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DISSERTATION


„Effect of probiotic supernatants on the metabolic activity and
survival of *Streptococcus mutans in vitro*"


„Wirkung von probiotischen Überständen auf die
Stoffwechselaktivität und das Überleben von *Streptococcus
mutans in vitro*"


zur Erlangung des akademischen Grades
Doctor medicinae dentariae (Dr. med. dent.)


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# Table of contents

# List of figures and tables

# List of abbreviations

| | |
|---|---|
| Arg | Arginine |
| BHI | Brain-heart-infusion |
| CFU | Colony forming units |
| Glu | Glucose |
| Gly | Glycerol |
| HPA | Hydroxypropionaldehyde |
| Lac | Lactic acid |
| Lox | Lactate oxidase |
| *L. reuteri* | *Limosilactobacillus reuteri* |
| MM | Minimal Medium |
| MRS | deMan-Rogosa-Sharpe |
| Pox | Pyruvate oxidase |
| *S. mutans* | *Streptococcus mutans* |
| *S. oligofermentans* | *Streptococcus oligofermentans* |

## Zusammenfassung

### Hintergrund

Zahnkaries ist mit einer Störung des dynamischen Gleichgewichts der oralen mikrobiellen Homöostase verbunden, die zur Dominanz von azidogenen und azidurischen Spezies wie *Streptococcus mutans* führt. Probiotika wie *Streptococcus oligofermentans* und *Limosilactobacillus reuteri* können die Stoffwechselaktivität und das Überleben von *S. mutans* hemmen, u.a. durch die Freisetzung von Substanzen wie Wasserstoffperoxid, Reuterin, Ammoniak und organischen Säuren. Ziel dieser Studie war es, die umgebungsspezifischen Mechanismen, mit denen *S. oligofermentans* und *L. reuteri* die Stoffwechselaktivität und das Überleben von *S. mutans* beeinflussen, zu untersuchen.

### Methoden

*L. reuteri* und *S. oligofermentans* wurden unter verschiedenen Umgebungsbedingungen kultiviert; Minimalmedium (MM), MM mit einem Glukosezusatz (MM+Glu), Glycerinzusatz (MM+Gly), Milchsäurezusatz (MM+Lac), Argininzusatz (MM+Arg) und Zusatz aller vier Substanzen (MM+all). Nach der Inkubation wurden der Metabolitengehalt (Wasserstoffperoxid, Reuterin, Ammoniak, Lactat) in den Kulturüberständen durch kolorimetrische Assays bestimmt. *S. mutans* wurde in ähnlicher Weise in den obigen sechs verschiedenen MM-Variationen kultiviert, wobei ein Zusatz von probiotischen Kulturüberständen erfolgte (Testgruppen) oder nicht erfolgte (Kontrollgruppen). Die von *S. mutans* produzierte Laktatmenge wurde bestimmt und das Überleben von *S. mutans* (koloniebildende Einheiten/mL) bewertet. Eine Einwege-ANOVA gefolgt von einem Dunnett-Test wurde zum statistischen Vergleich verwendet ($p = 0.05$).

### Ergebnisse

*L. reuteri* und *S. oligofermentans* erzeugten umgebungsspezifisch Wasserstoffperoxid, Reuterin, Ammoniak und Laktat. Wenn *S. mutans* in *S. oligofermentans*-Überständen kultiviert wurde, war seine Laktatausbeute signifikant verringert ($p<0.01$). In MM+Lac+Glu und MM+all war in den Testgruppen keinerlei Laktatnachweis mehr möglich; in den Kontrollgruppen betrug die durchschnittliche Laktatmenge hier 4.46 mM (Standardabweichung 0.41) und 6.00 mM (Standardabweichung 0.29) ($p<0.001$). Bei Kultivierung in *L. reuteri*-Überständen zeigten alle Gruppen außer MM+Lac+Glu eine ähnliche Verringerung der Laktatausbeute ($p<0.01$). Das Überleben von *S. mutans* war bei Kultivierung in den Überständen von *S. oligofermentans* in MM+Lac+Glu und MM+all um 0.6-$\log_{10}$ und 0.5-$\log_{10}$ signifikant verringert. Die Kultivierung mit dem Überstand von *L. reuteri* in MM+Gly+Glu und MM+all reduzierte die *S. mutans*-Zahl um 6.1-$\log_{10}$ und 7.1-$\log_{10}$.

### Schlussfolgerungen

Die Stoffwechselaktivität und das Überleben von *S. mutans* waren signifikant verringert,

wenn sie probiotischen Überständen ausgesetzt wurden, wobei umgebungsspezifisch verschiedene Effektpfade eingesetzt wurden.

# Abstract

## Background

Dental caries is associated with a disturbance in the dynamic balance of oral microbial homeostasis, which leads to the community dominance of acidogenic and acid-tolerating species like *Streptococcus mutans*. *Streptococcus oligofermentans* and *Limosilactobacillus reuteri* have been shown to inhibit the metabolic activity and survival of *S. mutans*, which may exert antibacterial effects through the release of substances like hydrogen peroxide, reuterin, ammonia, and organic acids. This study aimed to investigate the environment-specific mechanisms of metabolic activity and survival inhibition by *S. oligofermentans* and *L. reuteri* against *S. mutans*.

## Methods

*L.reuteri* and *S.oligofermentans* were cultivated in different environments: minimal medium (MM), minimal medium containing glucose (MM+Glu), glycerol (MM+Gly), lactic acid (MM+Lac), arginine (MM+Arg), and all four substances (MM+all) *in vitro*. After incubation, culture supernatants were obtained and metabolite contents (hydrogen peroxide, reuterin, ammonia, lactate) measured by colorimetric assays. *S.mutans* was similarly cultured in the above six various MM variations and glucose was additionally added to the MM+Gly, MM+Lac, and MM+Arg group (control samples), as well as the described probiotic supernatants of these groups (test samples). The lactate concentration produced by *S.mutans* was detected and its survival (as colony-forming units/mL) evaluated. One-way ANOVA followed by Dunnett's test was used for statistical comparison ($p=0.05$).

## Results

*L. reuteri* and *S. oligofermentans* environment-specifically generated hydrogen peroxide, reuterin, ammonia, and lactate. When *S.mutans* was cultivated in *S. oligofermentans* supernatants, its lactate yield was significantly reduced ($p<0.01$), especially in MM+Lac+Glu and MM+all. There was no detectable lactate yield compared to the control samples. The mean (SD) for the control samples were 4.46 (0.41) mM and 6.00 (0.29) mM, respectively ($p<0.001$). When cultivated in *L. reuteri* supernatants, all groups except MM+Lac+Glu showed a similar reduction in lactate yield ($p<0.01$). For *S. mutans*, when cultivated in the supernatants of *S. oligofermentans* in MM+Lac+Glu and MM+all, its survival was significantly reduced by 0.6-$\log_{10}$ and 0.5-$\log_{10}$, respectively. Cultivation with the supernatant of *L. reuteri* caused the survivability of *S. mutans* in MM+Gly+Glu and MM+all reduced by 6.1-$\log_{10}$ and 7.1-$\log_{10}$, respectively.

## Conclusions

The cultivation of *S. mutans* in the supernatants containing probiotic products may have an inhibitory effect on its metabolic activity and survival, and this effect was environment-specific through different pathways.

# 1. Introduction

Dental caries is one of the most prevalent worldwide diseases, which depends on sugar and biofilm. Oral microbial biofilms are highly structurally and functionally organized bacterial communities [2]. Frequent carbohydrates exposure, coupled with poor hygiene, low saliva flow, and other systemic problems will lead to a homeostatic imbalance in the dental biofilm environment and facilitate excessive multiplication of cariogenic pathogens. *Streptococcus mutans* has been identified as one major etiological agent of dental caries. It possesses some features associated with cariogenicity, including (1) adhesion firmly to the tooth surface by synthesizing insoluble glucan from sucrose, (2) production of large amounts of acid by fermenting sugar [3], (3) adaptation to the acidic environment through an agmatine deiminase system and F-ATPase [4]. Evidence suggests that *S. mutans* is selected under low pH conditions in dental biofilms [5]: Under homeostasis, dental biofilms contain only 2% of *S. mutans*. When this homeostasis is disturbed and converted to an acidic environment, the proportion of *S. mutans* substantially increases [6]. Acidogenic and aciduric bacteria like *S. mutans* hence generate an advantageous ecological condition for themselves to thrive and be competitive. The suppression of acid production by inhibiting the metabolic activity and survival of *S. mutans* could assist in maintaining a healthy microbial equilibrium in biofilms and decrease the incidence of dental caries.

Probiotics are health-beneficial microorganisms [6]. Potential probiotic pathways to exert beneficial effects in the oral cavity [7, 8] include, for example, (1) adhesion to oral tissues, occupying a place in oral biofilms, and the capacity to replace pathogenic bacteria, (2) production of antibiotic substances which inhibit the metabolic activity and survival of pathogenic bacteria, (3) resistance to low pH in the oral environment, (4) low acid production when fermenting dietary sugars, (5) modulation of the host's general and local immune response. In recent years, the anti-caries effect of probiotic bacteria on cariogenic bacteria has increasingly been investigated and probiotics are regarded as potentially useful for regulating microbial communities [9]. Probiotics are by now commercially available to combat oral disease treatments like caries, gingivitis, and periodontitis [10, 11].

*Streptococcus oligofermentans* is gram-positive, non-spore-forming, and non-motile coccus [12]. As a good probiotic candidate, *S. oligofermentans* has some favorable properties. It was isolated from healthy tooth surfaces or caries-free humans [13] and belongs to the "*mitis*" group of streptococci, which are considered the "pioneer" colonizers of the initial stages of oral biofilm formation [14]. This microorganism generates less lactic acid than *S. mutans* and inhibits the growth of cariogenic bacteria like *S. mutans* by the production of hydrogen peroxide ($H_2O_2$) [15]. The ability of *S. oligofermentans* to produce $H_2O_2$ from lactic acid, the main organic acid generated by dental biofilms, is particularly interesting. This property may have dual benefits: (1) by converting lactic acid into $H_2O_2$ to minimize the pH drop, and (2) through the production of $H_2O_2$ to inhibit *S. mutans* [15]. In addition to $H_2O_2$, *S. oligofermentans*

also produces ammonia from arginine, which can neutralize free acids and prevent the emergence of cariogenic microorganisms [16].

*Limosilactobacillus reuteri*, formerly known as *Lactobacillus reuteri* [17], has been used as a probiotic for oral administration and is currently considered generally safe [18]. An important mechanism by which it exerts its beneficial effects is the release of antimicrobial substances. Reuterin, a metabolite of glycerol, is a primary antibiotic multi-compound produced by *L. reuteri*. It is water-soluble and has a wide active pH range and antimicrobial activity against a variety of pathogens and food spoilage organisms, including gram-positive and gram-negative bacteria, fungi, moulds, yeasts as well as protozoa [19]. Moreover, $H_2O_2$, ammonia, and organic acids are also produced by *L. reuteri* [20]. Although many studies have demonstrated the ability of *L. reuteri* to generate different antimicrobial substances, little is known about whether different environmental conditions could modify its metabolic activity.

A range of studies on the bacteriology of dental caries has focused on the role of probiotics against cariogenic pathogens. However, the exact mechanisms by which probiotics exert their activity against *S. mutans* are still unknown. To gain more insight into the environment-specific metabolic mechanism of probiotics and their inhibitory effects on *S. mutans*, in the present study, we used a previously established *in vitro* model [21] and a modified saliva analogue to simulate different environmental conditions. We hypothesized that the metabolic activity and survival of *S. mutans* is significantly reduced by being cultured in the probiotic supernatants, and assumed this effect to be environment-specific.

## 2. Aims and objectives

The study aimed to investigate the metabolic activity of the two probiotics under specific environmental conditions and their inhibitory effects on the lactate production and survival of *S. mutans*. The following objectives were formulated:

1. To detect the concentration of the probiotic metabolites (reuterin, $H_2O_2$, ammonia, and lactate) under different environmental culture conditions.

2. To measure and compare the difference in lactate production of *S. mutans* between different environmental culture conditions (control groups) and the corresponding probiotic supernatants under different culture conditions (test groups).

3. To measure and compare the difference in survival of *S. mutans* between different environmental culture conditions (control groups) and the corresponding probiotic supernatants under different culture conditions (test groups).

## 3. Materials and methods

### 3.1. Materials

### 3.1.1 Bacterial strains

*S. mutans,* DSM 20523*, S. oligofermentans,* DSM 8249 (DMSZ, Braunschweig, Germany)

*L. reuteri,* ATCC PTA 5289 (BioGaia, Stockholm, Sweden)


### 3.1.2 Chemicals and reagents

| | |
|---|---|
| Brain-heart-infusion (BHI) broth | Carl Roth, Karlsruhe, Germany |
| Glucose | Carl Roth, Karlsruhe, Germany |
| Yeast extract | Carl Roth, Karlsruhe, Germany |
| Beef extract | Carl Roth, Karlsruhe, Germany |
| $KH_2PO_4$ | Merck, Darmstadt, Germany |
| $K_2HPO_4$ | Carl Roth, Karlsruhe, Germany |
| NaCl | Carl Roth, Karlsruhe, Germany |
| KCl | Merck, Darmstadt, Germany |
| $NH_4Cl$ | Merck, Darmstadt, Germany |
| $MgCl_2*6H_2O$ | Merck, Darmstadt, Germany |
| Glycerol | Sigma Aldrich, Taufkirchen, Germany |
| Lactic acid | Carl Roth, Karlsruhe, Germany |
| L-arginine | Sigma Aldrich, Taufkirchen, Germany |
| Tryptophan | Sigma Aldrich, Taufkirchen, Germany |
| Peroxide Assay Kit | Sigma Aldrich, Taufkirchen, Germany |
| Urease Activity Assay Kit | Sigma Aldrich, Taufkirchen, Germany |
| Lactate Assay Kit | Sigma Aldrich, Taufkirchen, Germany |
| Blood agar plates COLS+ | Oxoid, Wesel, Germany |
| deMan-Rogosa-Sharpe (MRS) agar | Oxoid, Wesel, Germany |
| 15 mL Falcon tubes | Corning, Kaiserslautern, Germany |
| Amicon Ultra-2mL centrifugal filter units | Merck, Darmstadt, Germany |
| 1.5 mL Eppendorf tubes | Eppendorf, Hamburg, Germany |
| 96-well microtiter plates | Corning, Kaiserslautern, Germany |

### 3.1.3 Instruments

| | |
|---|---|
| 5% $CO_2$-incubator | Binder GmbH, Tuttlingen, Germany |
| Centrifuge 5430R | Eppendorf AG, Hamburg, Germany |
| Spectrophotometer Multiskan Go | Thermo Scientific, Schwerte, Germany |
| Biophotometer plus 6132 | Eppendorf AG, Hamburg, Germany |
| Vortex Genie 2 | Bender & Hobein AG, Munich, Germany |
| pH Meters | Thermo Scientific, Schwerte, Germany |

## 3.2 Methods

### 3.2.1 Culture conditions

Bacteria used in this study were precultured in the modified BHI medium (BHI broth containing 1% glucose, 4 g/L yeast extract, and 8 g/L beef extract) at 37°C under aerobic conditions and subsequently transferred to different Minimal Medium (MM) variations. The MM was a chemical-based saliva analogue [22] with certain modifications. The composition of MM is shown in Table 1.

Table 1 The composition of the minimal medium.

| Substance | Concentration (mM) |
|---|---|
| $KH_2PO_4$ | 10 |
| $K_2HPO_4$ | 10 |
| NaCl | 1 |
| KCl | 3 |
| $NH_4Cl$ | 0.2 |
| $MgCl_2*6H_2O$ | 0.2 |

### 3.2.2 Growth curve determination

*S. oligofermentans, L. reuteri,* and *S. mutans* were cultured aerobically in the modified BHI medium at 37°C in 15 mL Falcon tubes, respectively. During the subsequent 2, 4, 6, 8, 10, 12, 18, 24 and 48 h incubation, bacterial growth was detected by measuring $OD_{600}$ nm.

### 3.2.3 Determination of products concentration

After 18 h of pre-culture, *S. oligofermentans* and *L. reuteri* were pelleted by centrifugation at room temperature at 7100 × g for 15 min. The cells were washed twice with 0.9% NaCl and resuspended in 0.9% NaCl solution, inoculated with an average of $3.58×10^7$ CFU/mL (SD 0.24) for *S. oligofermentans* and $3.67×10^7$ CFU/mL (SD 0.34) for *L. reuteri* as 1 mL-cultures in 1.5 mL Eppendorf tubes, respectively. After another centrifugation (7100 × g, 15 min), the supernatants were removed and 1 mL MM variation media was pipetted into each tube.

MM supplementation was performed as follows to create six different MM variations (Table 2), which were used to evaluate the association between different environmental conditions and the probiotic metabolic activity.

Table 2 Six different MM variation media.

| MM variation media | Final pH |
|---|---|
| MM | 6.95 |
| MM with 5 mM glucose (MM+Glu) | 6.90 |
| MM with 300 mM glycerol (MM+Gly) | 6.92 |
| MM with 5 mM Lactic acid (MM+Lac) | 6.61 |
| MM with 5 mM arginine (MM+Arg) | 7.14 |
| MM with 5 mM glucose, 300 mM glycerol, 5 mM Lactic acid and 5 mM arginine (MM+all) | 6.83 |

To boost the lactate production, 5 mM glucose was additionally added to the groups of MM+Gly, MM+Lac, and MM+Arg during the cultivation of *S. mutans*.

*S. oligofermentans* was incubated aerobically in six different MM variations at 37°C for 15 min, 30 min, 2 h, 4 h, and 18 h, respectively. The supernatants were attained following centrifugation (20800 × g, 10 min) at room temperature. *L. reuteri* was incubated in six different MM variations for 2 h, 4 h, and 18 h, respectively, and the supernatants were collected in the same process. Protein was removed from the supernatants by centrifugation (7500 × g, 20 min) at room temperature using Amicon Ultra-2mL centrifugal filters with nominal molecular weight limit of 10 kDa. The deproteinized supernatants were stored at -80°C for further usage. Probiotic-free control samples were established using the modified BHI medium followed by six different MM variation media processed in the same manner as in the probiotic culture groups. The flowchart shows the process of determining metabolites concentration in probiotic supernatant after different cultivation periods (Fig. 1).
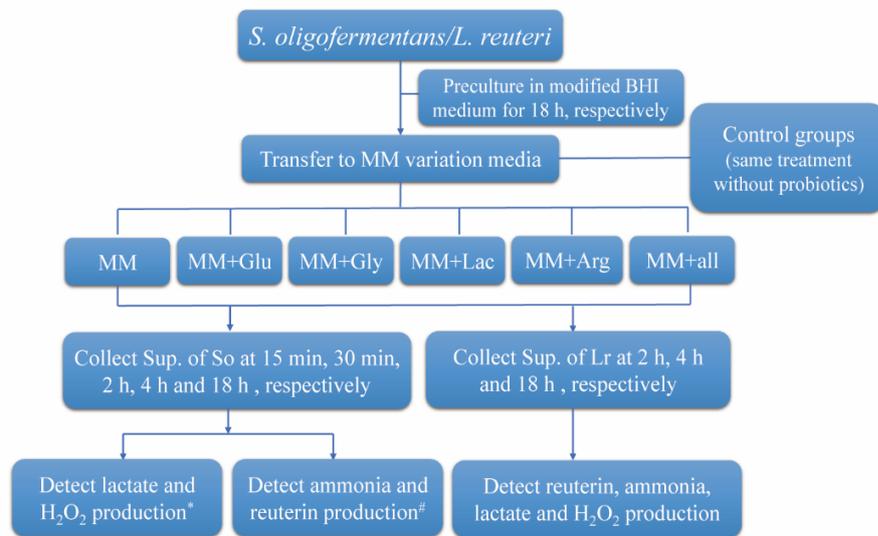
Fig. 1 Flowchart of determining the product concentration after different cultivation periods in the supernatants (Sup.) of *S. oligofermentans* (So) and *L. reuteri* (Lr)

[*] The samples of *S. oligofermentans* at 15 min, 30 min, 2 h, 4 h, and 18 h were used for measurement.

[#] The samples of *S. oligofermentans* at 2 h, 4 h, and 18 h were used for measurement.

### 3.2.4 Preparation of probiotic supernatants

The probiotics were precultured using the same method as described above, centrifugation was performed to pellet the cells. The cells were washed twice with 0.9% NaCl and resuspended with a total of 6 mL in 0.9% NaCl solution inoculated with an average of $3.96 \times 10^7$ CFU/mL (SD 0.37) for *S. oligofermentans* and $4.16 \times 10^7$ CFU/mL (SD 0.22) for *L. reuteri*, respectively. After another centrifugation ($7100 \times g$, 15 min), the supernatants were removed and 6 mL MM variation media were added to each tube.

Different incubation time was finally used to produce supernatants that were optimally enriched for these probiotic metabolites. Consequently, *S. oligofermentans* was cultivated under aerobic conditions at 37°C for 30 min in MM+Glu and MM+Lac, for 2 h in MM and MM+Gly, and 4 h in MM+Arg and MM+all. The supernatants were attained following centrifugation ($20800 \times g$, 10 min) at room temperature. *L. reuteri* was cultivated under the same conditions for 4 h in MM+Glu, MM+Gly, MM+Lac, and MM+all and 18 h in MM and MM+Arg. The supernatants were collected in the same process. The control samples without probiotics were established the same as above. Thereafter, the probiotic supernatants were collected and maintained as described above.

### 3.2.5 Preparation of *S. mutans* supernatants

*S. mutans* was precultured using the same method as described above, centrifugation was performed to collect the cells. The cells were rinsed twice with 0.9% NaCl and resuspended in 1 mL 0.9% NaCl solution in 1.5 mL Eppendorf tubes, with an average

inoculum of $4.35 \times 10^7$ CFU/mL (SD 0.39). After another centrifugation (7100 × g, 15 min), the supernatants were removed and 1 mL MM, MM+Glu, MM+Gly+Glu, MM+Lac+Glu, MM+Arg+Glu, and MM+all were added to each tube, respectively. *S. mutans* was further incubated for 18 h. Control samples without *S. mutans* were established using a modified BHI medium followed by six different MM variations media processed in the same manner as in the *S. mutans* culture groups, except that the same concentration of glucose as in MM+Glu and MM+all was added to MM+Gly, MM+Lac, and MM+Arg. Thereafter, the deproteinized supernatants of *S. mutans* were maintained as described above and the viable cells were calculated. The flowchart shows the process of collecting supernatants of *S. mutans* cultures and determining its survival in different MM variation media (Fig. 2).
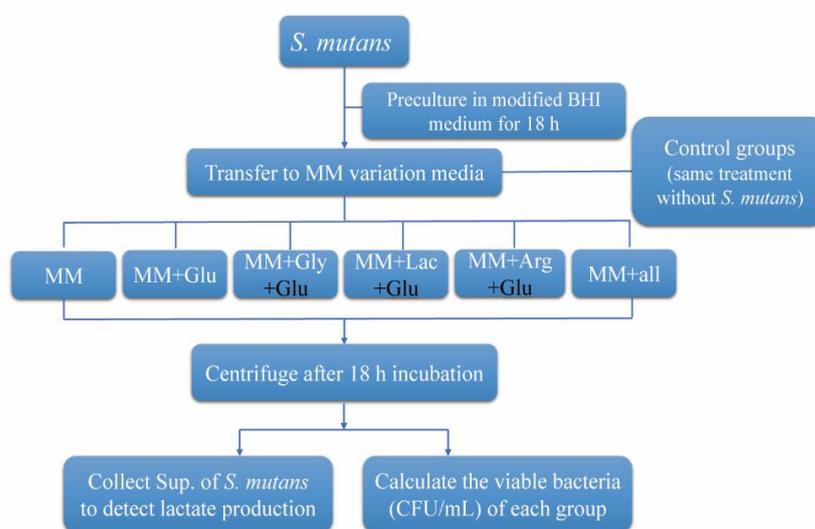


Fig. 2 Flowchart of collecting supernatants (Sup.) of *S. mutans* cultures

### 3.2.6 Cultivation of *S. mutans* with probiotic supernatants

*S. mutans* was precultured and treated using the same method as described above, with an average inoculum of $4.29 \times 10^7$ CFU/mL (SD 0.65). Centrifugation was performed to remove the supernatants and the probiotic supernatants (*S. oligofermentans* and *L. reuteri* cultivated in the six MM variation media as described) were added as 1 mL-cultures in 1.5 mL Eppendorf tubes respectively, with the addition of 5 mM glucose into MM+Gly, MM+Lac, and MM+Arg. *S. mutans* was further incubated for 18 h as before. The probiotic supernatant without *S. mutans* was used as control. The deproteinized supernatants of *S. mutans* were maintained as described above and the viable *S. mutans* cells were calculated. The flowchart shows the process of culturing *S. mutans* with probiotic supernatants (Fig. 3).
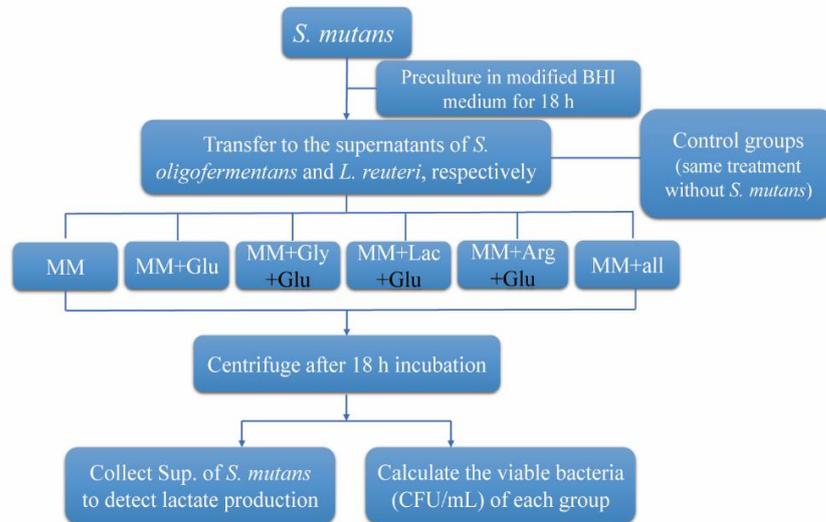
Fig. 3 Flowchart of culturing *S. mutans* with probiotic supernatants (Sup.)

### 3.2.7 Metabolite assays

Colorimetric assay kits were used to determine the metabolite concentration of $H_2O_2$, ammonia, and lactate in the probiotic supernatants according to the protocols of the manufacturer. The determination of reuterin in probiotic supernatants was measured based on the assay method described elsewhere [23] with some modifications. 250 μL of deproteinized samples were pipetted into 187.5 μL tryptophan, which was dissolved in 0.05 M HCl to a final concentration of 10 mM, followed by the addition of 750 μL of 37% HCl. After a 20 min-incubation at 37°C, the absorbance was detected at 560 nm. Acrolein was used as the calibration standard. A dilution series of 0, 2.5, 5, 7.5, 10, 12.5, and 15 mM of acrolein was used to establish a standard curve. Absorbance measurements were performed in the 96 well plate spectrophotometer.

### 3.2.8 Viable bacteria enumeration

After centrifugation, the culture supernatants were removed and the bacteria cells resuspended in 1 mL of 0.9% sodium chloride per tube by dispersing the cells with the assistance of a vortex mixer. A series of dilutions of bacterial suspensions were prepared and viable bacteria cells were calculated by splitting 100 μL aliquots of 1 mL serial dilutions on blood agar plates COLS+ for *S. oligofermentans* and *S. mutans* or on MRS agar plates for *L. reuteri.* After 24-48 h of incubation under aerobic conditions at 37°C, colony forming units per milliliter (CFU/mL) were calculated.

### 3.2.9 Statistical analysis

For descriptive analysis, mean (SD) were used. To test for statistically significant differences, one-way ANOVA followed by Dunnett's test was applied. SPSS Version 20.0 software was used for all analyses. *P* values <0.05 were considered statistically significant.

# 4. Results

## 4.1 Bacterial growth curve

When *S. oligofermentans*, *L. reuteri,* and *S. mutans* were cultured in the modified BHI medium, their growth curve was determined. For *S. oligofermentans*, the lag phase was short, less than 2 h and its growth entered the stationary phase after about 18 h (Fig. 4).
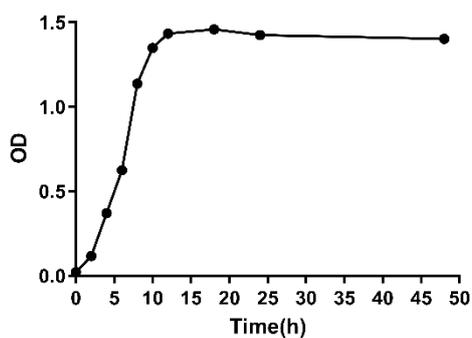


Fig. 4 The growth curve of *S. oligofermentans*

The growth of *S. mutans* was similar to that of *S. oligofermentans*, with a short lag phase of less than 2 h, and its growth reached a plateau after about 18 h (Fig. 5).
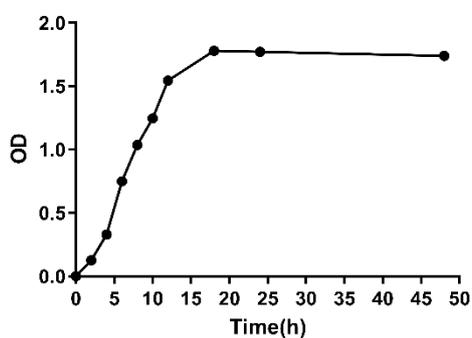


Fig. 5 The growth curve of *S. mutans*

The lag phase of *L. reuteri* was about 2 h; after about 24 h, the growth entered the stationary phase (Fig. 6). Based on the above results, 18 h was selected as the pre-culture time of the three bacteria in modified BHI medium, because *L. reuteri* was at the end of the exponential phase and the growth of *S. oligofermentans* and *S. mutans* just entered the stationary phase.

16
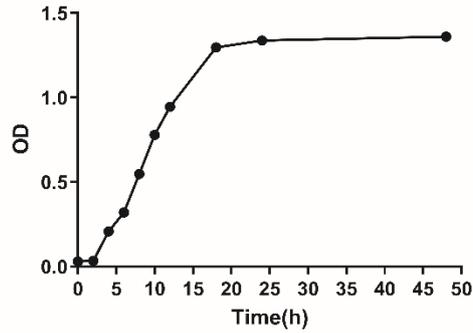
Fig. 6 The growth curve of *L. reuteri*

## 4.2 Metabolite contents in the supernatant of *S. oligofermentans* after different cultivation periods

At 2, 4, 18 h, *S. oligofermentans* produced almost no reuterin in each group and there was no obvious change with time (Fig. 7A). Ammonia was generated in MM+Arg and MM+all. At 2 h, it was mainly found in MM+Arg. From 2 h to 4 h, the ammonia concentration increased significantly; from 4 h to 18 h, the growth rate slowed. In MM+all, the ammonia concentration also increased significantly from 2h to 4h and then began to level off (Fig. 7B). Lactate was detectable mainly in MM+Glu and MM+all. In MM+Glu, the lactate production gradually increased from 15 min to 18 h. In MM+all, from 15 min to 30 min, the lactate concentration increased slowly, followed by a significant increase until 4 h, and then gradually decreased (Fig. 7C). $H_2O_2$ was generated in all groups. In MM+Lac and MM+Glu, the $H_2O_2$ concentration increased from 15 min to 30 min, then gradually decreased. For other groups, the $H_2O_2$ concentration increased from 15 min to 2 h and then decreased gradually (Fig. 7D). Therefore, to optimize the product harvest, *S. oligofermentans* was cultivated in MM+Glu and MM+Lac for 30 min, in MM and MM+Gly for 2 h, and in MM+Arg and MM+all for 4 h.
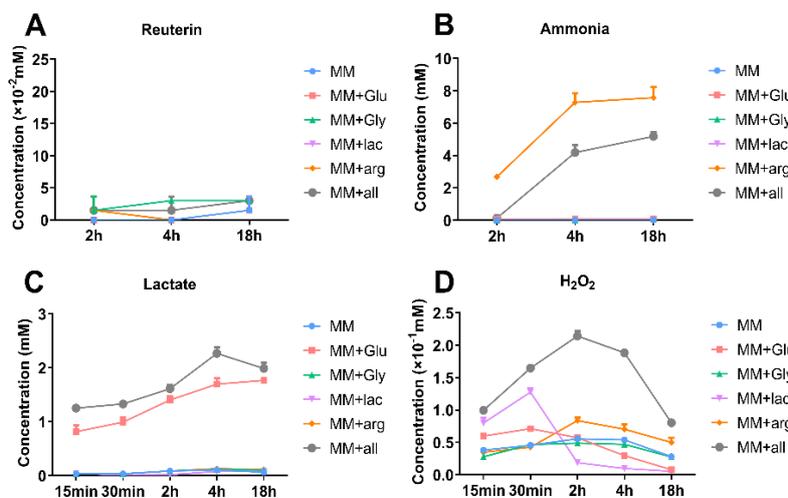


Fig. 7 Metabolite contents of *S. oligofermentans* in six MM variations after different cultivation periods (A) reuterin (B) ammonia (C) lactate (D) $H_2O_2$.

## 4.3 Metabolite contents in the supernatant of *L. reuteri* after different cultivation periods

Reuterin was produced in MM+Gly and MM+all, and its concentration gradually increased from 2h to 18h (Fig. 8A). Ammonia was only found in MM+Arg, and its concentration increased slowly from 2h to 4h. After 4h, the concentration increased significantly (Fig. 8B). Lactate was produced in MM+Glu and MM+all. Its concentration gradually increased from 2 h to 18 h (Fig. 8C). $H_2O_2$ was generated in all groups. From 2 h to 18 h, the concentration of each group gradually increased (Fig. 8D). As a consequence, *L. reuteri* was cultivated in MM+Glu, MM+Gly, MM+Lac, and MM+all for 4 h and MM and MM+Arg for 18 h.
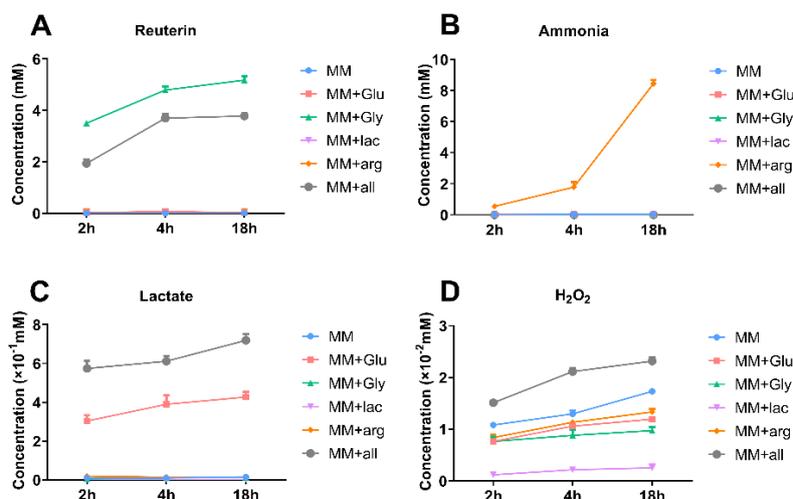


Fig. 8 Metabolite contents of *L. reuteri* in six MM variations after different cultivation periods (A) reuterin (B) ammonia (C) lactate (D) $H_2O_2$.

## 4.4 Metabolite contents of *S. oligofermentans* in six MM variations

The main antimicrobial substances produced by *S. oligofermentans* include ammonia and $H_2O_2$. Very minimal reuterin was found in MM+Gly and MM+all (Fig. 9A). Ammonia was found in both MM+Arg and MM+all, and the concentration in MM+Arg was higher than MM+all (Fig. 9B). Lactate was produced in MM+Glu and MM+all (Fig. 9C). $H_2O_2$ was detected in all groups, the maximum yield being measured in MM+all (Fig. 9D). The pH of the supernatants after the cultivation of *S. oligofermentans* is shown in Table 3.
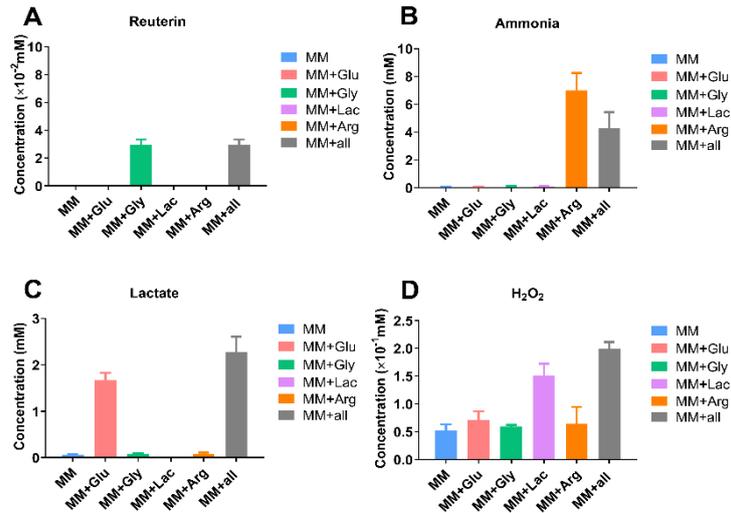
Fig. 9 Metabolite contents of *S. oligofermentans* in six MM variations.

Data are shown as mean (SD). Three independent biological replicates were performed. (A) Very minimal reuterin was found in *S. oligofermentans*. (B) Ammonia was generated in MM+Arg and MM+all. (C) Lactate was produced mainly in MM+Glu and MM+all. (D) $H_2O_2$ was found in all groups.

Table 3 The pH value of the supernatants after the cultivation of *S. oligofermentans*.

| MM variation media | Final pH |
| --- | --- |
| MM | 6.80 |
| MM+Glu | 6.70 |
| MM+Gly | 6.85 |
| MM+Lac | 6.40 |
| MM+Arg | 7.10 |
| MM+all | 6.70 |

## 4.5 Metabolite contents of *L. reuteri* in six MM variations

The main antimicrobial substances produced by *L. reuteri* include reuterin, ammonia, and $H_2O_2$. Reuterin was produced in MM+Gly and MM+all, and the concentration in MM+Gly was higher than MM+all (Fig. 10A). Ammonia was only found in MM+Arg (Fig. 10B). Lactate was produced in MM+Glu and MM+all (Fig. 10C). $H_2O_2$ was generated in all groups and the maximum yield was obtained in MM+all (Fig. 10D). The pH value of the supernatants after the cultivation of *L. reuteri* is shown in Table 4.
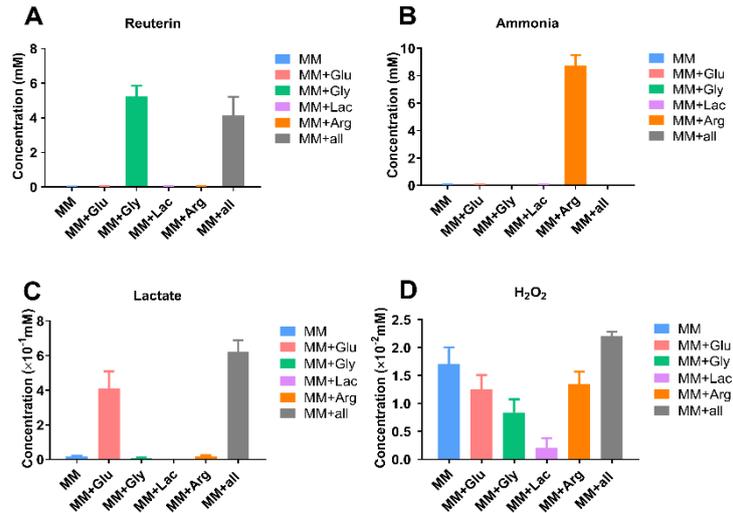
Fig.10 Metabolite contents of *L. reuteri* in six MM variations.

The figure displays the mean (SD). Three independent biological replicates were performed. (A) Reuterin was produced in MM+Gly and MM+all. (B) Ammonia was only found in MM+Arg. (C) Lactate was produced mainly in MM+Glu and MM+all. (D) $H_2O_2$ was generated in all groups.

Table 4 The pH value of the supernatants after the cultivation of *L. reuteri*.

| MM variation media | Final pH |
|---|---|
| MM | 6.85 |
| MM+Glu | 6.80 |
| MM+Gly | 6.80 |
| MM+Lac | 6.40 |
| MM+Arg | 7.20 |
| MM+all | 6.60 |

## 4.6 Inhibition of lactate production on *S. mutans* by probiotic supernatants

In the control MM without glucose, the lactate production of *S. mutans* was lowest, while in all control MM with glucose, the lactate production was significantly higher. The lactate production of *S. mutans* cultivated in the supernatant of *S. oligofermentans* was significantly decreased regardless of the MM (Table 5). In detail, there was ingestion of extracellular lactate from the culture medium by *S.mutans* cells in the MM+all and MM+Lac+Glu, respectively ($p<0.001$). The lactate production of *S. mutans* cultivated in the supernatant of *L. reuteri* also significantly decreased in all MM media except MM+Lac+Glu (Table 5).

Table 5. "Changes in lactate concentration (mM) of *S. mutans* (Sm) after cultivation in the supernatant (Sup.) of *S. oligofermentans* (So) and *L. reuteri* (Lr)" [1].

| Metabolite (mM) | Minimal Medium | Bacterial strain | | |
|---|---|---|---|---|
| | | Sm | Sm Sup. So[A] | Sm Sup. Lr[B] |
| Lactate | MM | $1.78 \times 10^{-2}$ (0.65) | $-5.14 \times 10^{-2}$ (0.50)*** | $-1.13 \times 10^{-2}$ (1.08)** |
| | MM+Glu | 6.52 (0.91) | 3.84 (0.40)** | 2.95 (0.31)** |
| | MM+Gly+Glu | 5.00 (0.42) | 3.01 (0.49)** | $3.65 \times 10^{-1}$ (0.33)*** |
| | MM+Lac+Glu | 4.46 (0.41) | -1.39 (0.25)*** | 4.42 (0.19) |
| | MM+Arg+Glu | 6.03 (0.48) | 1.69 (0.43)*** | 3.29 (0.26)*** |
| | MM+all | 6.00 (0.29) | -2.14 (0.35)*** | -0.46 (0.11)*** |

For the cultivation of *S. mutans*, 5mM glucose was added to MM+Gly, MM+Lac, and MM+Arg as well as the probiotic supernatants of these three groups. The lactate concentration of *S. mutans* cultured in probiotic supernatants (test groups) and that in probiotic supernatants without *S. mutans* (control groups) were analyzed. The lactate production of *S. mutans* cultivated in probiotic supernatants was calculated by subtracting the lactate yield in control groups from the corresponding test groups. Positive values indicate the formation of additional lactate, negative values indicate bacterial uptake of lactate. Data are shown as mean (SD). Each group of data was repeated three times. Dunnett's test: **$P < 0.01$, ***$P < 0.001$, versus Sm.

## 4.7 Inhibition of *S. mutans* survival by probiotic supernatants

In general, being cultured in the probiotic supernatants decreased the survival of *S. mutans* compared to the control groups (Fig. 11). In detail, incubation with the supernatant of *S. oligofermentans* yielded a 0.6-$\log_{10}$ reduction and a 0.5-$\log_{10}$ reduction in the viability of *S. mutans* in MM+Lac+Glu and MM+all, respectively. Similarly, a 6.1-$\log_{10}$ and 7.1-$\log_{10}$ reduction of *S. mutans* viability was found under the incubation of *L. reuteri* supernatant in MM+Gly+Glu and MM+all, respectively.
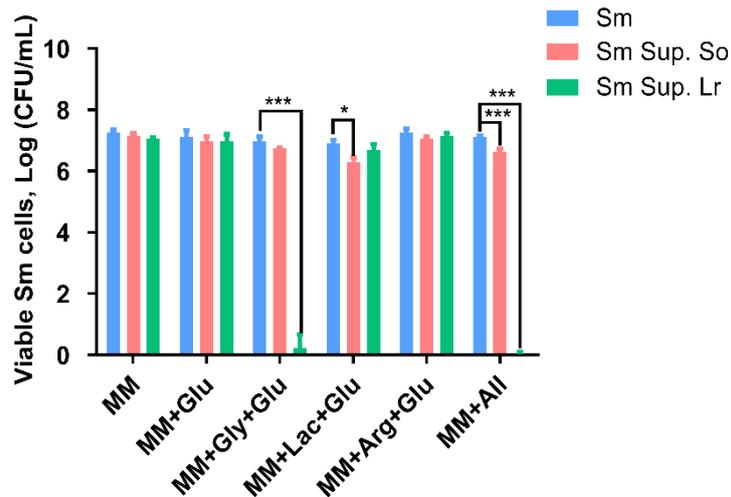
Fig. 11 Changes in CFU (Log CFU/mL) of *S. mutans* after being cultivated in the supernatant (Sup.) of *L. reuteri* (Lr) and *S. oligofermentans* (So).

*S. mutans* was cultivated in MM, MM+Glu, MM+Gly+Glu, MM+Lac+Glu, MM+Arg+Glu, and MM+all (control groups), as well as probiotic supernatants. 5 mM Glucose was added to MM+Gly, MM+Lac, and MM+Arg of *L. reuteri* and *S. oligofermentans* supernatants. The figure displays the mean (SD). Three separate experiments were performed. Dunnett's test: *$P < 0.05$, ***$P < 0.001$, versus controls.

## 5. Discussion

Understanding the mechanisms by which probiotics exert their inhibitory effects on *S. mutans* is crucial to the development of probiotics as clinical therapeutic alternatives for the prevention of dental caries. In this study, we established the important role of different culture conditions and found that $H_2O_2$ and reuterin have inhibitory effects on the lactate production and survival of *S. mutans*. This finding provides new therapeutic avenues for the inhibition of cariogenic pathogens with supernatants or specifically isolated compounds, which may be exploited for oral administration of probiotics.

### 5.1 $H_2O_2$ production in the probiotic supernatants

The oral cavity is a complex microecosystem, in which $H_2O_2$ is an important non-substitute antibacterial substance. $H_2O_2$ can be produced by lactobacillus and streptococcus colonized in oral biofilms, or it is derived from the host. $H_2O_2$ plays an important role in regulating microecological balance and the formation of biofilms. It is widely used as a biological weapon by lactic acid bacteria. It participates in the antagonistic competition of microorganisms by inhibiting the microbes which are sensitive to it, thereby helping those bacteria that are more resistant to $H_2O_2$ to gain a competitive advantage [24].

The antibacterial action of *S. oligofermentans* mainly depends on the production of $H_2O_2$. To shed new light on understanding the effects of different culture conditions on

the $H_2O_2$ production by *S. oligofermentans*, an *in vitro* model was employed in the present study. *S. oligofermentans* was cultured aerobically in different MM variations and its $H_2O_2$ production was measured after different cultivation periods. We found that within 30 min of cultivation, the $H_2O_2$ production in the MM+Glu, MM+Lac, and MM+all was much higher than that of the other groups. It was shown that the initial yield of $H_2O_2$ production was higher in the presence of sucrose or glucose than in the absence of carbohydrates. However, exogenous carbohydrates not only increased the initial rate of $H_2O_2$ production but also accelerated the metabolism of $H_2O_2$. This in turn resulted in no significant increase in the final $H_2O_2$ production compared to the sugar-free group [25]. These results are in line with our findings that after 30 min of incubation, the $H_2O_2$ production in MM+Glu gradually decreased.

*S. oligofermentans* employs three $H_2O_2$-forming enzymes: pyruvate oxidase (Pox), lactate oxidase (Lox), and L-amino acid oxidase [26]. It was found that $H_2O_2$ yields were most pronounced in MM+Lac and MM+all. Both sets of MM contained lactic acid, which could be used for Lox, indicating that Lox is at work here. Glucose can be converted into pyruvate and lactic acid by *S. oligofermentans*, thus acting as substrates for Pox and Lox. Compared with MM+Lac, the additional glucose contained in MM+all may further increase Pox and Lox activity. The synergetic effect of Pox and Lox maximizes the $H_2O_2$ production of *S. oligofermentans* [27].

The $H_2O_2$ yield produced by *S. oligofermentans* is also affected by different culture pH values. Studies demonstrated that the suitable pH value for the $H_2O_2$ production generated by *S. oligofermentans* was around a neutral pH and that with extreme acidic or alkalic conditions, the production ceases [28]. In this study, after 2h cultivation, in the sugar-free MM, MM+Gly, MM+Lac, and MM+Arg, the pH value of MM+Arg was closest to 7. We assume that this might be the reason for its higher $H_2O_2$ production compared with the other three groups.

*L. reuteri* also has the ability to produce $H_2O_2$ [29]. When grown under aerobic conditions, lactic acid bacteria typically accumulate $H_2O_2$ as a product of NADH oxidase and NADH peroxidase [30]. Another contributing factor for the $H_2O_2$ generation by lactobacillus has been proposed to be Pox, which oxidizes pyruvate through acetyl phosphate to acetate, producing $CO_2$, $H_2O_2$, ATP, and NADH [31]. Our study showed that from 2 h to 18 h, the $H_2O_2$ yield generated by *L. reuteri* in each group gradually increased, but which enzymatic pathway is involved in the production of $H_2O_2$ is currently unknown. Lactate dehydrogenase plays a dominant role in pyruvate metabolism during aerobic growth of *L. reuteri* and competes with Pox for the pyruvate pool [32]. Moreover, it has been shown that oxygen or $H_2O_2$ can induce Pox activity, while glucose can inhibit Pox activity [33]. In our study, the presence of glucose in the MM+Glu and MM+all does not seem to inhibit $H_2O_2$ production. Therefore, it is assumed that NADH oxidase plays a more important role in $H_2O_2$ production than Pox.

## 5.2 Reuterin production in the probiotic supernatants

Certain strains of *L. reuteri* have the capacity to produce reuterin. Although some strains of *L. reuteri* can also produce high-molecular-weight bacteriocin, reutericin or its low-molecular-weight compound, reutericyclin, their narrow and restricted antimicrobial activity make them unlikely to be prime candidates for synergistic antimicrobial compounds [34]. Reuterin is resistant to proteolytic and lipolytic enzymes and could retain its antibiotic activity at low pH and high NaCl concentration [35]. It is known to exist in three forms, monomeric, hydrated monomeric, and dimeric forms of 3-hydroxypropionaldehyde (3-HPA). In the glycerol-containing MM variations, *L. reuteri* catalyzes glycerol to produce 3-HPA by a $B_{12}$-dependent glycerol dehydratase [36].

From 2 h to 4 h, the production of reuterin increased rapidly and gradually stabilized after 4 h. In MM+Gly, reuterin was produced at about 5 mM while in MM+all, it was produced at about 4 mM. Although the yield of reuterin differed between these two groups, there was no difference in its inhibitory effect on lactate production and survival of *S. mutans*. The reduced production of reuterin in MM+all may be due to the presence of glucose. Since the MM solution could only provide ions to maintain bacterial survival, the bacteria only carried out limited glycolysis in it. Therefore, the addition of glucose in MM+all resulted in a further reduction of 3-HPA to 1,3-propanediol by NADH produced from limited glycolysis [36]. We found that very little reuterin was detected in *S. oligofermentans* supernatants. It is assumed that this low concentration of reuterin may originate from other compounds, which needs further experimental verification.

## 5.3 Ammonia production in the probiotic supernatants

Arginine is the main amino acid used by oral microorganisms to produce alkali [37]. Arginine degradation through the arginine deiminase pathway (ADS) generates ATP and ammonia, thereby counteracting the effect of bacterial glycolysis on biofilm acidification. Ammonia leads to a pH increase in the cytoplasm and the environment, and can be used by oral microbes for (1) prevention from acid killing, (2) bioenergy advantages, including the rise in ΔpH and ATP synthesis, (3) retaining a relatively neutral environmental pH which is conducive to enhancing the competitiveness of ADS-positive (ADS$^+$) bacteria against cariogenic bacteria [38]. In this study, for *S. oligofermentans,* ammonia was generated in MM+Arg and MM+all and its production increased rapidly from 2 to 4 h in both groups and leveled off after 4 h. Moreover, we found more ammonia was produced in MM+Arg than in MM+all, presumably due to the glucose contained in MM+all. Previously, studies have shown the relationship between arginine degradation and sugar metabolism in some lactic bacteria like streptococcus, and repression by glucose was observed [37]. For *L. reuteri*, ammonia was only detected in MM+Arg. The possible reason for the absence of ammonia in MM+all is that ammonia might react with the aldehyde group of reuterin in the medium [39].

### 5.4 Lactate production in the probiotic supernatants

Both *S. oligofermentans* and *L. reuteri* metabolize glucose to produce lactic acid, however, much less compared than *S. mutans*. Lactate production was detected in MM+Glu and MM+all, while the content of lactate in other groups was very low. In general, the amount of lactate produced by *S. oligofermentans* and *L. reuteri* increased with the extension of cultivation time. While they produced lactic acid, the production of other antimicrobial substances may inhibit the growth of *S. mutans*.

### 5.5 Impact on lactate generation and survival of *S. mutans*

The lactate production of *S. mutans* cultured in the supernatants of *S. oligofermentans* was significantly reduced in each MM variation group. *S. oligofermentans* produced large amounts of $H_2O_2$ in lactate-rich media, MM+Lac, and MM+all, and *S. mutans* survival was significantly reduced only in these two groups. Thus, $H_2O_2$ is likely to be the main reason for the inhibitory effect on the survival and lactate yield of *S. mutans*.

*S. mutans* cultured in *L. reuteri* supernatants also had significantly lower lactate production in all groups except MM+Lac+Glu. We assume the possible reason is that the amount of $H_2O_2$ in MM+Lac+Glu was so small that the effective concentration cannot be reached. By neutralizing the lactic acid in the solution, which changes the pH to a less cariogenic environment, ammonia produced by *S. oligofermentans* in MM+Arg and MM+all and *L. reuteri* in MM+Arg had an only moderate influence on the lactate production of *S. mutans*. After incubation in the *L. reuteri* supernatant containing reuterin, almost no *S. mutans* were detectable, suggesting that reuterin, a powerful bacterial inhibitor, has potentially more marked inhibitory influence on *S. mutans*.

### 5.6 Clinical significance

Certain probiotic strains used in dietary products have been investigated in clinical trials to prevent dental caries. However, the common usage of viable probiotics may also bring some problems, shelf life and safety among them. Also, some strains of probiotic lactobacillus are known to metabolize sucrose, possibly causing caries themselves [40]. Thus, there is growing interest in new-era products, like probiotic supernatants and purified key compounds to exert beneficial effects. They have an advantage over live probiotics in terms of their safety profile and might play an important role in replacing viable probiotics. Our findings provide a theoretical basis for understanding the metabolic activity of non-viable probiotics generated under different environmental conditions and identify key products that may play a role in inhibiting the lactate production and survival of *S. mutans*.

### 5.7 Future directions

The findings of our study have relevance for further research in the field, which should evaluate the ideal concentration and treatment time of each probiotic supernatant based on its efficacy in the control of specific cariogenic bacteria as well as the environment-specific probiotic effects on cariogenic pathogens. Future studies are required to determine the best vehicle, most effective probiotic bacteria, and their products. In

addition, clinical trials should also be conducted to explore the interaction and potential mechanisms of action between probiotic supernatants and cariogenic bacteria in the oral environment. Combining the anti-caries properties of different probiotics may play a more effective role in the probiotic agent development process.

**5.8 Conclusions**

The application of probiotics in the oral cavity to prevent dental caries has great potential for wide use in practice. In this study, we cultured *S. oligofermentans* and *L. reuteri* under different environmental conditions and *S. mutans* was cultivated in their supernatants only. Hence, we can draw the following conclusions:

1. *S. oligofermentans* is more likely to produce $H_2O_2$ in a lactate-rich environment, and *L. reuteri* produced reuterin in glycerol containing medium. Ammonia was generated by *S. oligofermentans* in arginine containing medium and was detected only in MM+Arg of *L. reuteri*. Both *S. oligofermentans* and *L. reuteri* metabolize glucose to produce lactic acid.

2. The $H_2O_2$ and ammonia generated by *S. oligofermentans* may have an inhibitory effect on the lactate production of *S. mutans*. The $H_2O_2$ and reuterin produced by *L. reuteri* may have an inhibitory effect on the lactate production of *S. mutans*, while the amount of $H_2O_2$ in MM+Lac+Glu was too small to reach the effective concentration.

3. *S. mutans* survival was significantly reduced only in lactate-rich media, which may be due to the high production of $H_2O_2$. After incubation in the *L. reuteri* supernatant containing reuterin, almost no *S. mutans* were detectable, suggesting that reuterin has potentially more marked inhibitory influence on *S. mutans.*

Thus, the application of environmentally specific probiotic supernatants to prevent dental caries is expected to have a good application prospect.

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# Eidesstattliche Versicherung

„Ich, Haiyue Yu, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: [Effect of probiotic supernatants on the metabolic activity and survival of *Streptococcus mutans in vitro*] [Wirkung von probiotischen Überständen auf die Stoffwechselaktivität und das Überleben von *Streptococcus mutans in vitro*] selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Datum                                    Unterschrift

## Anteilserklärung an den erfolgten Publikationen

Publikation

Autoren: **Haiyue Yu**, Petra Ganas, Falk Schwendicke

Titel: Environment-specific probiotic supernatants modify the metabolic activity and survival of *Streptococcus mutans in vitro*

Fachzeitschrift: Frontiers in Microbiology, 11**:** 1447, 2020

Beitrag im Einzelnen:

Haiyue Yu conceived and designed the study in collaboration with Petra Ganas and Prof. Falk Schwendicke. All the experiments were performed by her. She conducted the data and statistical analysis and prepared all the tables and figures. The manuscript was written by her and revised by all co-authors. The submission of the manuscript to the journal with the consent of the co-authors was done by her. Haiyue Yu conducted the proofreading and revised the manuscript according to the requirements of the reviewers, with the support of the co-authors.

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Unterschrift, Datum und Stempel des/der erstbetreuenden Hochschullehrers/in

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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 |

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| 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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# Environment-Specific Probiotic Supernatants Modify the Metabolic Activity and Survival of *Streptococcus mutans in vitro*

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A range of studies showed probiotics like *Streptococcus oligofermentans* and *Limosilactobacillus reuteri* to inhibit the cariogenic activity and survival of *Streptococcus mutans*, possibly via the production of substances like $H_2O_2$, reuterin, ammonia and organic acids. We aimed to assess the environment-specific mechanisms underlying this inhibition. We cultured *L. reuteri* and *S. oligofermentans* in various environments; minimal medium (MM), MM containing glucose (MM+Glu), glycerol (MM+Gly), lactic acid (MM+Lac), arginine (MM+Arg) and all four substances (MM+all) *in vitro*. Culture supernatants were obtained and metabolite concentrations (reuterin, ammonia, $H_2O_2$, lactate) measured. *S. mutans* was similarly cultivated in the above six different MM variation media, with glucose being additionally added to the MM+Gly, MM+Lac, and MM+Arg group, with (test groups) and without (control groups) the addition of the supernatants of the described probiotic cultures. Lactate production by *S. mutans* was measured and its survival (as colony-forming-units/mL) assessed. *L. reuteri* environment-specifically produced reuterin, $H_2O_2$, ammonia and lactate, as did *S. oligofermentans*. When cultured in *S. oligofermentans* supernatants, lactate production by *S. mutans* was significantly reduced ($p < 0.01$), especially in MM+Lac+Glu and MM+all, with no detectable lactate production at all (controls means ± SD: 4.46 ± 0.41 mM and 6.00 ± 0.29 mM, respectively, $p < 0.001$). A similar reduction in lactate production was found when *S. mutans* was cultured in *L. reuteri* supernatants ($p < 0.05$) for all groups except MM+Lac+Glu. Survival of *S. mutans* cultured in *S. oligofermentans* supernatants in MM+Lac+Glu and MM+all was significantly reduced by 0.6-$\log_{10}$ and 0.5-$\log_{10}$, respectively. Treatment with the supernatant of *L. reuteri* resulted in a reduction in the viability of *S. mutans* in MM+Gly+Glu and MM+all by 6.1-$\log_{10}$ and 7.1-$\log_{10}$, respectively. Probiotic effects on the metabolic activity and survival of *S. mutans* were environment-specific through different pathways.

**Keywords: caries, colony forming units, dental, lactate production, metabolites, probiotics**

# INTRODUCTION

The human oral cavity harbors more than 700 microbial species, which constitute a dynamic microbial community (Aas et al., 2005). The coexistence and competition between different species are central to the oral microbial homeostasis (Bao et al., 2015). A disturbance in this homeostasis, termed dysbiosis, is associated with dental diseases like dental caries or periodontitis (Exterkate et al., 2010). For dental caries, the dominance of acidogenic and aciduric species like *Streptococcus mutans*, triggered by the abundant intake of fermentable carbohydrates, is associated with a net mineral loss from dental hard tissues and the formation of a caries lesion (Marsh, 2006).

Contemporary caries management aims to rebuild a healthy microbial equilibrium within the dental biofilm (Marsh, 2006; Bao et al., 2015). One strategy supposedly supporting such rebalancing of the biofilm is the application of probiotics. Probiotics are microorganisms, mainly bacteria, that when administered in sufficient amounts, provide health benefits to the host (Ng et al., 2009; Bosch et al., 2012), for example by inhibiting the metabolic activity and survival of harmful microbiota as well as modulating the host's immune response, thereby helping to stabilize the local microecosystem (Meurman, 2005). Probiotics have been tested both *in vitro* and in clinical studies for their anti-caries effect, with mixed results (Jalasvuori et al., 2012; Gruner et al., 2016).

Certain probiotics have been tested more widely. *Streptococcus oligofermentans*, a synonym of *Streptococcus cristatus* (Jensen et al., 2016) was isolated from healthy tooth surfaces (Tong, 2003) and has anti-bacterial effects against pathogens like *S. mutans* (Liu et al., 2014). It produces hydrogen peroxide ($H_2O_2$) from lactic acid (Tong et al., 2007, 2008) as well as ammonia from arginine, which may both reduce the amount of free lactic acid, thereby increasing the local pH and preventing the initiation of a caries lesion or slowing down or stopping lesion progression (Burne and Marquis, 2000; Clancy et al., 2000). *Limosilactobacillus reuteri* (Zheng et al., 2020) is an obligate heterofermentative probiotic and most strains in its human lineages have the ability to excrete reuterin (Mu et al., 2018), a potent antibiotic substance, which exhibits broad-spectrum antimicrobial effect on Gram-positive and Gram-negative bacteria (Talarico and Dobrogosz, 1989; Doleyres et al., 2005). In addition to reuterin, *L. reuteri* also produces $H_2O_2$, organic acid (Kang et al., 2011) and ammonia (Mu et al., 2018; Zaura and Twetman, 2019), with possible impact on *S. mutans* metabolic activity and survival. Furthermore, some strains of *L. reuteri* generate a unique antagonistic activity, reutericyclin, which shows a broad inhibitory spectrum but has no effect on the growth of gram-negative bacteria (Ganzle et al., 2000; Lin et al., 2015).

The antibacterial effect of these probiotics hence relies, at least in parts, on the production of the described substances. This production, in turn, is likely to be dependent on the environmental conditions, especially the availability of certain educts required to produce reuterin, $H_2O_2$ etc., So far, it was not studied if different environments modify the probiotic effects on cariogenic pathogens like *S. mutans*. Deeper knowledge on such

environmental requirements is needed both for future research (setting up appropriate models considering these requirements) and for clinical applications. For example, it may be feasible to boost the probiotic anti-caries effect by supplementing probiotic products with certain substances required for a specific probiotic activity.

We aimed to assess the environment-specific activity and impact of two different probiotics, *S. oligofermentans,* and *L. reuteri,* on the metabolic activity and survival of *S. mutans*. We hypothesized that the metabolic activity and survival of *S. mutans* is significantly reduced when exposed to probiotic supernatants, and that this effect is environment-specific.

# MATERIALS AND METHODS

This study used an established *in vitro* model (Ganas and Schwendicke, 2019) to assess the environment-specific impact of probiotics on metabolic activity and survival of *S. mutans*. Different environmental conditions were simulated by using determined modifications of a saliva analog, allowing to deterministically vary the metabolic activity of the two different probiotics, *S. oligofermentans,* and *L. reuteri*. The supernatants resulting from the cultivation of probiotics in different environments were then used to assess their impact on *S. mutans* metabolic activity, measured via determining the lactate production, and survival, measured via the colony-forming-units/mL of *S. mutans*. Controls of *S. mutans* cultured in different environments, but without probiotic supernatant, were additionally used. All assays and tests were performed in three biological replications, each with two technical replications (measurements) whose average was used for statistical analysis.

## Bacterial Strains and Growth Conditions

Three bacterial strains *S. mutans,* DSM 20523, *S. oligofermentans,* DSM 8249 (DMSZ, Braunschweig, Germany) and *L. reuteri,* ATCC PTA 5289 (BioGaia, Stockholm, Sweden) were used. The strains *S. mutans* and *S. oligofermentans* were grown on blood agar plates COLS+ (Oxoid, Wesel, Germany) while the strain *L. reuteri* was maintained on deMan-Rogosa-Sharpe (MRS) agar (Oxoid) at 37°C aerobically for 1–2 days.

## Preparation of Deproteinized Supernatants From Probiotics Cultures

The two probiotics were precultured separately in brain-heart-infusion (BHI) broth (Carl Roth, Karlsruhe, Germany) supplemented with 1% glucose (Carl Roth), 4 g/L yeast extract and 8 g/L beef extract (Carl Roth) for 18 h under aerobic conditions at 37°C in 15 ml Falcon tubes (Corning, Kaiserslautern, Germany). After centrifugation at 7100 *g* for 15 min at room temperature, the supernatants were removed and the cells were transferred to 0.9% sodium chloride with an inoculum (means ± SD) of $3.96 \pm 0.37 \times 10^7$ cells/mL for *S. oligofermentans* and $4.16 \pm 0.22 \times 10^7$ cells/mL for *L. reuteri,* with a total of 6 ml-cultures in 15 ml Falcon tubes (Corning), respectively. After another centrifugation at 7100 *g*

for 15 min, the supernatants were discarded and 6 mL Minimal Medium (MM) was added.

The minimal medium was based on a chemically defined saliva analog (Wong and Sissions, 2001) and consisted of 10 mM $KH_2PO4$ (Merck, Darmstadt, Germany), 10 mM $K_2HPO4$ (Carl Roth), 1 mM NaCl (Carl Roth), 3 mM KCl (Merck), 0.2 mM $NH_4Cl$ (Merck), and 0.2 mM $MgCl_2 \times 6H_2O$ (Merck). The MM was supplemented as follows to generate six different MM variations, allowing to assess the relevance of the metabolic environment on the probiotic activity and its association with the inhibition of *S. mutans* survival and activity: (1) MM, (2) MM with 5 mM glucose (Carl Roth) (MM+Glu), (3) MM with 300 mM glycerol (Sigma-Aldrich, Taufkirchen, Germany) (MM+Gly), (4) MM with 5 mM Lactic acid (Carl Roth) (MM+Lac), (5) MM with 5 mM arginine (Sigma-Aldrich) (MM+Arg) and (6) MM with 5 mM glucose, 300 mM glycerol, 5 mM Lactic acid and 5 mM arginine (MM+all). The final pH of the six MM variations was MM 6.95, MM+Glu 6.90, MM+Gly 6.92, MM+Lac 6.61, MM+Arg 7.14, MM+all 6.83. For the culture of *S. mutans*, in order to enable it to produce lactate, glucose was added to MM+Gly, MM+Lac, MM+Arg group to a final concentration of 5 mM.

In a pre-experiment, the cultivation period of the probiotics was varied (15 min, 30 min, 2, 4, and 18 h) to gauge the impact of this period on the production of $H_2O_2$, lactate, reuterin and ammonia. Different probiotic cultivation periods were eventually used to generate supernatants optimally enriched with these metabolites. As a consequence, *S. oligofermentans*, was cultured in MM+Glu and MM+Lac aerobically at 37°C for 30 min, in MM and MM+Gly for 2 h, and in MM+Arg and MM+all for 4 h, followed by centrifugation at 20800 *g* for 10 min at room temperature to obtain the supernatants. *L. reuteri* was cultured in MM+Glu, MM+Gly, MM+Lac and MM+all for 4 h and in MM and MM+Arg for 18 h, followed by the same protocol to collect supernatants. Deproteinization was conducted using Amicon Ultra-2ml centrifugal filter units with molecular weight cut-off (MWCO) of 10 kDa (Merck) at 7500 *g* for 20 min at room temperature. The deproteinized supernatants were maintained at −80°C for later processing. Control samples without bacteria were established using BHI medium containing 1% Glucose, 4 g/L yeast extract and 8 g/L beef extract followed by six different MM variations treated in the same way as in the bacterial culture groups.

## Preparation of Deproteinized Supernatants From *S. mutans* Cultures

*S. mutans* was precultured in the BHI+1% glucose+4 g/L yeast extract+8 g/L beef extract medium for 18 h aerobically at 37°C in 15 mL Falcon tubes. After centrifugation at 7100 *g* for 15 min at room temperature, cultivation supernatants were removed. Cells were rinsed with 0.9% sodium chloride and after another centrifugation at 7100 *g* for 15 min, bacteria were transferred to MM, MM+Glu, MM+Gly+Glu, MM+Lac+Glu, MM+Arg+Glu and MM+all media, inoculated with $4.35 \pm 0.39 \times 10^7$ cells as 1 mL-cultures in 1.5 mL Eppendorf tubes (Eppendorf, Hamburg,

Germany), followed by incubation at 37°C aerobically for further 18 h. Control samples without bacteria were established using BHI medium containing 1% Glucose, 4 g/L yeast extract and 8 g/L beef extract followed by six different MM variations treated in the same way as in the bacterial culture groups, except that 5 mM glucose was additionally added to MM+Gly, MM+Lac, MM+Arg groups. Afterward, the deproteinized supernatants were collected and stored as described above.

## Culture of *S. mutans* With Probiotic Supernatants

After pre-incubation in BHI+1% glucose+4 g/L yeast extract+8 g/L beef extract medium for 18 h, *S. mutans* was treated in the same manner as above with an inoculum of $4.29 \pm 0.65 \times 10^7$ cells/mL. After centrifugation, the supernatants were discarded and 1 mL of the supernatants of *S. oligofermentans* and *L. reuteri* (cultured in the different MM as described) were pipetted into 1.5 mL Eppendorf tubes (Eppendorf). Glucose was additionally added to the MM+Gly, MM+Lac, and MM+Arg at a final concentration of 5 mM. *S. mutans* was cultured for further 18 h as before. The probiotic supernatant without bacteria was used as control. The deproteinized supernatants of *S. mutans* were collected and stored as described above.

## Metabolite Assays

The metabolite production of lactate, $H_2O_2$ and ammonia was measured via assessing their concentration in the deproteinized supernatants using colorimetric assay kits (Sigma-Aldrich) in accordance with the manufacturers' instructions. The determination of reuterin in the supernatants was analyzed as described elsewhere (Kang et al., 2011) with some modifications. In short, 250 µL of deproteinized probiotic supernatant samples were added to 187.5 µL of 10 mM tryptophan dissolved in 0.05 M HCl, followed by 750 µL of 37% HCl. Under acidic conditions, tryptophan and the aldehyde of reuterin form a β-carboline derivative which oxidizes to produce a purple pigment. After incubation at 37°C for 20 min, the absorbance was measured at 560 nm. Acrolein (Sigma) was used as the calibration standard. To obtain standard curve, 0–15 µmol of acrolein was added to 1 mL of distilled water. The detection of absorbance was performed by the 96 well plate spectrophotometer Multiskan Go (Thermo Fisher Scientific, Schwerte, Germany).

## Viable Bacteria Enumeration

Viable bacteria cells were determined by plating 100 µL aliquots of 1 mL serial dilutions on COLS+ agar plates for *S. oligofermentans* and *S. mutans* or on MRS agar plates for *L. reuteri*. After 1–2 days of aerobic incubation at 37°C, the colony forming units/mL (CFU/mL) were calculated.

## Statistical Analysis

Descriptive analysis was performed, and one-way ANOVA followed by Dunnett's test conducted, with $P < 0.05$ considered as statistically significant. SPSS Version 20.0 software (SPSS Inc., Chicago, IL, United States) was used for statistical analysis.

**FIGURE 1 |** Concentration of metabolites and pH of *S. oligofermentans* in six different MM media (means ± standard deviations, *n* = 3); minimal medium (MM), MM with glucose (MM+Glu), MM with glycerol (MM+Gly), MM with Lactic acid (MM+Lac), MM with arginine (MM+Arg) and all-full medium (MM+all). The left *Y* axis showed the concentration and the right *Y* axis showed the pH value. **(A)** Very little reuterin was produced in *S. oligofermentans*. **(B)** Ammonia was detected in MM+Arg and MM+all. **(C)** Lactate was detectable mainly in MM+Glu and MM+all. **(D)** $H_2O_2$ was produced in all groups.

## RESULTS

### Concentration of Metabolites of *S. oligofermentans* Under Different Environmental Conditions

*S. oligofermentans* produced very little reuterin in MM+Gly and MM+all (**Figure 1A**). Ammonia was detected in MM+Arg and MM+all (**Figure 1B**) and lactate was detectable mainly in MM+Glu and MM+all (**Figure 1C**). $H_2O_2$ was produced in all groups, with the highest concentration in MM+all (**Figure 1D**). The pH of the supernatants after the incubation of *S. oligofermentans* were MM 6.80, MM+Glu 6.70, MM+Gly 6.85, MM+Lac 6.40, MM+Arg 7.10, MM+all 6.70 (**Figure 1**).

### Concentration of Metabolites of *L. reuteri* in Six Different MM Medium

Reuterin was detected in both MM+Gly and MM+all (**Figure 2A**), while ammonia was only detected in MM+Arg (**Figure 2B**). Lactate was detected in MM+Glu and MM+all, with only very low concentrations in the other groups (**Figure 2C**).

$H_2O_2$ was produced in all groups, the highest concentration being measured in MM+all (**Figure 2D**). The pH of the supernatants after the incubation of *L. reuteri* were MM 6.85, MM+Glu 6.80, MM+Gly 6.80, MM+Lac 6.40, MM+Arg 7.20, MM+all 6.60 (**Figure 2**).

### Effect of Probiotic Supernatants on *S. mutans* Metabolic Activity

Lactate production of *S. mutans* was minimal ($1.78 \pm 0.65 \times 10^{-2}$ mM) in control medium without glucose, and significantly higher in control media with glucose. Cultivation in supernatant of *S. oligofermentans* significantly reduced the lactate production of *S. mutans* regardless of the medium (**Table 1**) and, via utilization of lactate by *S. mutans*, even decreased the concentration of lactate in MM+all (concentration decreased by $-2.14 \pm 0.35$ mM) and MM+Lac+Glu (concentration decreased by $-1.39 \pm 0.25$ mM) respectively, ($p < 0.001$). Cultivation in supernatant of *L. reuteri* also significantly reduced the lactate production of *S. mutans* in all media except MM+Lac+Glu (**Table 1**).

**FIGURE 2 |** Concentration of metabolites and pH of *L. reuteri* (means ± standard deviations, *n* = 3) in six different MM media; minimal medium (MM), MM with glucose (MM+Glu), MM with glycerol (MM+Gly), MM with Lactic acid (MM+Lac), MM with arginine (MM+Arg) and all-full medium (MM+all). The left *Y* axis showed the concentration and the right *Y* axis showed the pH value. **(A)** Reuterin was detected in both MM+Gly and MM+all. **(B)** Ammonia was only detected in MM+Arg. **(C)** Lactate was mainly produced in MM+Glu and MM+all. **(D)** $H_2O_2$ was produced in all groups, the lowest being in MM+Lac.

**TABLE 1 |** Changes (means ± standard deviations, *n* = 3/group) in lactate concentration (mM) of *S. mutans* (Sm) after cultivation in the supernatant (Sup.) of *S. oligofermentans* (So) and *L. reuteri* (Lr).

| Metabolite (mM) | Minimal medium | Bacterial strain | | |
|---|---|---|---|---|
| | | **Sm** | **Sm Sup. So[A]** | **Sm Sup. Lr[B]** |
| Lactate | MM | $1.78 \pm 0.65 \times 10^{-2}$ | $-5.14 \pm 0.50 \times 10^{-2}$*** | $-1.13 \pm 1.08 \times 10^{-2}$** |
| | MM+Glu | $6.52 \pm 0.91$ | $3.84 \pm 0.40$** | $2.95 \pm 0.31$** |
| | MM+Gly+Glu | $5.00 \pm 0.42$ | $3.01 \pm 0.49$** | $3.65 \pm 0.33 \times 10^{-1}$*** |
| | MM+Lac+Glu | $4.46 \pm 0.41$ | $-1.39 \pm 0.25$*** | $4.42 \pm 0.19$ |
| | MM+Arg+Glu | $6.03 \pm 0.48$ | $1.69 \pm 0.43$*** | $3.29 \pm 0.26$*** |
| | MM+all | $6.00 \pm 0.29$ | $-2.14 \pm 0.35$*** | $-0.46 \pm 0.11$*** |

*For the culture of S. mutans, in order to enable it to produce lactate, the same concentration of glucose (5 mM) as in MM+Glu and MM+all was additionally added to MM+Gly, MM+Lac, MM+Arg. Glucose was also added to the probiotic supernatants of MM+Gly, MM+Lac, and MM+Arg. S. mutans was cultivated in minimal medium (MM), MM with glucose (MM+Glu), MM with glycerol and glucose (MM+Gly+Glu), MM with Lactic acid and glucose (MM+Lac+Glu), MM with arginine and glucose (MM+Arg+Glu) and all-full medium (MM+all). The lactate concentration of S. mutans cultivated in probiotic supernatants (test samples) and the lactate concentration in probiotic supernatants without S. mutans (control samples) were measured. The lactate production of S. mutans cultured in probiotic supernatants was calculated by subtracting the amount of lactate in control samples from the amount of lactate in corresponding test samples. Positive values indicate additional lactate production, negative values indicate uptake of lactate by bacteria. Dunnett's test: *P < 0.05, **P < 0.01, ***P < 0.001, versus Sm.*

**FIGURE 3 |** Changes in colony-forming units (Log CFU/mL, means ± standard deviations) of *S. mutans* after being cultured in the supernatant (Sup.) of *S. oligofermentans* (So) and *L. reuteri* (Lr). *S. mutans* was cultivated in minimal medium (MM), MM with glucose (MM+Glu), MM with glycerol and glucose (MM+Gly+Glu), MM with Lactic acid and glucose (MM+Lac+Glu), MM with arginine and glucose (MM+Arg+Glu) and all-full medium (MM+all) (controls), as well as supernatants of the probiotics. Glucose was additionally added to the MM+Gly, MM+Lac, and MM+Arg group of probiotic supernatants as it is described in section "Culture of *S. mutans* With Probiotic Supernatants." Each experiment was repeated three times. Dunnett's test: *$P < 0.05$, ***$P < 0.001$, versus controls.

## Inhibitory Effect of the Probiotic Supernatants on the Survival of *S. mutans*

When cultured in supernatants of *S. oligofermentans* and *L. reuteri,* survival of *S. mutans* was reduced compared with the controls (**Figure 3**). Specifically, treatment with the supernatant of *S. oligofermentans* resulted in a reduction in the viability of *S. mutans* in MM+Lac+Glu and MM+all by 0.6-$\log_{10}$ and 0.5-$\log_{10}$, respectively. Moreover, in MM+Gly+Glu and MM+all group, supernatant of *L. reuteri* yielded a reduction in viability of 6.1-$\log_{10}$ and 7.1-$\log_{10}$, respectively.

## DISCUSSION

The present study investigated the potential of *S. oligofermentans* and *L. reuteri* to produce different antimicrobial substances under different environmental conditions, and tested the inhibitory effect of probiotic supernatants on lactate production and survival of *S. mutans*. We found that both probiotics produced $H_2O_2$ in different MM, and ammonia in MM+Arg. In MM containing glycerol, *L. reuteri* also produced reuterin. Both probiotics produced lactate when glucose was present. Cultivation in the supernatants of both probiotics reduced the metabolic activity and survival of *S. mutans* significantly and environment-specifically. It can be assumed that these effects were not associated with pH changes (which were minimal given the buffering capacity of the used media), but associated with the products generated by the probiotics (Castillo et al., 2000). We hence confirm our hypothesis.

The mechanism by which probiotics precisely interfere with cariogenic pathogens remains unknown. There are currently three possible explanations, including (1) release of bacteriocins (Martinez et al., 2013), (2) the antimicrobial effects through co-aggregation (Lang et al., 2010), (3) competition with cariogenic bacteria for nutrition and adhesion (Terai et al., 2015; Schwendicke et al., 2017). In this study, *S. mutans* was cultured in the supernatant of probiotics only. Hence, we can make certain assumptions as to how these supernatants interacted with *S. mutans*: In MM+Lac and MM+all, *S. oligofermentans* had produced large amounts of $H_2O_2$, which has likely impacted on the survival and lactate production of *S. mutans*. In MM+Gly and MM+all, *L. reuteri* produced large amounts of reuterin, with potentially even more pronounced effects on *S. mutans*. The production of ammonia by *S. oligofermentans* in arginine containing medium had only moderate inhibitory effects on *S. mutans* activity and survival. Note that we cannot fully exclude the observed effects to be associated with other, non-measured probiotic products present in the supernatant, but given the consistency and plausibility of the measured presence of $H_2O_2$ and reuterin and the observed effects on *S. mutans,* the outlined pathway of how probiotics impact on *S. mutans* seems likely.

$H_2O_2$ is the major antibacterial substance produced by *S. oligofermentans* (Zhang et al., 2010; Tong et al., 2020), as confirmed by our study. *S. oligofermentans* possesses three $H_2O_2$-forming enzyme: lactate oxidase (Lox), that catalyzes L-lactate and oxygen to produce $H_2O_2$ and pyruvate; pyruvate oxidase (Pox), that generates $H_2O_2$ by oxidizing pyruvate to acetate via acetyl coenzyme; L-amino acid oxidase, that catalyzes the production of $H_2O_2$ from amino acids and peptone (Tong et al., 2008; Liu L. et al., 2012). Our findings that $H_2O_2$ production was most pronounced in MM+Lac and MM+all, where lactic acid was available for Lox, suggest that Lox may play a role in $H_2O_2$ generation. The production of $H_2O_2$ in MM, MM+Glu, MM+Gly and MM+Arg was similar, presumably because *S. oligofermentans* had converted extracellular glucose into intracellular polysaccharides during precultivation in BHI+glucose. When cultivated in carbohydrate-limited MM variations, intracellular glucose or glycogen of *S. oligofermentans* was decomposed into pyruvate. Pyruvate can generate $H_2O_2$ and acetyl phosphate through Pox or can be converted into lactic acid by the lactate dehydrogenase. The additional availability of glucose in MM+all may further support Pox and Lox activity, as *S. oligofermentans* converted glucose into pyruvate and lactate, which act as substrates for Pox and Lox, respectively. Overall, *S. oligofermentans* requires a Pox-Lox synergy to produce the maximum amount of $H_2O_2$ (Liu L. et al., 2012).

Saliva and protein-rich foods contain abundant amounts of arginine. The arginine deiminase system degrades and metabolizes arginine to ammonia (Nascimento, 2018), which raises the pH (Liu Y. L. et al., 2012). For *S. oligofermentans,* we found that ammonia can be detected in MM+Arg and MM+all. The lower ammonia production in MM+all may be explained by excess glucose being present, with the arginine deiminase activity decreasing when glucose

concentrations exceed 2 mM (Crow and Thomas, 1982; Kanapka and Kleinberg, 1983). Ammonia had only moderate effect on *S. mutans* activity and survival, which may be expected. Anti-caries effects of ammonia will be relevant nevertheless via altering the pH to a less cariogenic environment, hence supporting to rebalance remineralization over demineralization and preventing net mineral loss (Zaura and Twetman, 2019).

Reuterin is a multi-compound dynamic equilibrium system composed of 3-hydroxypropionaldehyde (3-HPA), its hydrate and dimer (Spinler et al., 2008) as well as acrolein. 3-HPA is a product of glycerol dehydration in the propanediol utilization (Pdu) pathway and is catalyzed by glycerol dehydratase (Chen and Hatti-Kaul, 2017). In reuterin solutions, acrolein and 3-HPA are interconverted, with acrolein being the active antimicrobial compound (Engels et al., 2016). In this study, we found *S. oligofermentans* to produce very little reuterin in MM+Gly and MM+all. *L. reuteri*, however, is known to possess the Pdu pathway to produce reuterin via fermentation of glycerol. After cultivation in the reuterin-containing supernatant of *L. reuteri*, *S. mutans* survival was reduced nearly completely. Our results are in line with those from clinical studies finding *L. reuteri* to be an efficacious probiotic to combat oral pathogens (Lin et al., 2017; Geraldo et al., 2019).

It has also been shown that *L. reuteri* is capable of producing $H_2O_2$ (Kang et al., 2011; Basu et al., 2019), and in our experiments, *L. reuteri* produced $H_2O_2$ in each MM variation. $H_2O_2$ is mainly produced by Pox (as described) and NADH oxidase (Nox) (Hertzberger et al., 2014), while it is unclear which enzymatic pathway was relevant in our setting. It was found that Pox synthesis was inhibited when glucose was abundantly available, while Nox was not essentially affected (Sedewitz et al., 1984). This was not the case in MM+Glu in our study. Hence, we assume that $H_2O_2$ was largely produced through NADH-dependent reactions, as shown for *Lactobacillus delbrueckii*, too (Marty-Teysset et al., 2000).

To assess the influence of the products generated by the probiotics on the lactate production and survival of *S. mutans*, *S. mutans* was cultivated in the probiotic supernatants. When cultivated in *S. oligofermentans* supernatant, lactate production by *S. mutans* in each MM variation group was significantly reduced. Since the supernatants of each MM variation media contained $H_2O_2$, the reason for this decrease in lactate production may be related to $H_2O_2$. Furthermore, ammonia produced in MM+Arg+Glu and MM+all may also neutralize lactic acid produced by *S. mutans*, reminding us that adjustment the alkali-generation potential of oral microbial may also have great potential. A similar reduction in lactate production was found when *S. mutans* was cultured in *L. reuteri* supernatants for all groups except MM+Lac+Glu. We consider that it is because the amount of $H_2O_2$ in MM+Lac+Glu was too small to reach an effective concentration.

Our findings agree with the study of Rossoni et al. (2018) in which they found the growth of *S. mutans* in planktonic cultures was inhibited by the bioactive substances released by *Lactobacillus* strains. In our study, almost no CFU were detectable in MM+Gly+Glu and MM+all of *L. reuteri* supernatant, proving reuterin as a potentially powerful antibiotic substance. *S. mutans* showed significantly lower survival in the culture of the supernatants in MM+Lac+Glu and MM+all of *S. oligofermentans*, which indicated that large amounts of $H_2O_2$ produced in a lactate-rich environment may have an inhibitory effect on the survival of *S. mutans*.

Overall, our study demonstrated that the products generated by the probiotics in the supernatants may inhibit the metabolic activity and survival of *S. mutans* and this effect was environment-specific. While it may well be that additional benefits emerge from the usage of viable probiotic, e.g., via co-aggregation (probably between *L. reuteri* and *S. mutans*), competition with cariogenic bacteria for nutrients and adhesive surfaces, and isolation of substrate or metal ions, our findings open up new therapeutic avenues. Using supernatant or specific isolated compounds for inhibiting cariogenic pathogens comes with the advantage of being safer, easier to dose, and any product having an extended shelf-life compared with living probiotics. Understanding the interactions between probiotics and cariogenic bacteria in simulated oral environments and identifying the underlying molecular mechanisms may support more effective and safe applications.

This study has several limitations. First, the complex oral conditions cannot be completely simulated *in vitro*. The impact of other bacteria species and the relevance of further proteins being available for bacterial metabolization will likely modify our findings. For the sake of interpretability, however, a simplified model such as ours seems useful. Second, our method for detecting specific bacterial substances produced by probiotics using colorimetric assays was not comprehensive; a more detailed metabolomic analysis may yield further insights. Similar, determining the CFU/mL of *S. mutans* in planktonic bacterial cultures to assess survival inhibition comes with limitations and does not fully reflect that probiotic effects in a clinical setting should target dental biofilms. However, both the colorimetric assay and the enumeration of planktonic bacteria via CFU/mL were chosen as they are easy to operate, reproducible and sufficient for the purposes of this study. Third, the culture time used in this study were optimized to capture, in a limited amount of time, the specific impact of the different metabolites. Different culture periods will be associated with different degrees of bacterial interaction. Last, there may be other mechanism, like end-product inhibition, as a possible non-specific mechanism that leads to a decrease in lactate production of *S. mutans*. However, the actual impact of this mechanism on our experimental results requires further verification.

In conclusion and within these limitations, the probiotic effects of *S. oligofermentans* and *L. reuteri* supernatants on the metabolic activity and survival of *S. mutans* were environment-specific through different pathways. Future studies as well as clinical applications should consider environment-specific probiotic actions on cariogenic pathogens.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

## AUTHOR CONTRIBUTIONS

HY performed the experiments, data analysis, and wrote the manuscript. PG and HY conceived and designed the study. PG guided the experiments and revised the manuscript. FS conceived the study, guided the design of the experiments, reviewed and edited the manuscript. All authors had approved the final version of the work.

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43

## Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

# Complete list of publications

## Publications (in English):

1. **Yu, H**., P. Ganas, and F. Schwendicke. Environment-Specific Probiotic Supernatants Modify the Metabolic Activity and Survival of *Streptococcus mutans* in vitro. Frontiers in Microbiology 2020; 11**:**1447. (IF: 4.235)

2. **Yu, H.**, Zhang, X., Song, W., Pan, T., Wang, H., Ning, T., Wei, Q., Xu, H., Wu, B., Ma, D. Effects of 3-dimensional Bioprinting Alginate/Gelatin Hydrogel Scaffold Extract on Proliferation and Differentiation of Human Dental Pulp Stem Cells. Journal of Endodontics 2019; 45, 706-715. (IF: 3.118)

3. Zhang, X., Ning, T., Wang, H., Xu, S., **Yu, H.**, Luo, X., Hao, C., Wu, B., Ma, D. Stathmin regulates the proliferation and odontoblastic/osteogenic differentiation of human dental pulp stem cells through Wnt/beta-catenin signaling pathway. Journal of Proteomics. 2019; 202, 103364. (IF: 3.509)

4. Ma, D., **Yu, H.**, Xu, S., Wang, H., Zhang, X., Ning, T., Wu, B. Stathmin inhibits proliferation and differentiation of dental pulp stem cells via sonic hedgehog/Gli. Journal of Cellular and Molecular Medicine. 2018; 22, 3442-3451 **(co-first author)** (IF: 4.658)

5. Wang, H., Ci B., **Yu, H**., Qin, W., Yan, Y., Wu, B, Ma, D. Evaluation of Root and Canal Morphology of Maxillary Molars in a Southern Chinese Subpopulation: A Cone-Beam Computed Tomographic Study. International Journal of Clinical and Experimental Medicine, 2017; 10(4), 7030-7039. (IF: 0.901)

## Publications (in Chinese):

1. **Yu, H**., Ma, D., Wu, B. Gelatin/alginate hydrogel scaffolds prepared by 3D bioprinting promotes cell adhesion and proliferation of human dental pulp cells in vitro. Journal of Southern Medical University. 2017; 37, 668-672.

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