in getrennten Kammern (n = 21/Gruppe) verteilt und einmal täglich entweder mit *L. reuteri*-Lebendkultur (LC) oder zellfreiem Kulturüberstand (LS), mit *S. oligofermentans*-Lebendkultur (SC) oder zellfreiem Kulturüberstand (SS), oder nur mit Medium (Negativkontrolle, NC) umspült wurden, nachdem sie fünf Zyklen mit abwechselnder Zuführung von modifizierter Brain Heart Infusion (MBHI)-Boullion und definiertem Muzinmedium (DMM) erfahren hatten. Nach 10-tägiger Kultivierung wurde die Biofilmzusammensetzung (Gesamt- und stammspezifische Bakterienzahl) mittels Zählungen koloniebildender Einheiten (CFU) und quantitativer PCR (qPCR) ausgewertet (n = 5/Gruppe). Die Biofilmarchitektur wurde mittels Fluoreszenz-in-situ-Hybridisierung (FISH) an einer Zufallsstichprobe, die aus jeder Gruppe ausgewählt wurde, visualisiert. Die Kariesaktivität wurde durch pH-Messung und Bestimmung des Mineralverlustes (ΔZ) sowie der Läsionstiefe (LD) der biofilminduzierten Läsionen mittels transversaler Mikroradiographie ermittelt (TMR, n = 15 /Gruppe).

Ergebnisse: Im Vergleich mit NC waren die kariogene Bakterienmenge (*L. rhamnosus* und *S. mutans*) und die Gesamtbakterienzahl in der LC-Gruppe nach 10-tägiger Applikation signifikant vermindert ($p < 0,05$) und auch ΔZ (vol % × µm, Mittelwert ± SD) und LD (µm, Mittelwert ± SD) waren signifikant niedriger (1846,67 ± 317,89; 78,2 ± 13,13; $p < 0,05$). Zudem war in der SC-Gruppe die kariogene Bakterienmenge (*A. naeslundii* und *S. mutans*) und die Gesamtbakterienzahl sowie ΔZ (3315,87 ± 617,30)

und LD (101,35 ± 15,08) signifikant niedriger ($p < 0,05$). Die Applikation von Kulturüberständen hatte keine signifikante Verringerung der Bakterienzahl oder ΔZ bzw. LD zum Ergebnis ($p > 0,05$). Alle Biofilme zeigten die typische Architektur supragingivaler Plaque unabhängig von der Gruppenzuordnung.

Schlussfolgerung: Lebendkulturen von *L. reuteri* und *S. oligofermentans* konnten signifikant die Kariesaktivität von kariogenen Multispezies-Biofilm *in vitro* senken. Kulturüberstände zeigten keinen solchen Effekt.

(Diese Zusammenfassung wird mit Genehmigung der MDPI-Verlagsgruppe wiederveröffentlicht. Es ist Teil der folgenden Veröffentlichung: Chen Z, Schlafer S, Göstemeyer G and Schwendicke F. Probiotic Effects on Multispecies Biofilm Composition, Architecture, and Caries Acitvity *In Vitro*. Microorganisms. 2020 Sep,8(9),1272.)

1. Introduction

Caries is a multifactorial disease driven by dysbiosis of dental biofilm composition and activity. Triggered by frequent exposure to dietary carbohydrates, the acidic environment created by metabolites from cariogenic bacteria, the shift of predominant residents toward acidogenic and aciduric strains [1], and the ultimate ecological acid-adaptation may jointly promote the further damage of dental hard tissue [2].

Probiotics have been described as living microorganisms that are competent to restore microbial balance and confer a health benefit to the host [3, 4]. A considerable amount of studies has given clues to characterize two main strategies of probiotics on the modification of oral biofilm and their caries activity:

(1) On the one hand, probiotics increase the buffering capacity via the arginine deiminase system (ADS) activity, allowing arginolytic species to generate alkali from salivary arginine and hence buffering organic acids [5, 6], thereby countering the ecological acidification as well as the pathogenic shift of the microflora.
(2) On the other hand, probiotics tend to interfere with the growth and metabolic activity of pathogens after close physical contact using multiple antagonistic interactions, including nutrition competition, bacteriocins and bacteriocin-like substances attacks, the co-aggregation with indigenous microorganisms, and the competitive exclusion of binding sites on the tooth surface [7-10].

Based on a growing body of laboratory evidence involving single-strain biofilms developed by oral pathogenic bacteria [11-13], *Lactobacillus reuteri* and *Streptococcus oligofermentans* are armed with more than one of the aforementioned weapons and therefore suggested to be promising probiotic species that attract wide attention. *L. reuteri* produces reuterin, an antimicrobial component generated by glycerol fermentation, which has exhibited broad-spectrum antibacterial activity, e.g., proliferative suppression and disruption of biofilm formation on both periodontopathogens and caries-inducing *Streptococcus mutans* [14-16]. *S. oligofermentans*, as a newly discovered oral streptococci species with strong adhesive and low carbohydrates fermentative capacity, produces hydrogen peroxide by utilizing

lactic acid to inhibit pathogens such as *S. mutans* [17]. Apart from the antibacterial properties achieved by bacteriocins, both probiotics species have highly arginolytic activity, which, as described, is essential merit for candidate selection in caries treatment and prevention.

However, there remains a paucity of evidence to associate the modification of probiotics on microflora to the beneficial effects on mineral protection or illustrate how probiotic species interact with complex multispecies biofilm, which is truly encountered in the clinical setting [18-20]. In this study, we aimed to employ a validated multispecies biofilm model that simulates the dynamic oral environment and the formation of cariogenic biofilms on bovine enamel specimens, and to be more appropriately assess the potential effects of *L. reuteri* and *S. oligofermentans* on biofilm composition, caries activity, and architecture *in vitro*.

2. Materials and methods

2.1 Materials

2.1.1 Bacterial strains

Two probiotic strains, *Streptococcus oligofermentans* (DSM 8249) and *Lactobacillus reuteri* (ATCC PTA 5289) (BioGaia, Stockholm, Sweden), were assessed in this study. *Streptococcus mutans* (DSM 20523), *Lactobacillus rhamnosus* (DSM 20021), *Actinomyces naeslundii* (DSM 43013) were purchased from the German Collection of Microorganisms and Cell Cultures (Leibniz Institute DSMZ, Braunschweig, Germany) and employed as representative pathogenic species to generate the cariogenic biofilms. *Lactobacillus* spp. were cultured on de Man-Rogosa-Sharpe agar (MRS agar, Oxoid, Wesel, Germany) for 48h, while streptococci and *Actinomyces* were grown on Columbia Agar with 5% (*v*/*v*) Defibrinated Sheep Blood (Thermo Fisher Scientific, Vantaa, Finland) for 48h. All bacterial incubation steps, including the development of multispecies biofilm, were conducted aerobically at 37°C.

2.1.2 Enamel specimens

From the crowns of 50 bovine incisors of the second dentition, 105 enamel specimens with identical thickness (4.5 mm) and enamel surface area (4.5 × 4.5 mm) were prepared. In brief, specimens were cut (band saw EXAKT 300 CL, EXAKT Apparatebau, Norderstedt, Germany), grounded flat with P1200 to P2500 abrasive papers (WS FLEX 18C, Hermes, Hamburg, Germany) and polished with P4000 SiC grinding paper (MICROCUT 12IN, Buehler, Düsseldorf, Germany). Twenty-five specimens (n = 5/group) were prepared for biofilm compositional analysis. Except for the well-polished enamel surfaces (4.5 × 4.5 mm), the specimens were coated with acid-resistant nail polish (Rival de Loop, Rossmann, Burgwedel, Germany) (Figure 1a). In addition, a total of 80 specimens (n = 16/group) were prepared for the mineral loss and biofilm structural analysis. Before polishing (P4000 Sic grinding paper), the dental sections were embedded in acrylic resin (Technovit 4071, Heraeus Kulzer, Hanau, Germany) with the enamel surface exposed. Afterwards, part of the enamel surfaces of the latter specimens were covered with nail varnish (Rival de Loop, Rossmann) to serve as reference (sound area) for further mineral loss determination, leaving the well-polished enamel surfaces with surface area of 4.5 × 4.5 mm (Figure 1b). Specimens with fractures, hypomineralized areas, or other surface defects were excluded. Then the prepared specimens were sterilized (2.1 bar, 121°C, 34 min) and stored under 100% humidity at room temperature until use.

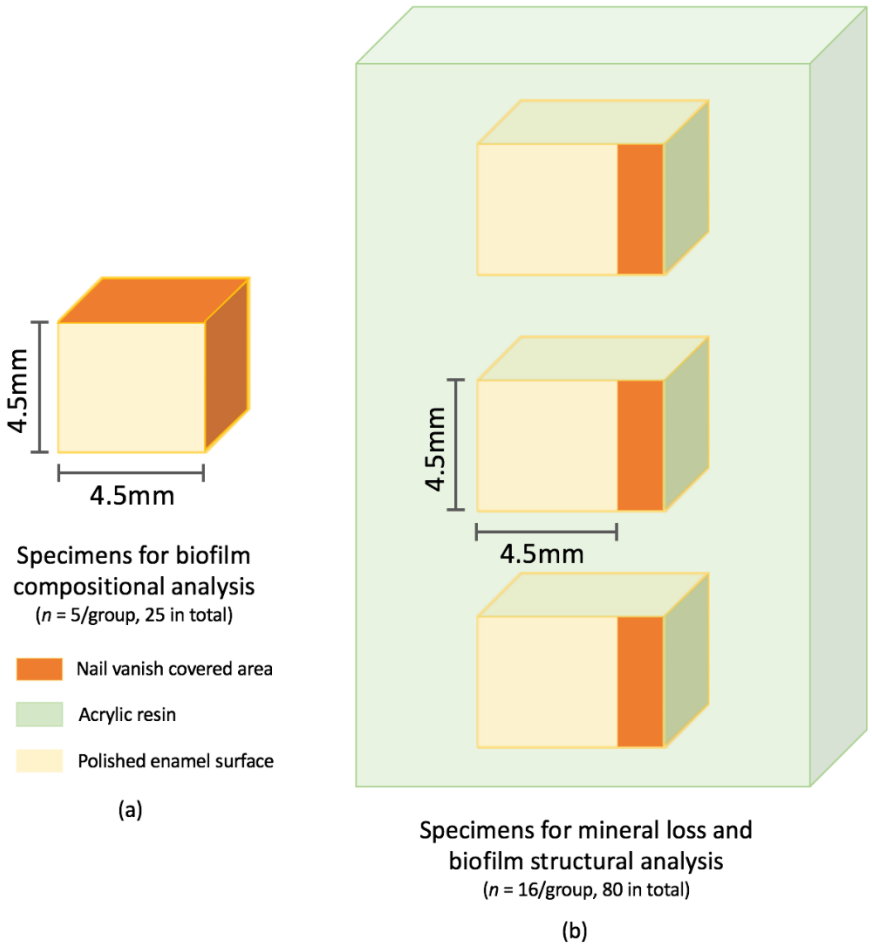

Figure 1. Enamel specimens prepared for different assays. (**a**) Enamel specimens for biofilm compositional analysis (n = 5/group, 25 in total) were covered with nail varnish (orange area) except the polished enamel surface (yellow area, 4.5 × 4.5 mm). (**b**) Enamel specimens for mineral loss and biofilm structural analysis (n = 16/group, 80 in total) were embedded in acrylic resin (green area) with the exposure of polished enamel surface (yellow area, 4.5 × 4.5 mm). The areas covered with nail varnish were served as the reference (orange area). n: sample size.

2.2 Methods

2.2.1 Multispecies biofilm model

To allow the formation of a salivary pellicle, sterilized enamel specimens were randomly allocated into five experimental groups (n = 21/group) in individual chambers and incubated in sterile-filtrated human saliva overnight at 37°C. In all experiments, the processed saliva was collected from a healthy 28-years-old donor (female, non-smoker, with low caries risk, without any periodontal or systemic disease, without any antibiotic therapy in the previous three months) following a protocol described by Wong et al. [21].

Saliva was sterilized by filtration through 0.22 μm syringe filters (Millex-GP Syringe Filter, Merck, Darmstadt, Germany) after centrifugation at 6,000 × *g* for 10 min. Prior to the onset of the biofilm development, colonies from three pathogenic species, *S. mutans*, *L. rhamnosus*, and *A. naeslundii*, were picked and inoculated into modified Brain Heart Infusion (MBHI) broth (Brain Heart Infusion broth supplied with 1% sucrose, 1% glucose, 8 g/L meat extract, 10mM L-arginine, and 250mM glycerol, pH 7.0; Carl Roth, Karlsruhe, Germany), respectively. Pure cultures of each pathogenic strain were collected in the exponential phase, diluted with fresh MBHI, and adjusted the turbidity to the optimal optical density at 600nm (OD_{600}). A volume of 250 mL of the bacterial suspension contained equivalent amounts (approximately 5×10^8 colony-forming units; CFU) of each strain was dispensed equally into five independent chambers (50 mL per chamber).

Initially, pellicle-coated specimens were immersed in the mixed bacterial suspension described above under static condition for 24 h to facilitate bacterial adhesion and cariogenic biofilms formation (Figure 2a). Following the preliminary incubation, a computer-controlled peristaltic pump (MS/CA pump head, ISM405 MCP-Z standard pump, ISMATEC, Wertheim, Germany) was then connected to the culture chambers as described previously [22]. The specimens were daily subjected to multiple MBHI/modified defined mucin medium (DMM) rinse cycles and different experimental treatments: (1) *L. reuteri* whole culture in MBHI (LC); (2) *L. reuteri* cell-free supernatant in MBHI (LS); (3) *S. oligofermentans* whole culture in MBHI (SC); (4) *S. oligofermentans* cell-free supernatant in MBHI (SS) and (5) fresh MBHI as the negative control (NC). *L. reuteri* and *S. oligofermentans* viable cultures were collected at a concentration of 1×10^9 CFU/mL in the logarithmic phase and provided to LC and SC groups for 40 min, with a volumetric flow rate at 5 mL/min (20 min inflow/outflow time with a 20 min rest interval). Instead of full cultures, cell-free supernatants were collected from the same concentration of probiotic inoculums and provided to the LS and SS groups in the same pattern after sterile filtration. In the NC group, plain MBHI medium rather than any probiotic solutions was provided to the chamber (Figure 2a). All groups were then exposed to fresh MBHI for 40 min with a volumetric flow rate at 5 mL/min (20 min inflow/outflow time with a 20 min rest interval), followed by rinsing (volumetric flow rate at 5 mL/min, 20 min inflow/outflow time) and resting (2 h) in DMM (0.5 mM K_2HPO_4, 1 mM NaCl, 3 mM KCl, 0.5 mM KH_2PO_4, 0.2 mM NH_4Cl,

0.5 mM $CO(NH_2)_2$, 0.2 mM $MgCl_2 \times 6H_2O$, 1 mM $CaCl_2 \times 2H_2O$, 2.5 g/L mucin, pH 7.0). The cycle of MBHI/DMM was repeated five times per day to mimic the dietary carbohydrate ingestion and the saliva clearance in the oral cavity, while the 8 h rest time at the end of the routine imitated the quiescent condition during the sleep period (Figure 2b). The attached biofilms on enamel specimen surfaces were always immersed in the provided solutions and developed at 100% humidity, except for certain intervals in every influx/efflux step for solution refreshment. After 10-day cultivation, specimens with matured biofilm were removed at the end of the daily schedule for further analysis. The study setup is summarized in Figure 2.

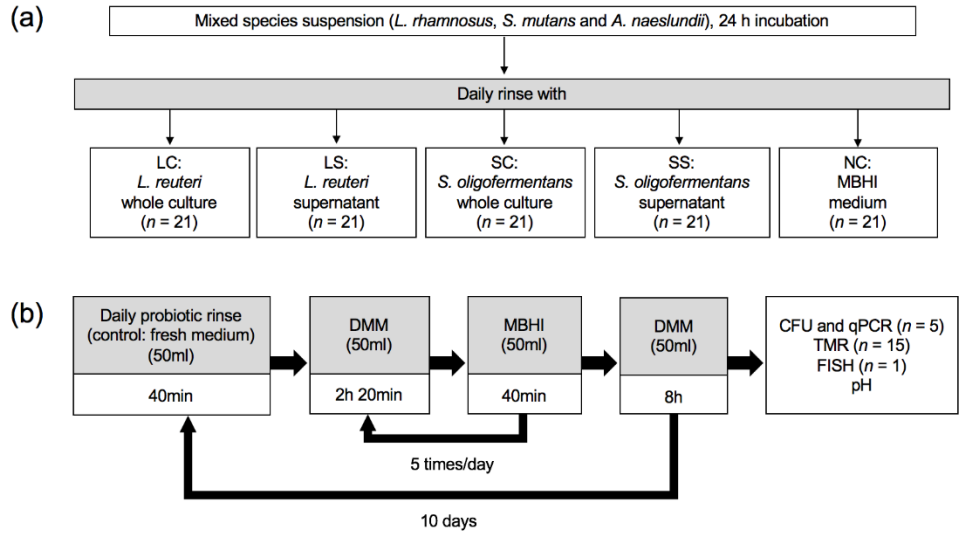

Figure 2. Study flow diagram of the multispecies biofilm model. (**a**) Initial cariogenic biofilms containing three pathogenic strains were cultured for 24 h and then submitted to daily flush with *L. reuteri* whole culture (LC), *L. reuteri* culture supernatant (LS), *S. oligofermentans* whole culture (SC), *S. oligofermentans* culture supernatant (SS) or fresh MBHI medium (NC). (**b**) The daily rinses of modified Brain Heart Infusion (MBHI) broth and Defined Mucin Medium (DMM) were repeated 5 times per day for 10 days before further analysis. CFU: colony-forming units; qPCR: quantitative polymerase chain reaction; TMR: transverse microradiography; FISH: fluorescence *in situ* hybridization; *n*: sample size [23]. Reproduced with permission from Chen Z, Schlafer S, Göstemeyer G and Schwendicke F. Probiotic Effects on Multispecies Biofilm Composition, Architecture, and Caries Acitvity *In Vitro*. Microorganisms. 2020 Sep,8(9),1272; published by MDPI.

2.2.2 Quantification of living bacteria via counting colony-forming units (CFU)

Following a continuous cultivation period, the co-culture chambers were detached from the pump and opened under sterile condition. The enamel specimens were carefully

taken out with sterile tweezers and washed twice using 1 mL 0.9% sodium chloride in 6-well plates to remove loosely bound cells. The adherent biofilms were harvested from specimens with identical surface areas (4.5 × 4.5 mm) using sterile curettes and transferred into 1 mL sterile 0.9% sodium chloride, respectively. One hundred microlitres of the microbial suspension of each specimen were serial diluted in 10 fold after vortex mixing and inoculated on Columbia Agar supplied with 5% sheep blood (Thermo Fisher Scientific) in triplicate. The numbers of colonies on agar plates were counted and determined after aerobic incubation at 37 °C for 48 h.

2.2.3 Quantification of cariogenic strains via quantitative polymerase chain reaction (qPCR)

The quantification of three cariogenic species was performed using quantitative polymerase chain reaction (qPCR), as described by Zhou et al. [24]. Briefly, the biofilm suspension (900 µL) was centrifuged at 6,000 × *g* for 20 min, after which the pellet was pre-treated with 500 µL enzymatic lysis buffer (20 mg/mL lysozyme, 1.2% Triton, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0) and incubated at 37 °C for 1 h with gentle shaking (80 rpm). After the lysis step, bacterial DNA of the collected biofilms was isolated and purified using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and concentration of the genomic DNA per biofilm were determined by spectrophotometer (Multiskan Microplate Spectrophotometer, Thermo Fisher Scientific) after eluted in 80 µL nuclease-free water. The genomic DNA was stored at -20 °C until further use. For quantitative detection, the strain-specific primer sets for amplification of 16S rRNA sequences were designed using the Primer3 software and checked the specificity using BLAST [25]. The primers specific for *L. rhamnosus* were forward primer AGG TGC TTG CAT CTT GAT TT and reverse primer CGC CAT CTT TCA GCC AAG AA, with the annealing temperature at 62 °C. The primers specific for *S. mutans* were forward primer GAC GCA AGG GAA CAC ACT and reverse primer TCA TGC AAT AAT TAA TAT TAT GCG GTA, with the annealing temperature at 62 °C. The primers specific for *A. naeslundii* were forward primer TTT GTG GGT CCT GGA TGA GT and reverse primer AAA AAG GCG CAA TCT TTCC, with the annealing temperature at 64 °C. Primers were synthesized by Metabion (Planegg, Germany). No cross-reactivity between the selected primer sets and non-target sequences or species was observed. The SYBR green-based qPCR

amplifications were performed using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, United States). Each reaction was carried out in triplicate in 96-well plates by using 1 μL template DNA, 2 μL of a mixture composed of 400 nm forward and reverse primers, 5 μL SYBR Select Master Mix (Applied Biosystems, Vilnius, Lithuania), and nuclease-free water to a final volume of 10 μL. The thermal cycling conditions were as follows: Initial denaturing at 95 °C for 2 min, followed by 40 cycles of amplification, with each cycle denaturing at 95 °C for 20 s and annealing for 60 s at the specific annealing temperature for primer sets. Specific amplification was verified by a single peak indicating in the melting curve analysis after each reaction. All the qPCR measurements were performed in triplicate. The genomic DNA of three cariogenic strains from the harvested biofilms was determined using standard curves constructed by 10-fold serial dilutions of the bacterial DNA, which was isolated from inoculums of the respective strains at the early stationary phase in a concentration of about 1×10^9 CFU/mL, as defined by OD_{600}. The corresponding quantification cycle values were converted into bacterial numbers based on the linear standard curves ranging from 1×10^4 to 1×10^9 CFU per mL.

2.2.4 Measurement of pH value

The acidity of the mature biofilms was evaluated by pH on the final day of the cultivation. The fluid was collected from each chamber 16 h and 24 h after the last probiotic flush, i.e., before and after the 8 h resting period. The collected liquid was filter-sterilized and measured the pH using a calibrated pH meter (GMH3510 Digital pH-/mV-/Thermometer, Greisinger, Regenstauf, Germany). The pH electrode was placed in each sample at room temperature for at least 2 min until the reading was stable, then the pH value was recorded. All measurements were performed in triplicate.

2.2.5 Measurement of mineral loss via Transverse Microradiography (TMR)

After gentle removal of the adherent biofilm, enamel specimens for Transverse Microradiography (TMR) assay were cut vertically, ground into thin slices, and polished (P4000 SiC grinding paper, Buehler) to a general thickness of 100 μm. The prepared specimens were examined by a nickel-filtered copper X-ray generator (PW 1730/10, Philips, Eindhoven, Netherlands) operating at 20 mA and 20 kV to generate

microradiographs. The films (Fine 71,337, Fujifilm, Tokyo, Japan) were exposed for 10 s and developed under standard conditions following the manufacturer's instructions. Digital analysis of the microradiographs was performed under an optical microscope connected to a CCD-video camera module (XC-77CE, Sony, Tokyo, Japan). Areas sized in 400 × 400 µm, without any cracks or fragments, were selected to represent the entire lesion and analyzed with TMR 2.0.27.2 software (Inspektor Research Systems, Amsterdam, Netherlands). As reference, the sound area covered by nail varnish was compared with the demineralized lesion to quantify the lesion depth (LD, in µm) and the integrated mineral loss (ΔZ, in vol % × µm) on the enamel surface.

2.2.6 Fluorescence in situ hybridization (FISH)

FISH was conducted for qualitative analysis of biofilm architecture. Five specimens with attached biofilms were selected at random from each co-culture chamber and gently washed three times with phosphate-buffered saline (PBS) in sterile 6-well plates. The specimens were fixated in 3% (*w*/*v*) paraformaldehyde solution in PBS (pH 7.2) for at least 16 h in separate containers. After washing three times in PBS for 20 min, the enamel specimens were then dehydrated for 1 h in each concentration of the increasing ethanol series (50%, 60%, 70%, 80%, 90%, 100%). Before fixation, specimens were transferred and infiltrated in pre-cooled polymerizing resin (Technovit 8100, Heraeus Kulzer) for at least three hours in a vacuum chamber (Exsikkator, Kartell S.p.A. LABWARE Division, Naviglio, Italy). The infiltration procedure was repeated twice before carefully embedding the specimens in resin for 16 h in polyethylene capsules (Flat Bottom, Electron Microscopy Sciences, Hatfield, PA, USA). The above processes were conducted at 4 °C without disrupting the overlaying biofilms. For decalcification, the embedded specimens were immersed in 17% EDTA for 21 days after being sectioned into 1 mm thin slices using a rotary saw microtome (Ernst Leitz, Wetzlar, Germany). After X-ray inspection, the decalcified slices were re-embedded in resin, cut into the ultrathin sections with an ultramicrotome (2 µm; Ultracut E, Reichert Jung Optische Werke, Wien, Austria), and mounted on glass slides with the adhesive coating (Polysine, Menzel-Gläser, Braunschweig, Germany).

As the positive controls for the specific probes and the negative controls for the non-specific probes, fixed cells of the five strains used in the co-culture model were used in

each experiment. Probe STR405 (5'-TAG CCG TCC CTT TCT GGT-3') [26], labeled with Alexa Fluor 488 at 5'-end (MWG Biotech, Ebersberg, Germany), was employed to visualize strain streptococci (*S. oligofermentans* and *S. mutans*). Probe ACT476 (5'-ATC CAG CTA CCG TCA ACC-3') [27], labeled with Atto 550 at 5'-end (IBA, Göttingen, Germany), was employed to visualize *A. naeslundii*. Probe EUB338 (5'-GCT GCC TCC CGT AGG AGT-3') [28] with 5'-end-labeled Atto 633 (IBA) was used to detect *Lactobacillus* spp. (*L. reuteri* and *L. rhamnosus*). The specificities of three oligonucleotide probes were tested and checked at 30% formamide in the hybridization buffer (Figure 3). Pre-treatment of bacterial cells with lysozyme (70 U/μL, Sigma, Brøndby, Denmark) was performed at 37°C for 9 min. The following hybridization and stringency wash procedures were conducted according to the previously described protocol [29].

After washing, the labeled biofilms on the enamel surface were examined using a LSM700 confocal laser scanning microscopy (Zeiss, Jena, Germany). The FISH probes were excited at the following wavelengths: Alexa 488, 488 nm; Atto 550, 543 nm; Atto 633, 633 nm. Filters were set to 300-550 nm for detecting Alexa 488, to 560-800 nm for Atto 550, and to 300-800 nm for Atto 633. Digital images (1048 × 1048 pixels) were acquired from representative sites (101.6 × 101.6 μm), with the pinhole size of 1 Airy Unit (AU) and a pixel time of 1.55 μs.

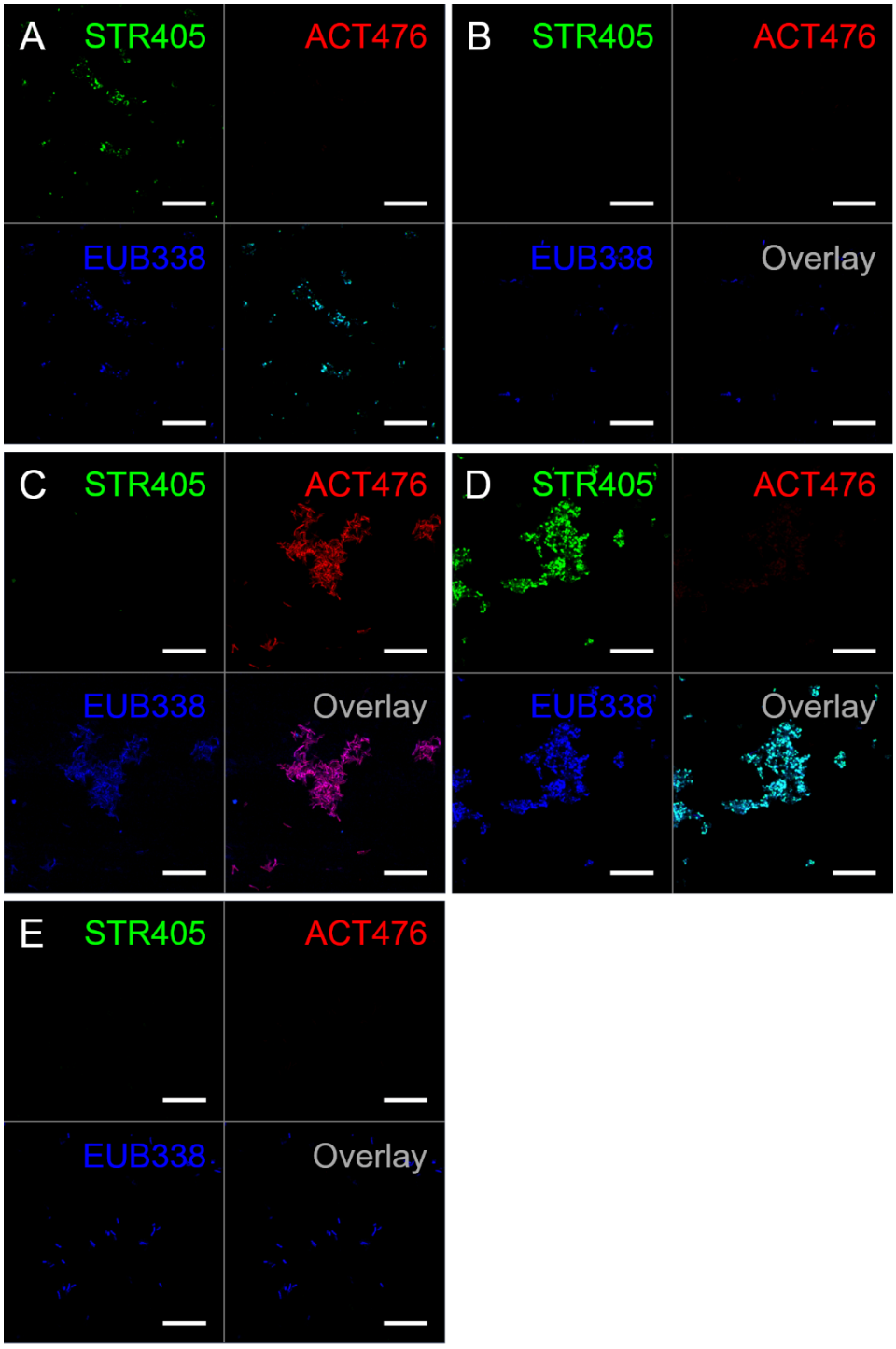

Figure 3. The specificity of the employed FISH probes. In the hybridization buffer with the formamide concentration of 30 %, all probes were specific for their target species. *S. mutans* (**A**) and *S. oligofermentans* (**D**) were detected by the streptococci-specific probe STR405 and the universal probe EUB338, but not by the Actinomyces-specific probe ACT476. *A. naeslundii* (**C**) was visualized by ACT476 and EUB338, rather than by STR405. Only probe EUB338 was able to target *L. rhamnosus* (**B**) and *L. reuteri* (**E**). Scale bars = 20 µm [23]. Reproduced with permission from Chen Z, Schlafer S, Göstemeyer G and Schwendicke F. Probiotic Effects on Multispecies Biofilm Composition, Architecture, and Caries Acitvity *In Vitro*. Microorganisms. 2020 Sep,8(9),1272; published by MDPI.

2.2.7 Statistical analysis

SPSS 25 (IBM, Armonk, United States) was used to perform the statistical analysis. Shapiro-Wilk and Levene's tests were used to examine the normal distribution and homogeneity of variances in all data sets, respectively. One-way analysis of variance (ANOVA) and Dunnett's pairwise comparison test were used to analyze the differences between groups on log-transformed CFUs, bacterial numbers of cariogenic strains quantified by qPCR, pH value, ΔZ, and LD. The level of significance for all statistical tests was set at $p < 0.05$.

3. Results

3.1 The total viable cell amount

Statistically significant differences in total viable cell amount [$\log_{10}$(CFU/mL), mean ± SD] were detected between experimental groups and presented in Figure 4. After 10 days of cultivation, LC group (4.88 ± 0.42, $p < 0.001$) and SC group (6.12 ± 0.05, $p < 0.05$) with daily vital probiotic applications showed significantly lower living bacterial numbers than NC (6.60 ± 0.05). Meanwhile, the cell-free supernatant groups LS (6.37 ± 0.29, $p > 0.05$) and SS (6.37 ± 0.09, $p > 0.05$) yielded similar numbers of living bacteria as in NC. Viable *L. reuteri* was superior to viable *S. oligofermentans* in reducing the number of living bacteria. In addition, difference in antibacterial effect was also observed between flushing with viable probiotics and with their cell-free supernatants.

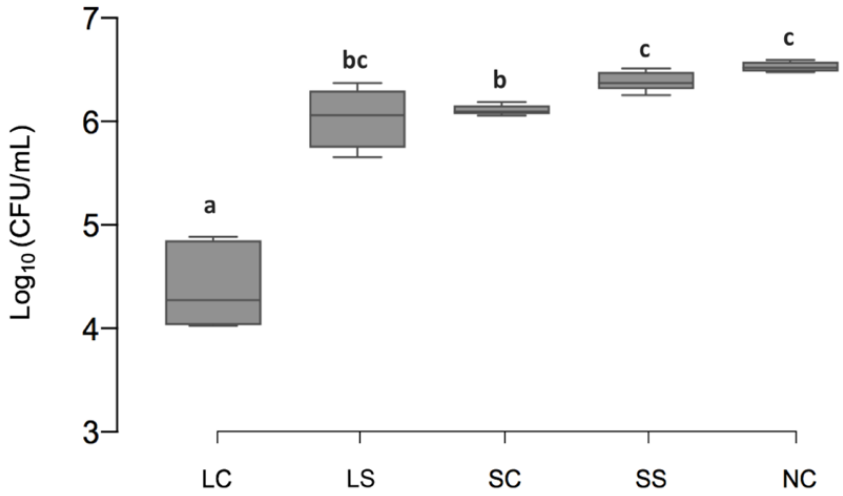

Figure 4. The total amount of viable bacteria in collected biofilms. The number of living microorganisms were determined on agar plates and presented in logarithmic forms [$\log_{10}$ (CFU/mL)]. Box and line = 25th/75th percentiles and median; error bars = maximum and minimum. Statistically significant differences between groups are indicated by different superscript letters ($p < 0.05$). LC: *L. reuteri* whole culture; LS: *L. reuteri* culture supernatant; SC: *S. oligofermentans* whole culture; SS: *S. oligofermentans* culture supernatant; NC: negative medium control. CFU: colony-forming units [23]. Reproduced with permission from Chen Z, Schlafer S, Göstemeyer G and Schwendicke F. Probiotic Effects on Multispecies Biofilm Composition, Architecture, and Caries Acitvity *In Vitro*. Microorganisms. 2020 Sep,8(9),1272; published by MDPI.

3.2 Bacterial numbers of three cariogenic species

The bacterial numbers of pathogenic strains within the multi-species biofilms were quantified by qPCR (Table 1). Daily application of living probiotic cells resulted in notable inhibition of the pathogen growth: *S. mutans* and *L. rhamnosus*, rather than *A. naeslundii*, had five to ten fold decrease in the LC group compared with NC ($p < 0.05$); *A. naeslundii* and *S. mutans*, rather than *L. rhamnosus*, demonstrated the varying extent of reduction in the SC group compared with NC ($p < 0.05$). Only limited impact of the applications of both supernatants on the pathogenic population was detected compared with NC ($p > 0.05$).

Table 1. Bacterial numbers of each cariogenic strain in different treatment groups.

Group	*L. rhamnosus*	*S. mutans*	*A. naeslundii*
LC	0.55 ± 0.10^{a}	0.37 ± 0.11^{a}	0.19 ± 0.04^{a}
LS	4.03 ± 1.87^{ab}	3.65 ± 1.86^{abc}	0.36 ± 0.20^{ab}
SC	2.23 ± 0.86^{ab}	0.22 ± 0.14^{ab}	0.09 ± 0.04^{b}
SS	3.84 ± 1.61^{ab}	2.76 ± 0.57^{c}	0.43 ± 0.07^{a}
NC	3.13 ± 0.87^{b}	3.69 ± 1.38^{c}	0.45 ± 0.10^{a}

Three cariogenic strains ($\times 10^{6}$ cells/mL, mean ± SD) were determined by quantitative polymerase chain reaction (qPCR) ($n = 5$). Statistical differences between experimental groups within each column are indicated by different superscript letters ($p < 0.05$) [23]. Reproduced with permission from Chen Z, Schlafer S, Göstemeyer G and Schwendicke F. Probiotic Effects on Multispecies Biofilm Composition, Architecture, and Caries Acitvity *In Vitro*. Microorganisms. 2020 Sep,8(9),1272; published by MDPI.

3.3 pH value

The efficacy of the probiotics compounds in modifying the cariogenic activity of the multispecies biofilm was determined by pH measurement at the end of the experiment after the last probiotic application. The pH of the LC group was significantly higher than NC group and remained at 5 after 16 h ($p < 0.001$), while the rest groups had been lower than 4 and well below the critical point of demineralization. After 24 h, the LC group still maintained the highest pH level of all groups. Also, in the SC group, the pH was significantly higher than in the NC group after 24 h ($p < 0.01$). Conversely, rinsing with probiotic supernatants resulted in a slight drop of the pH after 16 h compared to NC, even ending with more acidic pH after 24 h ($p < 0.05$). The SS group had the lowest pH value after 24 h ($p < 0.001$).

Table 2. Final pH values of the liquids collected 16 h and 24 h after the final probiotic flush.

Group	16 h	24 h
LC	5.00 ± 0.02 [a]	3.90 ± 0.03 [a]
LS	3.90 ± 0.02 [b]	3.53 ± 0.01 [b]
SC	3.83 ± 0.01 [cd]	3.75 ± 0.00 [a]
SS	3.73 ± 0.03 [d]	3.44 ± 0.01 [c]
NC	3.94 ± 0.03 [bc]	3.70 ± 0.01 [d]

The pH values (mean ± SD) of liquids collected from five co-culture chambers were measured in triplicate. Statistically significant differences between experimental groups measured at the same time point are indicated within each column by different superscript letters ($p < 0.05$) [23]. Reproduced with permission from Chen Z, Schlafer S, Göstemeyer G and Schwendicke F. Probiotic Effects on Multispecies Biofilm Composition, Architecture, and Caries Acitvity *In Vitro*. Microorganisms. 2020 Sep,8(9),1272; published by MDPI.

3.4 Mineral loss ΔZ and LD

Biofilm-induced lesions on enamel specimens surface were assessed using TMR. Corresponding to the pH results, ΔZ (vol% × μm, mean ± SD) and LD (μm, mean ± SD) (Figure 5, 6) in the LC (1846.67 ± 317.89; 78.20 ± 13.13) were considerably reduced by about 50% compared to NC (4681.48 ± 495.18; 122.39 ± 15.72, $p < 0.05$). Also, vital *S. oligofermentans* inoculum significantly decreased ΔZ and LD in the SC group (3315.87 ± 617.30; 101.35 ± 15.08, $p < 0.05$). Probiotic supernatants provided in the LS group (4290.11 ± 924.60, 118.69 ± 27.89) and SS group (5288.09 ± 963.03, 123.35 ± 19.37) did not have any significant impact on ΔZ and LD ($p > 0.05$).

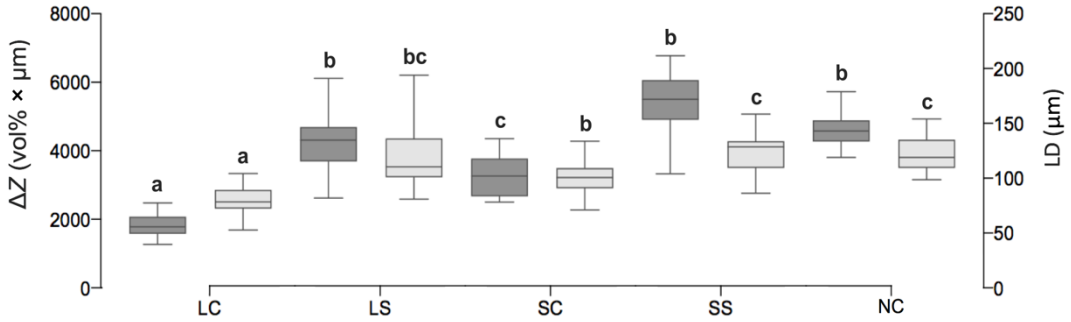

Figure 5. Mineral loss (ΔZ) and lesion depth (LD) of enamel lesions after 10 days of continuous incubation. Statistically significant differences in ΔZ (dark grey boxes, left y-axis) and LD (light grey boxes, right y-axis) between groups are indicated by different superscript letters ($p < 0.05$). Box and line = 25th/75th percentiles and median; error bars = maximum and minimum. LC: *L. reuteri* whole culture; LS: *L. reuteri* culture supernatant; SC: *S. oligofermentans* whole culture; SS: *S. oligofermentans* culture supernatant; NC: negative medium control [23]. Reproduced with permission from Chen Z, Schlafer S, Göstemeyer G and Schwendicke F. Probiotic Effects on Multispecies Biofilm Composition, Architecture, and Caries Acitvity *In Vitro*. Microorganisms. 2020 Sep,8(9),1272; published by MDPI.

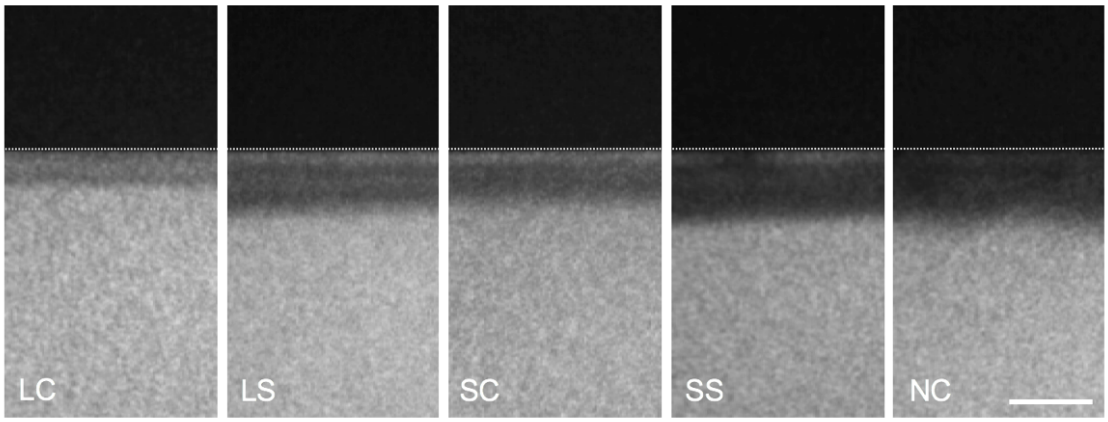

Figure 6. Representative microradiographs of the biofilm-induced enamel lesions. The white dotted line indicates the lesion interface. The mean lesion depths ranged from 78.2 µm (LC) to 122.4 µm (NC). Scale bar = 100 µm. LC: *L. reuteri* whole culture; LS: *L. reuteri* culture supernatant; SC: *S. oligofermentans* whole culture; SS: *S. oligofermentans* culture supernatant; NC: negative medium control [23]. Reproduced with permission from Chen Z, Schlafer S, Göstemeyer G and Schwendicke F. Probiotic Effects on Multispecies Biofilm Composition, Architecture, and Caries Acitvity *In Vitro*. Microorganisms. 2020 Sep,8(9),1272; published by MDPI.

3.5 Multispecies biofilm visualized by FISH

After fixation and permeabilization, the organization of multispecies biofilms attached to the enamel surface was revealed by the FISH assay. Each genus was labeled with specific probes carrying different fluorescence-tags. No specific differences in biofilm architecture were detected between groups. Typical architectural features of supragingival biofilms with a general thickness of 150-250 μm were observed from all experimental groups. The bulged surfaces of the attached biofilms were revealed, as well as the compact cell clumps and cell-free areas. Streptococci and *Lactobacillus* spp. predominated, while as the minority group, characteristic branched colonies of *A. naeslundii* were identified at the biofilm bases (Figure 7).

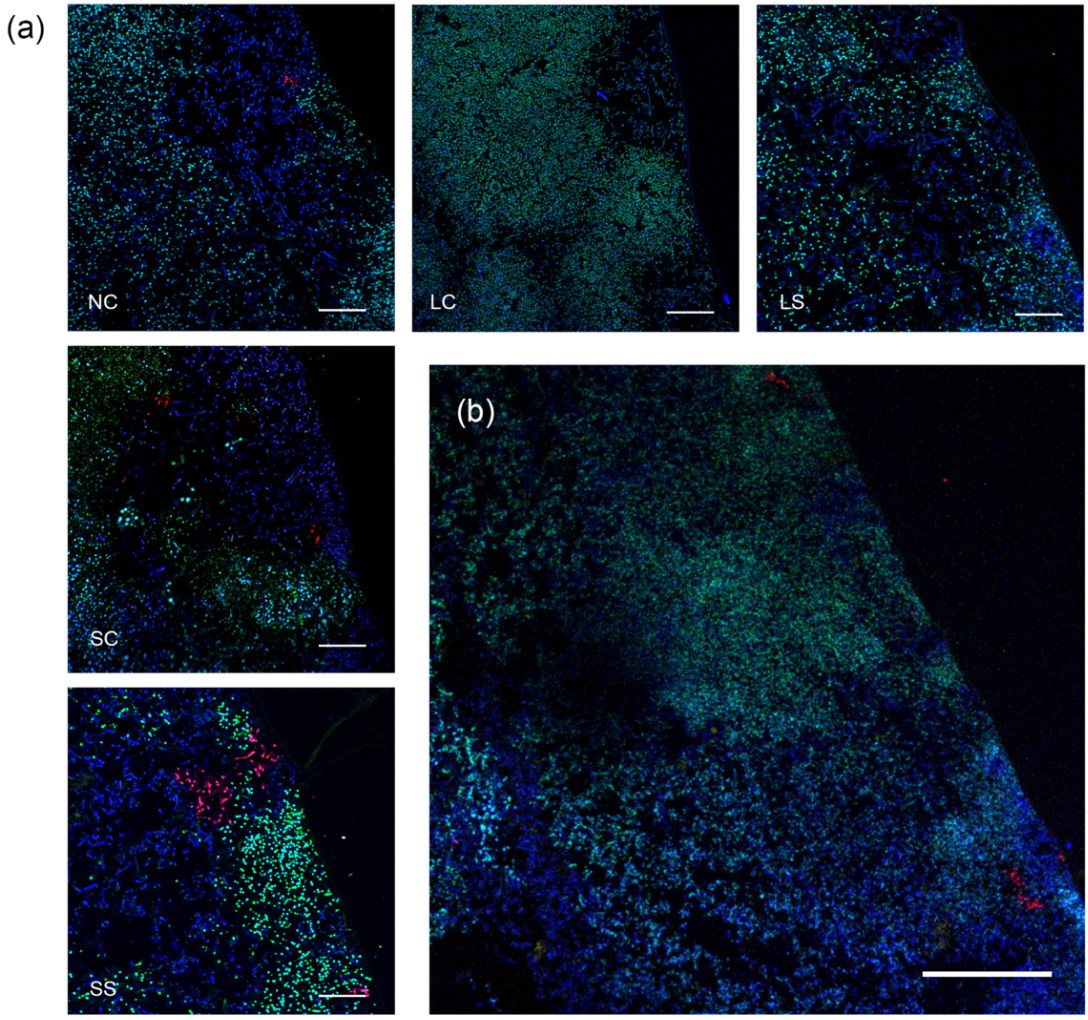

Figure 7. Biofilm architecture visualized by fluorescence *in situ* hybridization (FISH). (**a**) Multispecies biofilms were developed on the enamel surface and fixed for FISH staining after 10 days of continuous cultivation. Clustered streptococci (green; Alexa-488-labeled) and *Lactobacillus spp*. (blue; Atto-633-labeled) were observed to predominate in the compact biofilms in all five treatment groups. *A. naeslundii* (red; Atto-550-labeled) was detected in low numbers as branched colonies within all biofilms, typically located at the base of the biofilm. Scale bars = 20 µm. (**b**) Representative image (from LS) showed the complex biofilm architecture with cell clusters, cell-free regions, and bulged surface, which resembled the structure of supragingival biofilm. There were no significant structural changes in the biofilms between experimental groups. Scale bar = 60 µm. Dark areas represent the enamel surface of the specimens. LC: *L. reuteri* whole culture; LS: *L. reuteri* culture supernatant; SC: *S. oligofermentans* whole culture; SS: *S. oligofermentans* culture supernatant; NC: negative medium control [23]. Reproduced with permission from Chen Z, Schlafer S, Göstemeyer G and Schwendicke F. Probiotic Effects on Multispecies Biofilm Composition, Architecture, and Caries Acitvity *In Vitro*. Microorganisms. 2020 Sep,8(9),1272; published by MDPI.

4. Discussion

Probiotics have demonstrated broad potential in conferring health benefits on the host. However, data from previous studies on probiotics as effective agents on caries management and prevention remain controversial to derive a substantive conclusion [18]. This study set out to assess the possible effect of both vital cultures and the cell-free supernatants of probiotic strains *L. reuteri* and *S. oligofermentans* in a multispecies biofilm model. We found that the vibrant bacterial populations, the cariogenic activity of the complex biofilms, and the mineral loss in enamel specimens were significantly reduced by vital probiotic applications. Overall, in our experimental setting, *L. reuteri* displayed superior competence against caries compared to *S. oligofermentans*, whereas both kinds of supernatants failed to exert perceptible effects on caries management *in vitro*.

4.1 Biofilm cariogenic activity was modified after the application of vital probiotics

Our findings can be interpreted from several perspectives. As the most widely used assay to quantify the loss of small amounts of minerals *in vitro* [30], TMR offered compelling evidence for the suppressive effects of vital probiotics on the collective caries activity of the consortium. Moreover, even though daily challenged with living microbes at a concentration of 10^9 CFU/mL, we detected a lower bacterial load, higher pH level, and consequently, lower mineral loss in the vital probiotic groups compared to the cell-free rinse groups. This suggested that, in addition to the excretion of antibacterial compounds, the anti-caries properties of probiotics may be primarily attributed to the alteration of inherent ADS activity. Evidence from clinical studies suggested that caries-free subjects encounter more alkaline biofilm [6, 31]. The ADS pathway can potentially achieve pH equilibrium by the catabolism of arginine to produce alkali, thus averting the accumulation of acidic end-products to disrupt the vicious circle between microflora acidification and the adaptation of endogenous bacteria [32, 33]. The colonization of highly arginolytic strains, such as *L. reuteri* and *S. oligofermentans*, can enhance the stability of the microbial community under such acid stress by constantly countering the acidic products via this ADS route [34]. As for the supernatant groups, without the intervention of the metabolically active probiotic species, the biofilms had no way to reverse the detrimental pH milieu, thereby

progressing even further in the pathological course as well as the dysbiosis exacerbation. The probiotic inhabitants can strengthen biofilm resilience over time, empowering the system to bounce back from disease state and offering an additional chance to rebuild a host-compatible community [35-38].

By contrast, supernatants that are supposed to mediate the indirect interactions of probiotics through various antimicrobial compounds (e.g., bacteriocins) turned out to have a relatively minor impact on the observed biofilms. In the previous study, we have explored these indirect actions with a particular emphasis on *S. mutans* monospecies biofilms and identified reuterin as a promising antimicrobial component [39], which seems to conflict with the current results. We ascribe this difference to the fact that multispecies biofilms tend to be polymicrobial synergy, e.g., nutritional cross-feeding between intra- and inter-species [40-43]. Simultaneously, the expression of virulence genes and the pathogenicity of the communities can be upregulated as the consequence of the synergistic interactions [44]. Besides, the polymeric matrix produced by complex cariogenic pathogens, constructed from extracellular polymeric substances (EPS) such as soluble glucans and polysaccharides, offers a favorable habitat for metabolic competition/synergy, surface adhesion, antimicrobial tolerance as well as resistance to mechanical shearing forces [45]. The extracellular matrix also functions as a barrier against the penetration of extraneous chemicals, thus making the community more robust to the external shocks, such as the supernatant rinses in the present study.

Based on our previous study indicating that the environmental specificity of probiotic potency is achieved through specific metabolic pathways [39], certain nutritions are conducive for probiotics to fulfill their anti-caries tasks [46]. The L-arginine is crucial for the synthesis of urea and alkali through the ADS pathway, whereas glycerol is an essential substance for the production of reuterin by *L. reuteri* [47, 48]. The current study supplemented the culture medium with low concentrations of these two common nutrients, which can generally be obtained from saliva and diet, in order to allow the proper functions of probiotics in the simulated system.

4.2 The bacterial composition of cariogenic biofilm was substantially affected by viable probiotics

The impacts of the probiotic bacteria on biofilm composition were species-specific: *S. mutans* and *L. rhamnosus*, but not *A. naeslundii*, were specifically reduced by viable *L. reuteri*. In contrast, *A. naeslundii* and *S. mutans*, but not *L. rhamnosus*, had been seen reductions in bacterial numbers by the application of viable *S. oligofermentans*.

Hence, *L. reuteri* was likely to restrain the biofilm-induced lesion by effectively suppressing the two dominant strains in our system, *S. mutans* and *L. rhamnosus*, which are competitively and reproductively benefit from highly acidic conditions. Surprisingly, regardless of the probiotic applications, streptococci and *Lactobacillus spp.* remained predominant genera within the biofilms, which was verified by qPCR and the unaffected biofilm visualized by FISH. These results indicate that the intra-genus antagonism of probiotics was stronger than the inter-genus one, which means *L. rhamnosus* was mainly replaced by *L. reuteri*, and *S. mutans* was mainly replaced by *S. oligofermentans*. Inevitably, due to the close physical proximity of microorganisms, bacterial interactions occur constantly within the oral communities, either synergistically or antagonistically. In fact, the interspecies antagonism between *S. mutans* and other oral streptococci, e.g., *Streptococcus sanguinis*, *Streptococcus gordonii*, and *S. oligofermentans*, has been well-characterized [49, 50]. Likewise, *L. rhamnosus* probably had experienced intense competition with *L. reuteri*, which share parallel metabolic interests and thus compete for limited carbohydrate resources, leading to its exclusion from the tight-knit community [51, 52]. By the desirable replacement of the highly acidogenic species with congenic probiotics, dental plaque is collectively transformed into the less aggressive one. From a clinical perspective, it might be a wise choice to combine both genera of probiotics rather than monotherapy.

4.3 Strengths and limitations

There are several strengths and limitations in our study. First of all, we have assessed the anti-plaque as well as anti-caries actives of two promising probiotic strains by employing an established biofilm model *in vitro.* This model is committed to simulate the natural processes of plaque accumulation and intimate the interplays within multiple

pathogenic/probiotic species under fluctuating conditions while maintaining practical feasibility [22, 53, 54]. Evidently, the anti-caries efficacy of viable probiotic interventions was observed, as suggested by the results of the demineralization assay. Second, a complementary set of parameters (e.g., CFU, qPCR, pH, and FISH) were included to characterize the probiotic effects (e.g., alternations on biomass, biofilm composition, and architecture). Species-specific qPCR quantification is competent to enumerate analogous bacteria from the same genus, while the impact of any intervention on viable bacteria is better reflected by CFU. Third, and as a limitation, *in vitro* biofilm models cannot fully represent the highly complex microflora encountered in the human mouth. This can only be a simplification of natural process instead of an accurate reflection of the dynamic and polymicrobial oral microbiome, the host defense system, individual oral hygiene behaviors, or dietary factors on dental caries. Fourth, our FISH analysis focused only on the qualitative assessment of biofilm structure and composition, rather than species-specific quantification. Last, this study cannot allow precise inferences for the clinical application. Further research should be carried out to establish the therapeutic effects of viable *S. oligofermentans* and *L. reuteri* cultures at varying concentrations in the biofilm model before applying probiotics as caries preventive agents in long-term clinical trials. Another important aspect to be considered in future studies is the analysis of the environment-specific effects of probiotics on caries management.

5. Conclusion

In the present study, both viable cultures of *L. reuteri* and *S. oligofermentans* exhibited anti-caries potential. *L. reuteri* was superior to *S. oligofermentans* in terms of biomass reduction and the inhibition of cariogenic activity in the used multispecies biofilm model. These findings may provide clues for the future establishment of biotherapeutic countermeasures against dental caries.

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Statutory Declaration

"I, Zhihui Chen, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic 'Effects of Selected Probiotic Strains *Lactobacillus reuteri* and *Streptococcus oligofermentans* on Composition and Caries Activity of Multispecies Biofilm *in Vitro* / Auswirkungen der probiotischen Stämme *Lactobacillus reuteri* und *Streptococcus oligofermentans* auf die Zusammensetzung und Kariesaktivität von Multispezies-Biofilmen *in vitro*', independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité-Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Signature, date of the doctoral candidate

Declaration of own contribution to the publications

Zhihui Chen has contributed to the following publications:

Publication 1:

Zhihui Chen, Sebastian Schlafer, Gerd Göstemeyer, Falk Schwendicke. Probiotic effects on multispecies biofilm composition, architecture and caries activity *in vitro*. *Microogranisms.* 2020 Sep, 8 (9), 1272. DOI: 10.3390/microorganisms8091272.

Contribution in detail:

- Participated in project conception;
- Designed and tested the protocol;
- Generated the growing curves and standard curves of three cariogenic pathogens;
- Participated in qPCR primers design;
- Primers optimization;
- Tested the primer specificity;
- Optimized bacterial DNA extraction method;
- Performed the pre-experiment;
- Prepared experimental materials and performed the formal experiments, including specimen preparation, bacteria and biofilm cultivation, CFU count, bacterial DNA extraction, qPCR analysis, pH measurement, TMR analysis and FISH sample fixation;
- Data collection, data statistically analysis and data visualization of the CFU, qPCR, pH, TMR assay;
- Figure 1, 2, 4, 5, 6, 7 and Table 1, 2 preparation and edition on the basis of statistical evaluation;
- Drafted and revised the manuscript.

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Signature, date and stamp of the supervising members

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Signature, date of the doctoral candidate

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI
Selected Categories: **"MICROBIOLOGY"** Selected Category Scheme: WoS
Gesamtanzahl: 133 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS MICROBIOLOGY	29,637	34.648	0.054610
2	CLINICAL MICROBIOLOGY REVIEWS	19,194	17.750	0.021180
3	Cell Host & Microbe	17,787	15.753	0.063260
4	MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS	11,790	15.255	0.010610
5	Nature Microbiology	4,996	14.300	0.027420
6	TRENDS IN MICROBIOLOGY	12,514	11.974	0.020700
7	FEMS MICROBIOLOGY REVIEWS	12,528	11.524	0.015960
8	Microbiome	4,428	10.465	0.018070
9	Annual Review of Microbiology	10,068	10.242	0.009680
10	ISME Journal	23,603	9.493	0.051330
11	CLINICAL INFECTIOUS DISEASES	64,031	9.055	0.119010
12	Gut Microbes	3,203	7.823	0.008110
13	CURRENT OPINION IN MICROBIOLOGY	10,298	6.916	0.020360
14	mBio	16,500	6.747	0.069130
15	mSystems	1,003	6.519	0.004190
16	PLoS Pathogens	43,751	6.463	0.118940
17	CLINICAL MICROBIOLOGY AND INFECTION	17,929	6.425	0.036730
18	npj Biofilms and Microbiomes	475	6.333	0.001800
19	Emerging Microbes & Infections	1,941	6.212	0.006720
20	CRITICAL REVIEWS IN MICROBIOLOGY	2,694	5.697	0.004000

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
21	ENVIRONMENTAL MICROBIOLOGY	22,923	5.147	0.034820
22	JOURNAL OF ANTIMICROBIAL CHEMOTHERAPY	30,927	5.113	0.048620
23	JOURNAL OF INFECTIOUS DISEASES	45,452	5.045	0.076010
24	JOURNAL OF CLINICAL MICROBIOLOGY	52,963	4.959	0.053360
25	Microbial Biotechnology	3,576	4.857	0.006790
26	Microbial Genomics	573	4.853	0.003040
27	Virulence	3,557	4.775	0.009120
28	ANTIMICROBIAL AGENTS AND CHEMOTHERAPY	65,138	4.715	0.086660
29	INTERNATIONAL JOURNAL OF ANTIMICROBIAL AGENTS	11,529	4.615	0.017010
30	mSphere	1,732	4.447	0.007090
31	CELLULAR MICROBIOLOGY	9,048	4.288	0.013250
32	Frontiers in Microbiology	40,275	4.259	0.118000
33	Microbiology Spectrum	2,955	4.218	0.013220
34	Microorganisms	731	4.167	0.002000
35	Advances in Microbial Physiology	1,164	4.120	0.000780
36	FEMS MICROBIOLOGY ECOLOGY	15,674	4.098	0.016380
37	FOOD MICROBIOLOGY	10,786	4.089	0.012130
38	APPLIED AND ENVIRONMENTAL MICROBIOLOGY	105,845	4.077	0.064410
39	INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY	26,840	4.006	0.019610
40	MICROBIOLOGICAL RESEARCH	4,610	3.701	0.006580
41	Advances in Applied Microbiology	1,695	3.700	0.001850

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Probiotic Effects on Multispecies Biofilm Composition, Architecture, and Caries Activity *In Vitro*

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Abstract: While probiotics have been tested for their anti-caries effect in vitro and also clinically, there is a lack of understanding of their effects on complex dental biofilms. We assessed two probiotics, *Lactobacillus reuteri* and *Streptococcus oligofermentans*, on a continuous-cultured model containing *Streptococcus mutans*, *Lactobacillus rhamnosus* and *Actinomyces naeslundii*. Cariogenic biofilms were grown on bovine enamel specimens and daily challenged with *L. reuteri* or *S. oligofermentans* whole culture (LC/SC) or cell-free supernatant (LS/SS) or medium only (negative control, NC) (n = 21/group) for 10 days. Biofilm was assessed via counting colony-forming units, quantitative polymerase chain reaction, and fluorescence in situ hybridization. Caries activity was determined by pH measurements and by assessing mineral loss (ΔZ) using transverse microradiography. Both LC and SC significantly reduced total and strain-specific cariogenic bacterial numbers ($p < 0.05$). ΔZ was reduced in LC (mean ± SD: 1846.67 ± 317.89) and SC (3315.87 ± 617.30) compared to NC (4681.48 ± 495.18, $p < 0.05$). No significant reductions in bacterial numbers and ΔZ was induced by supernatants. Biofilm architecture was not considerably affected by probiotic applications. Viable probiotics *L. reuteri* and *S. oligofermentans*, but not their culture supernatants, could reduce the caries activity of multi-species biofilms *in vitro*.

Keywords: biofilm model; demineralization; dental caries; enamel; transverse microradiography

1. Introduction

Caries is a multifactorial disease characterized by an imbalance ("dysbiosis") in the dental biofilm composition and activity, triggered by available fermentable carbohydrates allowing cariogenic (i.e., acidogenic and aciduric) bacterial strains to metabolize these to organic acids. The resulting pH decrease generates an ecological advantage for pathogens and results in mineral loss from dental hard tissues [1].

Probiotics have been described as microorganisms that provide a health benefit and that have the potential to modify the microflora and re-balance the described dysbiosis [2,3]. However, the association between this modification and any clinically relevant effects on dental caries (lesion prevention or arrest) has not been unambiguously established [4–6]. Hence, a range of in vitro studies has been conducted to better understand the effects of widely available probiotic strains on dental pathogens [7–9], demonstrating probiotic effects mainly based on three mechanisms—an enhanced activity of the arginine deiminase system (ADS), direct inhibition of pathogens, and indirect reduction of pathogen colonization by competition for surface receptors. ADS activity contributes to

pH homeostasis by generating alkali from L-arginine, thereby reducing the ecological advantage of aciduric pathogens while preventing dental hard tissue demineralization [10,11]. Direct inhibition of pathogens has been demonstrated via competing for common nutrients or by inducing the production of inhibitory substances, i.e., bacteriocins and metabolic products, or by co-aggregation with cariogenic organisms [12–14]. Additionally, probiotic organisms have been shown to compete for binding sites on surfaces or host cells and thus, exclude the adherence of pathogenic bacteria [15].

Lactobacillus reuteri and *Streptococcus oligofermentans* are two probiotic species that have been shown to harness one or more of these strategies: *L. reuteri* exhibits significant antibacterial activity against both periodontopathic and cariogenic bacteria, mainly through reuterin, an antimicrobial component produced by glycerol fermentation [7,16–18]. *S. oligofermentans* possesses strong adhesion and low carbohydrate fermentation capacity, and produces hydrogen peroxide by utilizing lactic acid, thereby inhibiting pathogens such as *Streptococcus mutans* [19]. However, there is a paucity of data illustrating how these strains interact not with single pathogens, but with complex multispecies biofilms, which is a better reflection of the clinical situation. This limitation in modeling probiotic effects might explain the gap between promising in vitro results and contradictory clinical data on the anti-caries efficacy of probiotics.

Here, we aimed to employ a validated multi-species cariogenic biofilm model of *S. mutans*, *Lactobacillus rhamnosus*, and *Actinomyces naeslundii* to assess the effects of *L. reuteri* and *S. oligofermentans* on biofilm composition, architecture and caries activity *in vitro*.

2. Materials and Methods

2.1. Sample Preparation

From the crowns of 50 bovine incisors of the second dentition, without cracks, fractures, caries, or any defects, 105 enamel specimens were prepared (4.5 × 4.5 × 4 mm; band saw EXAKT 300 CL, EXAKT Apparatebau, Norderstedt, Germany). Specimens were ground flat (abrasive paper 1200–2500 grit, Hermes WS FLEX 18C, Buehler, Düsseldorf, Germany) and polished (silicon carbide grinding paper 4000 grit, Buehler). Specimens used for assessing biofilm composition (n = 5/group, 25 in total) were covered with nail varnish (Rival de Loop, Rossmann, Burgwedel, Germany) except for the polished enamel surface. Specimens used for assessment of mineral loss and biofilm structure (n = 16/group, 80 in total) were embedded in acrylic resin (Technovit 4071, Heraeus Kulzer, Hanau, Germany) before polishing, after which approximately one-third of the surface was covered with nail varnish to protect it from biofilm-induced demineralization (sound control). Specimens were then sterilized (121 °C, 2.1 bar, 34 min) and stored at room temperature for further experiment.

2.2. Bacterial Strains and Culture Conditions

Lactobacillus reuteri ATCC PTA 5289 and *Streptococcus oligofermentans* DSM 8249 (BioGaia, Stockholm, Sweden) were employed as probiotic strains; *Lactobacillus rhamnosus* DSM 20021, *Streptococcus mutans* DSM 20523, *Actinomyces naeslundii* DSM 43013 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) as cariogenic ones. *Lactobacillus* strains were cultured on de Man-Rogosa-Sharpe agar (MRS agar, Oxoid, Wesel, Germany), all other strains for 48 h on Columbia Agar with 5% Defibrinated Sheep Blood (Thermo Fisher Scientific, Vantaa, Finland). Single colonies were transferred into modified Brain Heart Infusion (MBHI) broth (BHI supplied with 8 g/L meat extract, 1% glucose, 1% sucrose, 250 mM glycerol, and 10 mM L-arginine, pH 7.0; Carl Roth, Karlsruhe, Germany) and incubated for 24 h. Incubation for all experimental steps, including the continuous-culture biofilm model, was conducted at 37 °C under aerobic conditions.

2.3. Multispecies Biofilm Model

The sterilized enamel specimens (n = 21/group) were randomly allocated to five experimental groups in different chambers and pre-coated overnight with sterile-filtrated human saliva to allow

for pellicle formation. Human saliva was collected from a healthy donor (28-years old female with low caries risk, without any caries, periodontal or systemic disease, who had not received antibiotic therapy for at least three months before the study) following a previously described protocol [20]. After the collection, the saliva was centrifuged (10 min, 6000× *g*) and filter-sterilized by 0.22 µm syringe filter (Millex-GP Syringe Filter, Merck, Darmstadt, Germany). Overnight cultures of the three cariogenic strains were adjusted and diluted to the optimal optical density at 600nm (OD_{600}) in fresh MBHI. Fifty-milliliter mixed suspension containing the equal amounts of each strain (approx. 1×10^8 colony-forming units; CFU) was delivered into each chamber to create cariogenic biofilms (Figure 1a).

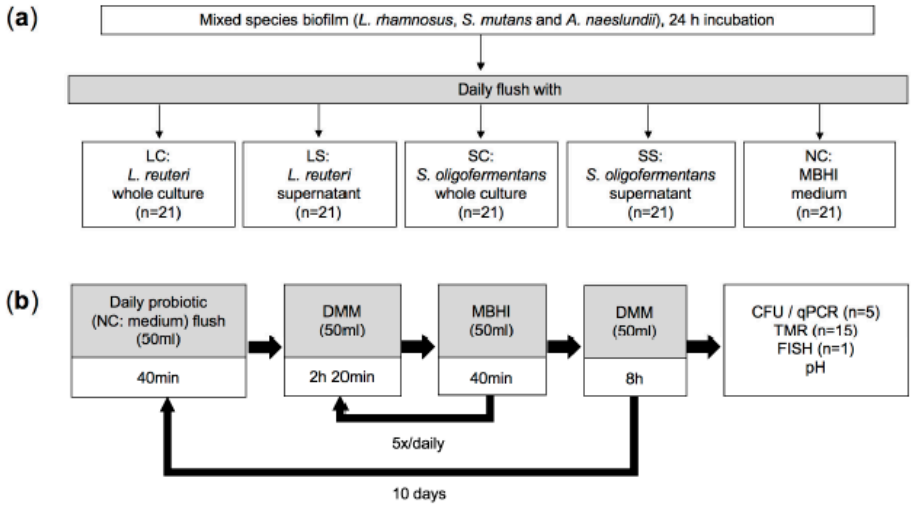

Figure 1. Study flow. (**a**) Multispecies biofilms were cultured for 24 h and then submitted to daily rinses with *L. reuteri* whole culture (LC), *L. reuteri* culture supernatant (LS), *S. oligofermentans* whole culture (SC), *S. oligofermentans* culture supernatant (SS) or fresh MBHI medium (NC). (**b**) The daily provision of modified Brain Heart Infusion (MBHI) broth and Defined Mucin Medium (DMM) was repeated over 10 days before submitting biofilms and specimens to further analysis. CFU: colony-forming units; qPCR: quantitative polymerase chain reaction; TMR: transverse microradiography; FISH: fluorescence in situ hybridization; n: sample size.

After 24 h of incubation, the chambers were connected to computer-controlled pumps (ISM405 MCP-Z standard pump with MS/CA pump head, ISMATEC, Wertheim, Germany) as previously described [21]. This continuous-cultured system was then challenged by different experimental interventions: (1) *L. reuteri* whole culture (LC); (2) *L. reuteri* culture supernatant (LS); (3) *S. oligofermentans* whole culture (SC); (4) *S. oligofermentans* culture supernatant (SS) and (5) negative medium control (NC) (Figure 1a). In LC and SC groups, *L. reuteri* or *S. oligofermentans* full cultures in MBHI, collected in the late exponential phase at a concentration of 1×10^9 CFU/mL, were provided for 40 min (5 mL/min, 20 min influx/efflux time, 20 min rest) once per day. In LS and SS groups, sterile-filtrated culture supernatants were provided in the same manner, instead of full cultures. NC group was rinsed with plain MBHI medium instead of full cultures or supernatants. Afterwards, all groups received fresh MBHI for 40 min (5 mL/min, 20 min influx/efflux time, 20 min rest), followed by rinsing (5 mL/min, 20 min influx/efflux time) and resting (2 h) in modified defined mucin medium (DMM; 3 mM KCl, 0.5 mM K_2HPO_4, 0.5 mM KH_2PO_4, 1 mM NaCl, 0.2 mM NH_4Cl, 1 mM $CaCl_2 \times 2H_2O$, 0.2 mM $MgCl_2 \times 6H_2O$, 0.5 mM $CO(NH_2)_2$, 2.5 g/L mucin, pH 7.0). This medium/DMM cycle was repeated five times daily to simulate the consumption of food and saliva clearance. The day was concluded with an 8-h resting period (Figure 1b). After 10 days of cultivation at 100% humidity, samples were removed and processed for analysis at the end of the daily schedule of the final day. The study setup is summed up in Figure 1.

2.4. Quantification of Bacterial Numbers via CFU

Following the incubation period, enamel samples were removed and gently washed with 0.9% sterile saline twice. The attached biofilms on the sample surface were carefully collected from identical surfaces (4.5 × 4.5 mm) and transferred into 1 mL sterile saline using a sterile curette. After vortex mixing, 100 µL of the microbial suspensions were plated out in triplicate on Columbia Agar with Sheep Blood (Thermo Fisher Scientific) in serial 10-fold dilutions. Total viable bacteria numbers were determined after incubation for 48 h at 37 °C aerobically.

2.5. DNA Isolation and Quantitative Polymerase Chain Reaction (qPCR)

For the DNA extraction from collected biofilms, the collected biofilm suspension (900 µL) was centrifuged (20 min, 6000× *g*), after which the pellet was re-suspended in 500 µL enzyme solution (20 mg/mL lysozyme, 1.2% Triton, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0) and incubated at 37 °C for 1 h. The DNA isolation was performed using the QIAamp mini DNA Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total genomic DNA was quantified by spectrophotometer (Multiskan Microplate Spectrophotometer, Thermo Fisher Scientific) and stored at 20 °C before further processing. The three cariogenic strains were quantified by qPCR as described previously [22]. In brief, strain-specific primers for 16S rRNA gene amplicons were designed using the Primer3 software [23], checked for specificity using BLAST, and synthesized by Metabion (Planegg, Germany). The primers specific for *L. rhamnosus* were forward primer AGGTGCTTGCATCTTGATTT and reverse primer CGCCATCTTTCAGCCAAGAA, and the annealing temperature was 62 °C. The primers specific for *S. mutans* were forward primer GACGCAAGGGAACACACT and reverse primer TCATGCAATAATTAATATTATGCGGTA, and the annealing temperature was 62 °C. The primers specific for *A. naeslundii* were forward primer TTTGTGGGTCCTGGATGAGT and reverse primer AAAAAGGCGCAATCTTTCC, and the annealing temperature was 64 °C. No cross-reactivity of the primers with other participating strains was observed. Quantification of *L. rhamnosus*, *S. mutans*, and *A. naeslundii* in the biofilms was performed by qPCR using a CFX Connect Thermal Cycler with a 96-well reaction module (Bio-Rad, Hercules, United States). Each reaction mixture (final volume 10 µL) contained 1 µL template DNA, 5 µL SYBR Select Master Mix (Applied Biosystems, Vilnius, Lithuania), 2 µL 400 nm forward/reverse primers and 2 µL RNase-free water (Braun Biotech, Melsungen, Germany). The applied thermal profile consisted of an initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturing at 95 °C for 20 s and annealing for 60 s at the specific annealing temperature. Melting curve analysis was performed for all primer sets to ensure a single peak, which was indicative of primer specificity. All experiments were performed in triplicate. The genomic DNA of the three bacterial species in the unknown samples was quantified using standard curves constructed by 10-fold serial dilution of bacterial DNA, which was extracted from pure cultures in MBHI of each strain collected at early stationary phase, with the optical density adjusted to a concentration of about 1×10^9 CFU/mL. Following the same extraction method described for the qPCR reaction, the DNA content was determined and serially diluted for the generation of standard curves correlating quantification cycle values to CFU per mL range from 1×10^4 to 1×10^9.

2.6. Caries Activity Determination (pH Measurements and Transverse Microradiography)

On the final day of the continuous biofilm culture, liquid waste in each chamber was collected and sterile filtrated, respectively, before and after the resting period, i.e., 16 h and 24 h after the last probiotic rinse. The pH value in the liquid was determined in triplicate measurements using a pH-meter (GMH3510 Digital pH-/mV-/Thermometer, Greisinger, Regenstauf, Germany).

For the transverse microradiography (TMR), samples were cut vertically to the surface into thin sections, ground to a mean thickness of 100 ± 10 µm, and polished up to 4000 grit as described. Microradiographs of the thin sections were obtained by a nickel-filtered copper X-ray source operating at 20 kV and 20 mA with an exposure time of 10 s. Films (Fine 71,337, Fujifilm, Tokyo, Japan)

were developed according to the manufacturer's recommendation under standardized conditions. The radiographic images were digitally analyzed using a light microscope connected to CCD-video camera module (XC-77CE, Sony, Tokyo, Japan). A digital image analysis software (TMR 2.0.27.2, Inspektor Research Systems, Amsterdam, Netherlands) was used to analyze the integrated mineral loss (ΔZ, in vol % × µm) and lesion depth (LD, in µm). Enamel areas covered with nail varnish served as sound reference.

2.7. Fluorescence In Situ Hybridization (FISH)

Five specimens were randomly selected from each of the five experimental groups and subjected to FISH for a qualitative analysis of the biofilm architecture. The samples were fixated in 3% (*w*/*v*) paraformaldehyde/PBS for at least 16 h, washed three times in PBS for 20 min and then dehydrated in an ethanol series (50%, 60%, 70%, 80%, 90%, 100%) for 1 h each. Then the specimens were transferred to a vacuum chamber (Exsikkator, Kartell S.p.A. LABWARE Division, Noviglio, Italy) and infiltrated with cold polymerizing resin (Technovit 8100, Heraeus Kulzer) for 10 min with the vacuum on, followed by 3 h with the vacuum turned off. The infiltration procedure was performed twice. Samples were then embedded in resin for 16 h using polyethylene embedding molds (Flat Bottom Embedding Capsules, Electron Microscopy Sciences, Hatfield, PA, USA). All of the above procedures were carried out at 4 °C. Before decalcification in 17% EDTA for 21 days, the specimens were cut into thin slices of 1 mm with a rotary saw microtome (Ernst Leitz, Wetzlar, Germany). Decalcification was checked by X-ray analysis, after which the slices were re-embedded in resin (Technovit 8100, Heraeus Kulzer), sectioned on an ultramicrotome (2 µm; Ultracut E, Reichert Jung Optische Werke, Wien, Austria) and mounted on glass slides with adhesive coating (Polysine, Menzel-Gläser, Braunschweig, Germany).

The probes STR405 [24], 5′-labeled with Alexa Fluor 488 (MWG Biotech, Ebersberg, Germany) and ACT476 [25], 5′-labeled with Atto 550 (IBA, Göttingen, Germany) were used to detect streptococci (*S. mutans* and *S. oligofermentans*) and *A. naeslundii*, respectively. Additionally, EUB338 [26], 5′-labeled with Atto 633 (IBA), was employed to visualize the remaining bacteria (*L. rhamnosus* and *L. reuteri*). For each experiment, fixed cells of all strains employed in the study were used as positive and negative controls for the three probes. All probes were specific at 30% formamide in the hybridization buffer (Figure S1). Permeabilization, hybridization, stringency washes, and mounting were performed according to the protocol described previously [27].

After mounting, the biofilms were analyzed by confocal laser scanning microscopy (Zeiss LSM700, Zeiss, Jena, Germany). Dyes were excited sequentially at 488 nm (Alexa 488), 543 nm (Atto 550), and 633 nm (Atto 633) and detected from 300–550 nm (Alexa 488), 560–800 nm (Atto 550) and 300–800 nm (Atto 633). Images were acquired at representative sites with a 63× objective (Plan-Apochromat; NA = 1.4; oil; Zeiss) objective, with the pinhole set to 1 Airy Unit, a pixel time of 1.55 µs and an image size of 1048 × 1048 pixels (101.6 × 101.6 µm^2).

2.8. Statistical Analysis

The statistical analysis was performed with SPSS 25 (IBM, Armonk, Armonk, NY, USA). Normal distribution and homogeneity were checked using Shapiro–Wilk and Levene's tests, respectively. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test for assessing differences between groups regarding the log-transformed CFU data, numbers of cariogenic bacteria quantified by qPCR, pH value, LD and ΔZ. The level of significance was set at $p < 0.05$ for all statistical tests.

3. Results

3.1. Viable Cell Counts of Biofilms

The total amount of viable bacteria [$\log_{10}$ (CFU/mL), mean ± SD] was significantly lower in LC (4.88 ± 0.42) and SC (6.11 ± 0.05, $p < 0.001$) than NC (6.60 ± 0.05, $p < 0.001$). For LS and SS (6.37 ± 0.29 and 6.37 ± 0.10, respectively) no significant difference to NC was observed ($p > 0.05$) (Figure 2).

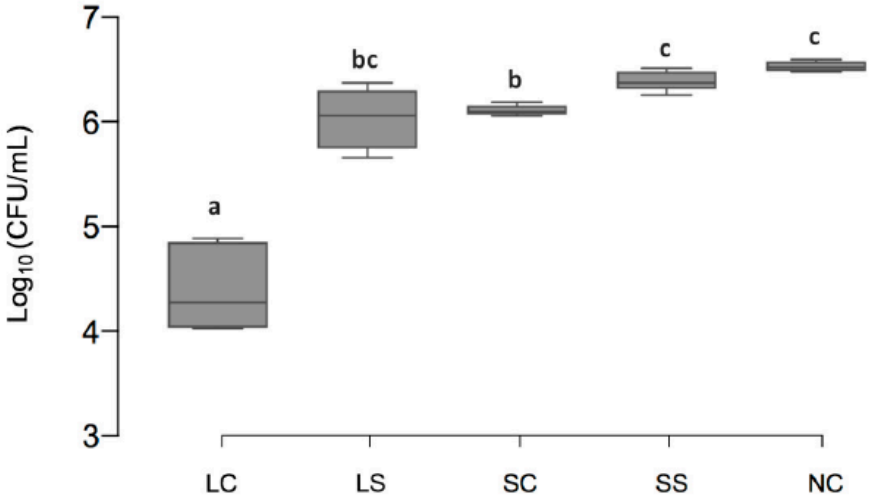

Figure 2. The total amount of viable bacteria. Line and box = median and 25th/75th percentiles; error bars = minimum and maximum. LC: *L. reuteri* whole culture; LS: *L. reuteri* culture supernatant; SC: *S. oligofermentans* whole culture; SS: *S. oligofermentans* culture supernatant; NC: negative medium control. Different superscript letters indicate statistically significant differences between groups ($p < 0.05$); if boxes share minimum one letter there was no significant difference detected ($p > 0.05$).

3.2. Cariogenic Bacterial Amounts

In the LC group, *L. rhamnosus* and *S. mutans*, but not *A. naeslundii* were significantly reduced compared with NC ($p < 0.05$). For SC group, *S. mutans* and *A. naeslundii*, but not *L. rhamnosus* were significantly reduced compared with NC ($p < 0.05$). LS and SS did not show any significant differences compared with NC ($p > 0.05$) (Table 1).

Table 1. Bacterial numbers (× 10^6 cells/mL, mean ± SD) of each cariogenic strain in the different treatment groups, as determined by qPCR ($n = 5$).

Group	*L. rhamnosus*	*S. mutans*	*A. naeslundii*
LC	0.55 ± 0.10 ^{a}	0.37 ± 0.11 ^{a}	0.19 ± 0.04 ^{a}
LS	4.03 ± 1.87 ^{ab}	3.65 ± 1.86 ^{abc}	0.36 ± 0.20 ^{ab}
SC	2.23 ± 0.86 ^{ab}	0.22 ± 0.14 ^{ab}	0.09 ± 0.04 ^{b}
SS	3.84 ± 1.61 ^{ab}	2.76 ± 0.57 ^{c}	0.43 ± 0.07 ^{a}
NC	3.13 ± 0.87 ^{b}	3.69 ± 1.38 ^{c}	0.45 ± 0.10 ^{a}

Different superscript letters indicate the statistically significant difference between experimental groups within each column measured on the same time point ($p < 0.05$); if groups share minimum one letter there was no significant difference detected ($p > 0.05$).

3.3. pH Value

The pH value in the LC group was significantly higher than that in most other groups, specifically NC, after both 16 and 24 h of the last probiotic rinse, respectively ($p < 0.05$). SC did not provide obvious pH alterations compared with NC. For LS and SS, a significant decrease compared to NC was observed after 24 (but not 16) h ($p < 0.05$) (Table 2).

Table 2. Final pH values (mean ± SD) of the fluid in different biofilm chambers for five experimental groups 16 and 24 h after the last probiotic rinse.

Group	16 h	24 h
LC	5.00 ± 0.02 ^{a}	3.90 ± 0.03 ^{a}
LS	3.90 ± 0.02 ^{b}	3.53 ± 0.01 ^{b}
SC	3.83 ± 0.01 ^{cd}	3.75 ± 0.00 ^{a}
SS	3.73 ± 0.03 ^{d}	3.44 ± 0.01 ^{c}
NC	3.94 ± 0.03 ^{bc}	3.70 ± 0.01 ^{d}

Different superscript letters indicate the statistically significant difference between experimental groups within each column measured at the same time point ($p < 0.05$); if groups share minimum one letter there was no significant difference detected ($p > 0.05$).

3.4. Mineral Loss of Enamel Lesions

ΔZ (vol% × µm, mean ± SD) and LD (µm, mean ± SD) (Figures 3 and 4) was significantly lower in LC (ΔZ: 1846.67 ± 317.89, LD: 78.20 ± 13.13) and SC (3315.87 ± 617.30, 101.35 ± 15.08) than NC (4681.48 ± 495.18, 122.39 ± 15.72, $p < 0.01$). In LS and SS, no significant differences to NC were observed ($p > 0.05$).

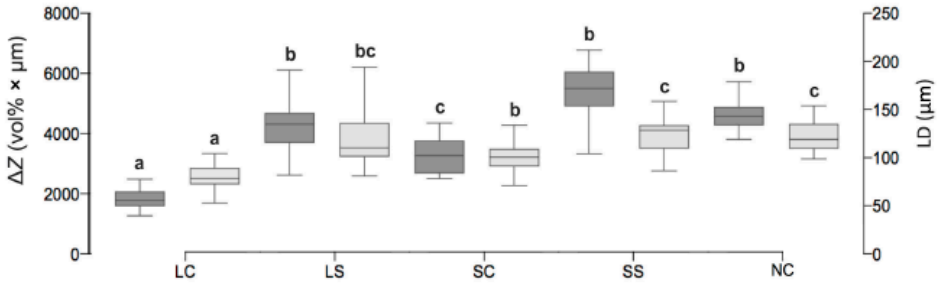

Figure 3. Mineral loss ΔZ (dark grey boxes, left y-axis) and lesion depth LD (light grey boxes, right y-axis) of enamel caries lesions after 10 days of biofilm cultivation. Line and box = median and 25th/75th percentiles; error bars = minimum and maximum. LC: *L. reuteri* whole culture; LS: *L. reuteri* culture supernatant; SC: *S. oligofermentans* whole culture; SS: *S. oligofermentans* culture supernatant; NC: negative medium control. Different superscript letters indicate statistically significant differences between treatment groups ($p < 0.05$); if boxes share minimum one letter there was no significant difference detected ($p > 0.05$).

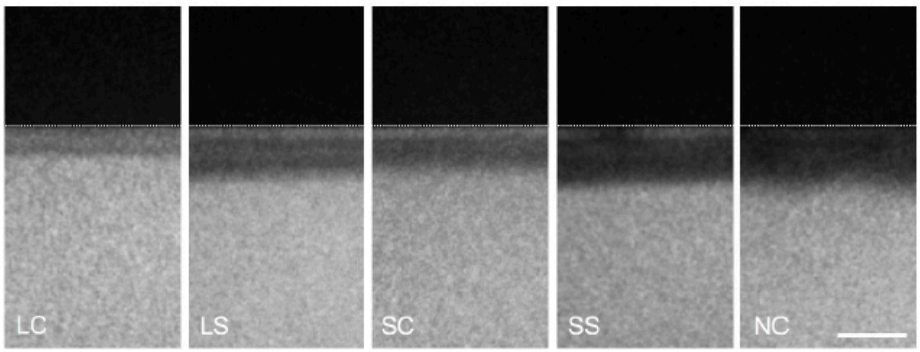

Figure 4. Representative microradiographs of the carious lesions on enamel surface induced by the multispecies biofilms after 10 days. The dotted white line indicates the lesion interface. The mean value of lesion depths differed from 78.2 µm (LC) to 122.4 µm (NC). LC: *L. reuteri* whole culture; LS: *L. reuteri* culture supernatant; SC: *S. oligofermentans* whole culture; SS: *S. oligofermentans* culture supernatant; NC: negative medium control. Scale bar = 100 µm.

3.5. Biofilm Structure and Composition

FISH analysis of biofilms attached to the enamel specimens revealed the presence of compact structures in all treatment groups. The biofilms had a thickness of 150–250 µm and showed typical architectural features of supragingival biofilms with a bulged surface. In all treatment groups, the biofilms were dominated by streptococci and *Lactobacillus* spp. *A. naeslundii* was identified in low numbers in all biofilms, as characteristic branched colonies typically located at the biofilm bases (Figure 5).

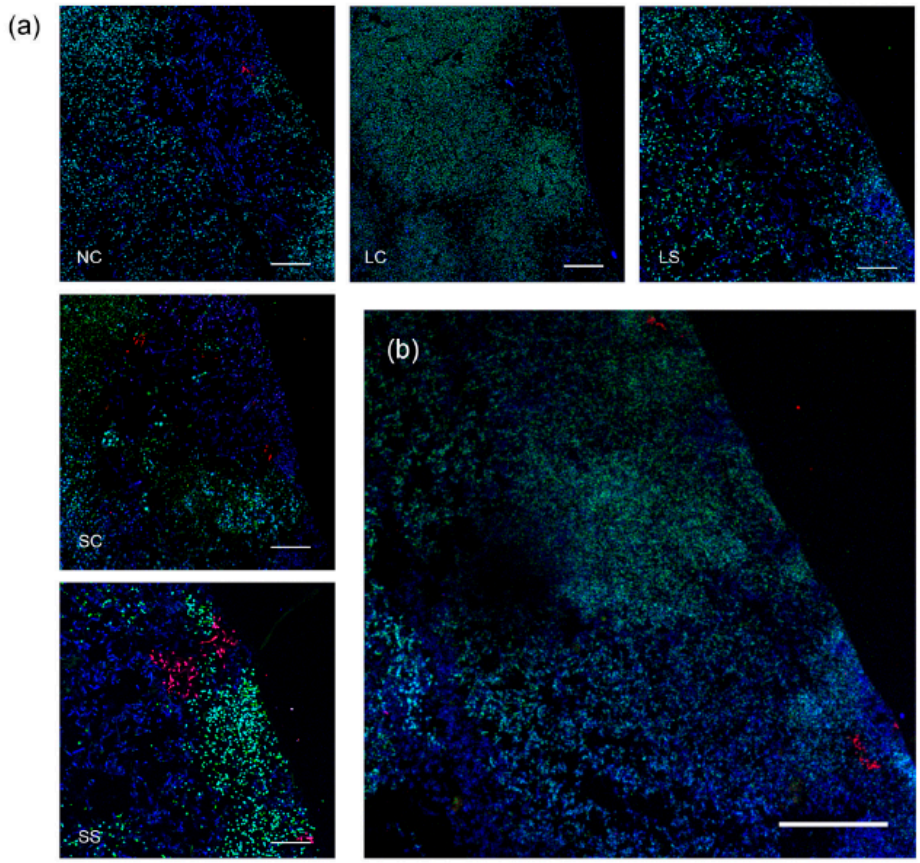

Figure 5. Biofilm architecture, visualized by fluorescence in situ hybridization (FISH). (**a**) In all five treatment groups, compact biofilms were observed, dominated by clusters of streptococci (STR405-Alexa-488, green) and *Lactobacillus spp* (EUB338-Atto-633, blue). *A. naeslundii* (ACT476-Atto-550, red) was identified in low numbers in all biofilms, as branched colonies typically located at the biofilm base. Scale bars = 20 µm. (**b**) Representative image (from LS group) demonstrates the complex biofilm architecture with cell clusters, cell-free areas, and a bulged surface, which resemble the structure of supragingival biofilm. No significant structural modification was observed between experimental groups. Scale bar = 60 µm. Dark areas represent the enamel surface of the specimens. LC: *L. reuteri* whole culture; LS: *L. reuteri* culture supernatant; SC: *S. oligofermentans* whole culture; SS: *S. oligofermentans* culture supernatant; NC: negative medium control.

4. Discussion

Probiotics have been demonstrated as a potentially useful agent for the prevention or treatment of oral diseases. However, data on the efficacy of probiotics on caries prevention and management remains ambiguous [4]. The present study assessed the application of viable *L. reuteri* and *S. oligofermentans* cultures as well as their supernatants on cariogenic multispecies biofilms. We found the vital probiotics

to significantly decrease the enamel mineral loss, bacterial numbers, and cariogenic activity of the biofilm. *L. reuteri* tended to show superior anti-caries potential compared with *S. oligofermentans*, while the supernatants exerted no evident beneficial effects.

Our findings can be interpreted from a range of perspectives. First of all, the presence of viable probiotics could significantly affect the composition and caries activity of the biofilm, as reflected by changes in CFU and bacterial numbers, pH and mineral loss. We likely ascribe this contribution to the modification of inherent ADS activity: Evidence from both clinical and laboratory studies suggest a positive relationship between ADS activity and dental health. ADS contributes to pH homeostasis by generating alkali from arginine metabolism, and thereby reduces the ecological advantage of aciduric pathogens, which is grounded in a positive regulation between microflora acidification and aciduric bacterial selection [28–31]. The arginolytic strains *L. reuteri* and *S. oligofermentans* were able to constantly neutralize acid products via the ADS route and slow down the caries process. In the supernatant groups, without the persistent participation of active probiotic bacteria, biofilms had no opportunity to reverse the disadvantaged pH milieu and hence remained in a pathogenically metabolic pattern. The addition of probiotic microorganisms could prevent further ecological shifts toward disease and improve biofilm resilience [32–35]. For dysbiotic biofilms, probiotics could offer a chance to reconstruct a host-compatible community, while for healthy ones, to maintain health status and improve the resilience of the ecosystem.

In contrast, any indirect actions of probiotics that have been mediated by supernatant, e.g., bacteriocins (such as reuterin), are likely to play a minor role in the observed microbial communities. This seems to conflict with our previous study, which described reuterin in the supernatant as a promising antimicrobial factor [36]. However, our previous study only assessed *S. mutans* monospecies biofilms. Multi-species biofilm tends to be more aggressive, unite [37,38], and better recover from external shocks, especially those provided only once per day (as in the present study). The polymeric matrix, containing extracellular polymeric substances (EPS) such as polysaccharides, and soluble glucans that are produced by cariogenic pathogens, offers a robust habitat for surface adhesion, synergistic/competitive interactions, and antimicrobial tolerance against external chemical and physical fluctuations, e.g., probiotic bacteriocins [39]. Besides, the polymicrobial synergy could modulate virulence gene expression and therefore elevate the pathogenicity of the bacterial community [40]. Note that we did account for the environment-specific probiotic actions, i.e., certain external conditions required for probiotics to exert their anti-cariogenic talent [41]. Therefore, we supplemented our media with low concentrations of glycerol and arginine, both common nutrients that could be acquired from diet and saliva, to assure that the probiotics share adequate nutrition for correlative metabolic pathways in the simulation system. Glycerol is required by *L. reuteri* to generate reuterin, and arginine is an essential educt for the ADS system [42,43].

Species-specific effects were also observed: *L. reuteri* reduced the bacterial numbers of *L. rhamnosus* and *S. mutans*, but not *A. naeslundii*. *L. reuteri* also exhibited more pronounced capacity than *S. oligofermentans* in modification on the cariogenic activity of biofilms. The latter, however, impacted more on the bacterial numbers of *S. mutans* and *A. naeslundii* rather than *L. rhamnosus*, which may explain the divergence in caries activity reduction between LC and SC. *L. reuteri* probably achieves successful lesion control through the significant inhibition of dominant strains in our complex biofilm model, *L. rhamnosus* and *S. mutans*, which are fast-growing and competitive under severely acidic conditions. Though both probiotic strains inhibit the reproduction of *S. mutans*, *L. reuteri* is suggested via bacteriocin and *S. oligofermentans* via hydrogen peroxide products [7,19], which is also a powerful agent specifically against facultative anaerobes such as *A. naeslundii*. Moreover, the genera streptococci and lactobacilli remained dominant in the biofilms regardless of which probiotic intervention was employed, and biofilm architectures visualized by FISH were largely unaffected. This suggests that, as described, the probiotics had larger intra-genus than inter-genus effects, i.e., *L. reuteri* had mainly replaced *L. rhamnosus* and *S. oligofermentans* mainly replaced *S. mutans*. The congeneric antagonism between *S. mutans* and other common streptococci, such as *S. oligofermentans*, *Streptococcus sanguinis*,

and *Streptococcus gordonii* has been well studied [44,45]. The interspecies competition probably exists also between *L. reuteri* and *L. rhamnosus*, which share similar metabolic behavior and, therefore, fight for limited carbohydrate resources in tight-knit communities, which may lead to the starvation of the competing pathogen *L. rhamnosus* [46,47]. This replacement of highly acidogenic species from the same genus is likely to be one of the strategies for probiotic to moderate the dental plaque aggressiveness. From the clinical perspective, the combined application of vital probiotics from both genera, streptococci and lactobacilli, would be a wise choice.

This study has several strengths and limitations. Firstly, and as a strength, we employed a biodiverse and complex caries model to assess the anti-caries effects of both viable cultures and supernatants of *L. reuteri* and *S. oligofermentans*. Our model aims to mimic natural conditions as best as possible while retaining operational feasibility [21,48,49]. Secondly, we have proven the favorable efficacy of the probiotic intervention on the caries process, as the gold standard for assessing mineral loss in vitro [50], TMR, provided direct evidence of the anti-caries potential of the two probiotic strains. A range of outcome parameters (e.g., the biofilm composition, architecture) were also acquired via a diverse set of measures (CFU, qPCR, FISH, pH). For example, species-specific quantification using qPCR allowed us to overcome the limits of differential sensitivity of CFU when enumerating bacteria from the same genus, while CFU is more suitable to reflect the impact of interventions on vital bacteria with metabolic activity. Serial assays with mutual complementation provided us a comprehensive interpretation of biofilm changes under probiotic therapy. Thirdly, as a limitation, our model remained a simplification of natural processes. It does not permit to reflect the impact of more complex and dynamic oral microbiome, host responses, oral hygiene behaviors, or diets on cariogenicity. Additionally, our FISH analysis allowed only for a qualitative assessment of biofilm structure and composition rather than quantification, and also did not focus on cariogenic versus probiotic strains. We accepted that, partially as further methods were employed to obtain information in these directions. Further studies are required to elucidate why supernatant did not, as described, have any significant impact on cariogenic biofilms, and should also assess how environment-specific conditions modify the anti-caries efficacy of two probiotic strains in more detail.

5. Conclusions

Within the limitations of this in vitro study, viable probiotics *L. reuteri* and *S. oligofermentans*, but not their culture supernatants, had the potential to reduce the cariogenic effects of multi-species biofilms in vitro. Viable probiotics had species selective effects on cariogenic strains in a complex multispecies biofilm model.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/9/1272/s1, Figure S1: Specificity of the employed FISH probes.

Author Contributions: Z.C. and F.S. conceived the study. F.S., Z.C. and G.G. designed and planned the study. Z.C. prepared specimens and conducted the study. S.S. prepared samples, acquired data and figures of FISH analysis. Z.C. performed statistical analysis, drafted the manuscript. F.S., S.S. and G.G. critically revised the manuscript and all authors approved the present paper as the final submitted version.

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