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Limosilactobacillus reuteri inhibits the acid tolerance response in oral bacteria

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ABSTRACT

Probiotic bacteria show promising results in prevention of the biofilm-mediated disease caries, but the mechanisms are not fully understood. The acid tolerance response (ATR) allows biofilm bacteria to survive and metabolize at low pH resulting from microbial carbohydrate fermentation. We have studied the effect of probiotic strains: *Limosilactobacillus reuteri* and *Lacticaseibacillus rhamnosus* on ATR induction in common oral bacteria. Communities of L. *reuteri* ATCC PTA5289 and *Streptoccus gordonii, Streptococcus oralis, Streptococcus mutans* or *Actinomyces naeslundii* in the initial stages of biofilm formation were exposed to pH 5.5 to allow ATR induction, followed by a low pH challenge. Acid tolerance was evaluated as viable cells after staining with LIVE/DEAD®BacLight™. The presence of *L. reuteri* ATCC PTA5289 caused a significant reduction in acid tolerance in all strains except *S. oralis*. When *S. mutans* was used as a model organism to study the effects of additional probiotic strains (*L. reuteri* SD2112, *L. reuteri* DSM17938 or *L. rhamnosus* GG) as well as *L. reuteri* ATCC PTA5289 supernatant on ATR development, neither the other probiotic strains nor supernatants showed any effect. The presence of *L. reuteri* ATCC PTA5289 during ATR induction led to down-regulation of three key genes involved in tolerance of acid stress (*luxS*, *brpA* and *ldh*) in Streptococci. These data suggest that live cells of probiotic *L. reuteri* ATCC PTA5289 can interfere with ATR development in common oral bacteria and specific strains of *L. reuteri* may thus have a role in caries prevention by inhibiting development of an acid-tolerant biofilm microbiota.

1. Introduction

Oral biofilms in health are usually highly diverse, with over 700 different bacterial species identified to date [1] and single individuals hosting 100–200 species [2]. Biofilm composition differs throughout the oral cavity due to variation in the ecological niches at different sites. The supragingival microbiota is dominated by facultative anaerobic, saccharolytic species belonging to the genera *Streptococcus*, *Actinomyces*, *Veillonella*, *Granulicatella* and *Rothia* whereas the subgingival microflora is often dominated by Gram-negative, anaerobic and proteolytic species [3,4]. The microbiota is important for maintaining oral health through prevention of colonization by exogenous pathogens, but is also the cause of the major biofilm-related diseases; caries and periodontitis. The ecological plaque hypothesis describes oral disease as arising from changes in the composition and/or properties of biofilms (dysbiosis) due to sustained environmental perturbations and any bacteria in the community with relevant traits can contribute to the disease process [5]. Thus, in contrast to previously favoured preventive measures such as elimination of bacteria, recent years have seen an increased focus on promoting oral health through maintenance of a balanced, eubiotic microbiome [6].

Probiotic bacteria have been defined by the Food and Agriculture Organization of the United Nations and the World Health Organization as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [7]. They have traditionally been used to treat infections in the gastrointestinal (GI) tract as well as for promoting a healthy gut microbiota [8]. The effects have been proposed to include immunomodulation and inhibition of pathogen growth, as well as the promotion of health-related bacteria [9,10]. Over recent decades, the effect of probiotics in the prevention and treatment of oral biofilm-mediated diseases has been investigated in a number of clinical studies. A systematic review including fifty studies revealed

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evidence that the administration of probiotics could significantly reduce the clinical signs of periodontal inflammation and supported their use for the management of periodontal disease [11]. Clinical trials on the prevention and treatment of dental caries have shown a reduction in caries activity in children after probiotic intervention with *L. reuteri* ATCC 55730 [12] or a combination of *Streptococcus uberis* KJ2™, *Streptococcus oralis* KJ3™ and *Streptococcus rattus* JH145™ [13], and administration of probiotic strains in a range of different vehicles has been shown to reduce counts of *Streptococcus mutans* in saliva [14–16]. These promising results are reinforced by a range of *in vitro* studies investigating the mechanisms of action of probiotic strains in the oral cavity. *Limosilactobacillus reuteri* strain ATCC PTA5289 has been shown to inhibit binding of *S. mutans* to saliva-coated surfaces [17] and a range of probiotic strains including *L. reuteri* ATCC PTA5289 and *Lacticaseibacillus rhamnosus* GG coaggregate with, and inhibit growth of, mutans streptococci [18–20]. More recently, challenge with cultures of live *L. reuteri* ATCC PTA5289, but not culture supernatants, has been demonstrated to reduce the cariogenic activity of *in vitro* biofilms [21] and addition of *L. reuteri* ATCC PTA5289 to a multispecies biofilm model significantly reduced *S. mutans* levels [22]. These results suggest that further investigation into potential mechanisms by which these probiotic strains could contribute to managing dental caries are warranted.

Bacterial carbohydrate fermentation by members of the oral biofilm results in acidic end-products such as lactic and acetic acids, which lower the pH in the biofilm and can lead to dissolution of tooth enamel and dentine [23]. The buffering capacity of the saliva as well as bacterial generation of alkaline end-products helps to neutralize this fall in pH, resulting in a dynamic balance between de- and remineralization of dental hard tissues [24]. However, frequent intake of readily fermentable carbohydrates will result in increased duration of low pH. These changes in the biofilm environment can lead to dysbiosis and alterations in composition, resulting in selection of bacteria with an acid-tolerant phenotype [5]. As low intracellular pH denatures proteins and inhibits metabolic processes, members of the oral biofilm have developed mechanisms to protect themselves from low external pH. The acidogenic species *S. mutans*, that can be isolated from cariogenic biofilms, has been shown to induce an acid tolerance response (ATR) when exposed to a sub-lethal pH [25], including upregulation of genes involved in regulation of intracellular pH and tolerance of acid stress (26, 27). *In vitro* the development of an ATR has been shown to include factors that could contribute to a caries-promoting microbiota *in vivo*, including enhanced ability to metabolize and carry out cellular functions at a lower pH, which is not seen in non-adapted cells [28]. It is therefore likely that the ATR is an important step in the development of an acid tolerant microbiota that promotes net hard tissue demineralization and development of caries. In *S. mutans*, cells adhered to a surface showed an increased acid tolerance, which is further enhanced after adaptation after only 4 h of biofilm growth, indicating an ability to induce an ATR during the initial stages of biofilm development [29]. Recently we have shown that other abundant members of oral biofilms, often associated with health, are also able to induce an ATR in the early stages of biofilm formation, indicating that they might play a role in caries initiation by driving the development of an acid tolerant microbiota [30]. Intervention in ATR induction could thus be an effective measure for the prevention of caries and the aim of this study was therefore to investigate the effect of probiotic strains of *L. reuteri* as well as one probiotic strain of *L. rhamnosus* on ATR induction in four oral strains of streptococci as well as *Actinomyces*.

2. Materials and methods

2.1. Bacterial strains and media

Four strains of oral streptococci were used in this study; a type culture strain of *S. mutans* UA159 (ATCC 700610) and three archived strains originally isolated from supragingival dental plaque of healthy individuals [*S. mutans* (B4B), *Streptococcus gordonii* (CW) and *S. oralis* (CW)]. Following isolation on blood agar, Gram-positive, catalase-negative, facultative anaerobic cocci growing in chains were identified using phenotypic tests [31,32], and their identities confirmed with 16 S rRNA sequencing (*S. oralis*) or PCR (*S. mutans* and *S. gordonii)*. One strain of *Actinomyces naeslundii* (CW) was also used. This was isolated on blood agar from healthy supragingival plaque, as Gram-positive, facultative anaerobic branched rods and identified to species level using phenotypic tests [33] and PCR.

The probiotic bacteria used were: *L. reuteri* ATCC PTA5289 (FJ1; isolated from the oral cavity of a Japanese woman with no signs of oral disease), *L. reuteri* SD2112 (ATCC 55730; isolated from the breast milk of a Peruvian woman), *L. reuteri* DSM17938 (a daughter strain of *L. reuteri* SD2112 lacking the resistance plasmids against tetracycline and lincomycin) (BioGaia, Stockholm, Sweden) and *L. rhamnosus* GG ATCC 53103 (isolated from the intestinal tract of a healthy individual). Bacteria were inoculated separately into either Todd Hewitt Broth (oral strains) or MRS Broth (probiotic strains) and incubated at 37 °C in 5% CO_2 overnight. Aliquots of the overnight cultures were grown to exponential growth phase (OD_{600} = 0.5–0.8) in fresh growth medium at 37 °C in 5% CO_2. Minimal medium, MM4 [34] containing 20 mM glucose, buffered with 40 mM phosphate/citrate buffer at pH 3.5, 5.5 and 7.5 was used for the acid-tolerance response experiments.

2.1.1. Initial biofilm formation in mini flow-cell systems

Cultures of each strain in exponential growth phase (OD_{600} = 0.6–0.8) were transferred to MM4, pH 7.5 through two rounds of centrifugation at 2400×*g*, 5 °C, for 5 min (Thermo Scientific Heraeus Fresco 17 Centrifuge, Gothenburg, Sweden) and resuspension in MM4, pH 7.5. The effect on ATR induction was then investigated by inoculating a mixture of *L. reuteri* ATCC PTA5289 (50 μl) and an equal volume of *S. gordonii*, *S. oralis*, *S. mutans* (B4B or UA159) or *A. naeslundii* suspensions into mini flow-cells compatible with inverted confocal laser scanning microscopy (IbiTreat μ-Slide VI, Ibidi, Germany). The flow-cells were then incubated in a humid chamber in 5% CO_2 at 37 °C for 2 h to allow the cells to attach and initiate biofilm formation. Single-species biofilms of *S. gordonii*, *S. oralis*, *S. mutans* (UA159, B4B), or *A. naeslundii* were initiated in the same manner by inoculating aliquots (100 μl) into the mini-flow cells and maintaining in a humid chamber in 5% CO_2 at 37 °C for 2 h.

2.1.2. ATR development in oral strains

After 2 h, excess medium was removed and an ATR induced by exposing the bacterial cells to an adaptation pH, 5.5. Channels were gently washed twice with MM4 pH 5.5, fresh MM4 pH 5.5 added and the flow-cells incubated in a humid chamber for 2 h in 5% CO_2 at 37 °C. Control cells (non-adapted) were washed with MM4 pH 7.5 and fresh MM4 pH 7.5 added for 2 h. After this period, acid tolerance development was assessed by subjecting control and adapted bacteria to a low pH challenge by washing twice with MM4 pH 3.5 and incubating with MM4 pH 3.5 for 30 min in 5% CO_2 at 37 °C. Viability was then assessed by staining with LIVE/DEAD®BacLight™ stain (Molecular Probes, Eugene, Oregon, USA) according to manufacturer's instructions, and visualizing with confocal laser scanning microscopy (CLSM) using a Nikon Eclipse TE200 inverted microscope [35]. To confirm viability measurements from the BacLight assay, control cells in MM4 pH 7.5 and cells challenged with MM4 pH 3.5 for 30 min as above were plated onto blood agar and incubated at 37 °C in 5% CO_2 for 48 h, after which the number of colony-forming units (CFU) was counted.

To ensure that the presence of *L. reuteri* ATCC PTA5289 did not affect viability of the oral strains during the adaptation period, an additional experiment was conducted where the cells were exposed to the adaptation pH (pH 5.5) in the presence of *L. reuteri* ATCC PTA5289 for 2 h followed by a further 30 min at pH 5.5. Viability was assessed as above.

2.1.3. Effects of other probiotic strains on acid tolerance development

To investigate whether other probiotic strains affected ATR induction, exponential growth phase cells of the model organism, *S. mutans* UA159 were inoculated into the mini flow-cells with equal volumes of *L. reuteri* SD2112, *L. reuteri* DSM17938 or *L. rhamnosus* GG and maintaining in a humid chamber in 5% CO_2 at 37 °C for 2 h. Development of an ATR was then studied by subjecting control and adapted bacteria to a low pH challenge and assessing viability as above.

2.1.4. Collection of L. reuteri ATCC PTA5289-derived supernatant

L. reuteri ATCC PTA5289 were allowed to adhere to the surface of mini flow-cells for 2 h in MM4 pH 7.5. To maximize the concentration of *L. reuteri*-derived products in the supernatant, twice the number of cells were used compared to when *L. reuteri* was grown together with an oral strain. Following adaptation in MM4 pH 5.5 for a further 2 h and a low pH challenge of 30 min in MM4 pH 3.5, supernatants (130 μl) were collected and centrifuged (2400×*g*, 5 °C for 5 min). The pellet was then discarded and the pH of the supernatant adjusted to 7.5, 5.5 or 3.5 using HCl. Aliquots were stored at −20 °C until use.

2.1.5. ATR development in S. mutans UA159 during exposure to L. reuteri ATCC PTA5289-derived supernatant

Exponential growth phase cells of S. *mutans* UA159 washed in MM4 pH 7.5 were suspended in *L. reuteri* ATCC PTA5289-derived supernatant, pH 7.5 and inoculated into mini flow-cells which were kept in a humid chamber in 5% CO_2 at 37 °C for 2 h. Following attachment, the medium was removed and lanes were washed twice with MM4 pH 5.5. *L. reuteri*-derived supernatant pH 5.5 was added to each lane and the flow-cells were incubated in a humid chamber with 5% CO_2 at 37 °C. After 2 h of adaptation, the medium was removed and the flow-cells washed with MM4 pH 3.5. *L. reuteri* derived supernatant, pH 3.5 was added to each lane and incubated in a humid chamber with 5% CO_2 at 37 °C for 30 min. Control cells were exposed to MM4 at the equivalent pH without the addition of the *L. reuteri*-derived supernatant. Viability was then assessed as described above.

2.2. Image- and statistical analysis

All experiments were performed in triplicate, using three independent biological replicates of each strain. For analysis of acid tolerance of the oral strains, images from ten areas of the biofilm (image area = 0.05 mm^2), selected randomly using the Nikon NIS elements software, were captured and saved for subsequent analysis. The percentage of live cells was calculated after counting the green (live) and red (dead) cells in each image manually, while distinguishing between the strains on the basis of morphology (Fig. 1). All images were printed at high resolution and analyzed independently by four calibrated investigators. Mean values obtained were analyzed with the Mann-Whitney *U* test using Prism 9.3.1 software (GraphPad San Diego, USA). *P*-values of less than 0.05 were considered statistically significant.

2.2.1. Effect of L. reuteri ATCC PTA5289 on the acid stress related gene expression of S. mutans ATCC UA159

Log phase suspensions (OD_{600} = 0.6–0.8) of *S. mutans* ATCC UA159 and *L. reuteri* ATCC PTA5289 were mixed and washed twice with MM4 pH 7.5 by centrifugation at 2400×*g*, at 5 °C for 5 min (Beckman Coulter GS-6R Centrifuge, California, USA). The cells were resuspended in MM4 pH 7.5 and inoculated in a Petri dish at 37 °C in 5% CO_2 for 2 h to allow bacterial adhesion. The adhered cells were then washed three times with MM4 pH 5.5, resuspended in MM4 pH 5.5 and incubated at 37 °C in 5% CO_2 for 2 h to allow bacterial adaptation. Excess medium was then discarded and bacterial cells were collected by scraping (Sarstedt, Newton, USA) and addition of new MM4 pH 5.5. The number of viable cells was determined as CFU after serial dilution in 10 mM phosphate-buffered saline and 0.07 M NaCl, pH 7,2 (PBS), and plating on blood agar. RNAprotectÒ Bacteria Reagent was added to the remaining bacterial suspensions followed by centrifugation at 2540×*g*, at 5 °C for 10 min. The supernatants were decanted and the pellets were stored at −80 °C for RNA Extraction. Single species biofilms of *S. mutans* ATCC UA159 were prepared and treated in the same manner. All experiments were carried out in triplicate using independent biological replicates.

2.2.2. RNA extraction, reverse transcriptase and quantitative RT-PCR

RNA was extracted using a Qiagen, RNeasy® Protect Bacteria kit (Hilden, Germany) according to the manufacturer's instructions, with the addition of 15 mg/ml lysozyme (62,971-10G-F, Sigma-Aldrich, Steinheim, Belgium) to the proteinase K/Tris-EDTA buffer followed by mechanical disruption with glass beads. Total RNA was quantified using

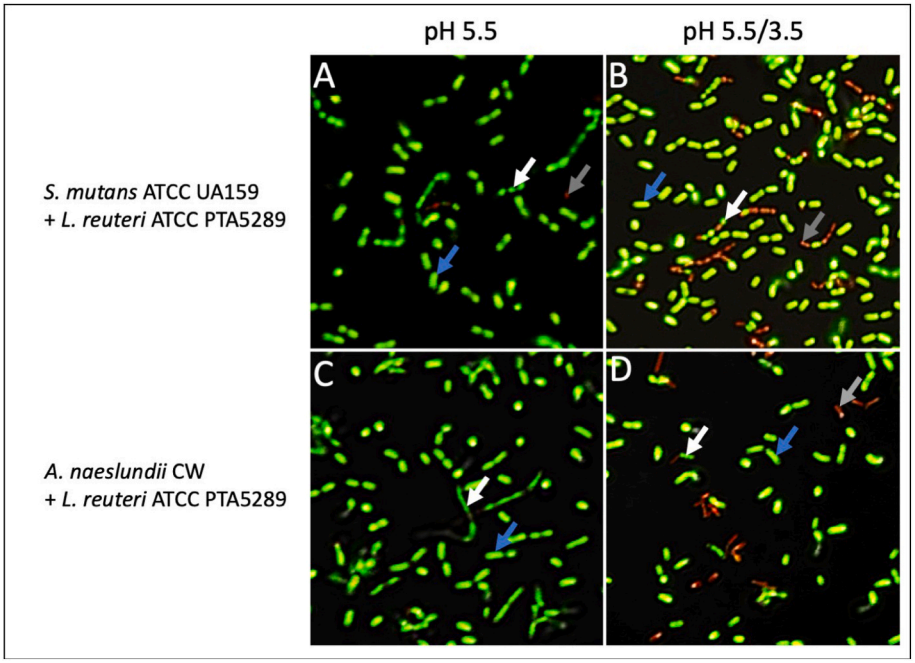

Fig. 1. Representative images of *L. reuteri* ATCC PTA5289 (blue arrow) in the initial stages of biofilm formation with *S. mutans* ATCC UA159 or *A. naeslundii* CW. Cells were exposed to pH 5.5 for 2 h, followed by exposure to pH 5.5 for a further 30 min (1 A and 1C) or a challenge of pH 3.5 for 30 min (1 B and 1D). They were then visualized with LIVE/DEAD®BacLight™ staining and CLSM. White arrows show live cells of *S. mutans* ATCC UA159 or *A. naeslundii* CW, and grey arrows show dead cells.

a Qubit® RNA BR Assay Kit, Oregon, USA) and stored at −80 °C. Equal amounts of RNA were transcribed to cDNA using Superscript IV Vilo master mix containing ezDNase Enzyme according to the manufacturer's instructions (Thermo Fisher Scientific, California, USA). qPCR was carried out on a QuantStudio 3 (Applied Biosystems, Thermo Fisher Scientific, California, USA) using PowerTrack SYBRGreen master mix according to the manufacturer's instructions. The primers used are shown in Table 1. Values were normalized to the ratio of *S. mutans* CFU to the total CFU in the dual-species biofilms.

3. Results

3.1. L. reuteri ATCC PTA5289 inhibits ATR induction in oral bacteria

Dual species communities of *L. reuteri* ATCC PTA5289 and *S. mutans*, *S. gordonii*, *S oralis* or *A. naeslundii* in the initial stage of biofilm formation were created in mini flow-cells. After the 2 h adaptation period at pH 5.5, all of the oral strains as well as *L. reuteri* ATCC PTA5289 showed over 95% viability, as demonstrated by green staining with LIVE/DEAD® BacLight™ (Fig. 1A and C), and the oral strains represented 53–65% of the total cell count of the biofilms. The induction of acid-tolerance during the adaptation period was then evaluated by exposing the cells to a low pH challenge (pH 3.5) for 30 min (Fig. 1B and D). In the presence of *L. reuteri* ATCC PTA5289, the number of acid tolerant cells was significantly reduced in all strains except *S. oralis*, with the most prominent effect seen for *S. mutans* ATCC UA159, where there was an almost 3-fold reduction ($p < 0.01$, Fig. 2). The effect was similar, although less strong in *S. mutans* B4B and *A. naeslundii*, with a 2-fold decrease in the number of acid tolerant cells compared to control ($p < 0.01$, Fig. 2). For *S. gordonii*, there was also a significant decrease in the number of acid tolerant cells in the presence of *L. reuteri* ATCC PTA5289, although the effect was less pronounced than for the other bacteria [1.3-fold decrease ($p < 0.01$)]. Overall, these data show that the presence of *L. reuteri* ATCC PTA5289 significantly reduces development of an ATR in four of the five strains of oral bacteria tested.

3.2. Inhibition of ATR induction in S. mutans (UA159) by L. reuteri is strain-dependent

To test whether the effect seen on ATR development in the oral bacteria was specific to *L. reuteri* ATCC PTA5289, two additional strains of *L. reuteri; L. reuteri* DSM17938 and *L. reuteri* SD2112, as well as *L. rhamnosus GG* were tested. Since the effect of *L. reuteri* ATCC PTA5289 on ATR development was greatest for *S. mutans* UA159, this was used as a model organism in these experiments. In the control, containing only *S. mutans* UA159, 52% of the cells induced an ATR after exposure to pH 5.5 for 2 h and this was significantly reduced by the presence of *L. reuteri* ATCC PTA5289. However, no statistically significant reduction in the number of acid tolerant cells was seen in the presence of any of the other tested strains; *L. reuteri* DSM17938 ($p = 0.153$), *L. reuteri* SD2112 ($p = 0.859$) or *L. rhamnosus* GG ($p = 0.265$) (Fig. 3). These data thus show that, amongst the probiotic strains tested here, the inhibitory effect on ATR development was specific to *L. reuteri* ATCC PTA5289.

Table 1
Primers used for qPCR [36].

Primer	DNA sequence (5′ T 3′)	Length
luxS-Fw	ACTGTTCCCCTTTTGGCTGTC	21 bp
luxS-Rv	AACTTGCTTTGATGACTGTGGC	22 bp
brpA-Fw	CGTGAGGTCATCAGCAAGGTC	21 bp
brpA-Rv	CGCTGTACCCCAAAAGTTTAGG	22 bp
ldh-Fw	TTGGCGACGCTCTTGATCTTAG	22 bp
ldh-Rv	GTCAGCATCCGCACAGTCTTC	21 bp

Fw = Forward, Rv = Reversed, T = To, bp = Base pair.

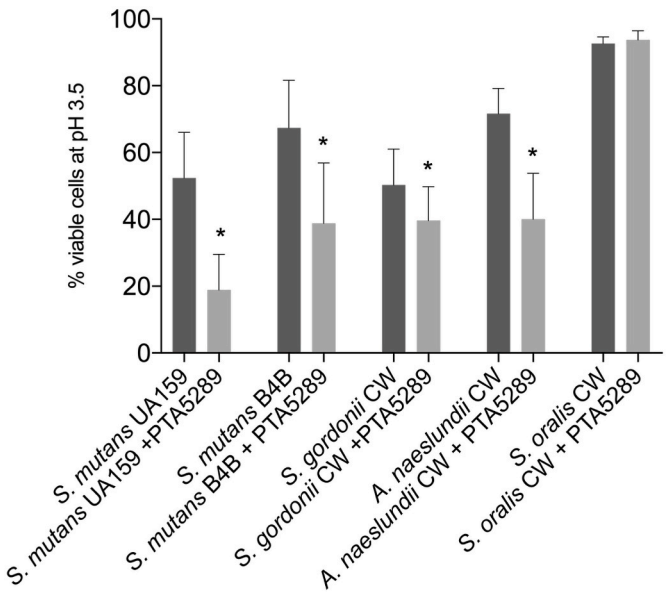

Fig. 2. Viability of oral streptococci and *Actinomyces* in initial biofilms alone or together with *L. reuteri* ATCC PTA5289 (+PTA5289). Cells were subjected to adaptation at pH 5.5 for 2 h followed by an acid challenge of pH 3.5 for 30 min. Viability was assessed using CLSM after staining with LIVE/DEAD® BacLight™. Percentage viability of oral strains was evaluated by counting the cells manually and graphs show the mean and standard deviation of three independent biological replicates, *$P < 0.05$.

3.3. Supernatants of L. reuteri ATCC PTA5289 do not affect bacterial adhesion or ATR induction

To further investigate their effect on ATR induction, supernatants of sessile *L. reuteri* ATCC PTA5289 cells were prepared and their effects on the initial stages of biofilm formation and ATR induction tested in *S. mutans* UA159. The concentration of *L. reuteri*-derived products in the supernatant was optimized by increasing the cell number. Adhesion to the flow-cell surfaces was not affected by the *L. reuteri* supernatant with a mean surface coverage of 57% (SEM ± 3.5%) in the control and 54% (SEM ± 6.1%) in the presence of the supernatant. After exposure to pH 5.5 for 2 h followed by a low pH challenge (pH 3.5) for 30 min, surface coverage in the presence of the *L. reuteri* supernatant did not differ from control ($p = 0.245$). A somewhat lower surface coverage was seen after exposure to pH 3.5 compared to pH 7.5 and 5.5 respectively, but this was the same for both conditions. In contrast to when *L. reuteri* ATCC PTA5289 was physically present, the supernatant had no effect on ATR development, with these cells showing a small significant increase in survival at pH 3.5 (Fig. 3).

3.4. L. reuteri ATCC PTA5289 causes a reduction in expression of luxS, brpA and ldh in S. mutans UA159

To investigate the effects of *L. reuteri* ATCC PTA5289 on acid tolerance at the cellular level, expression of three genes associated with the response to acid stress (*luxS, brpA* and *ldh)*, was investigated in *S. mutans* UA159. This showed that expression levels of *luxS, brpA* and *ldh* were significantly reduced (14-fold, 5-fold and 15-fold, respectively) in *S. mutans* in the presence of *L. reuteri* ATCC PTA5289 compared to when *S. mutans* was grown alone (Fig. 4).

4. Discussion

In recent years, interest in promotion of a healthy oral microbiome and prevention of disease through the use of probiotic bacteria has increased. In caries, the ecological plaque hypothesis and its

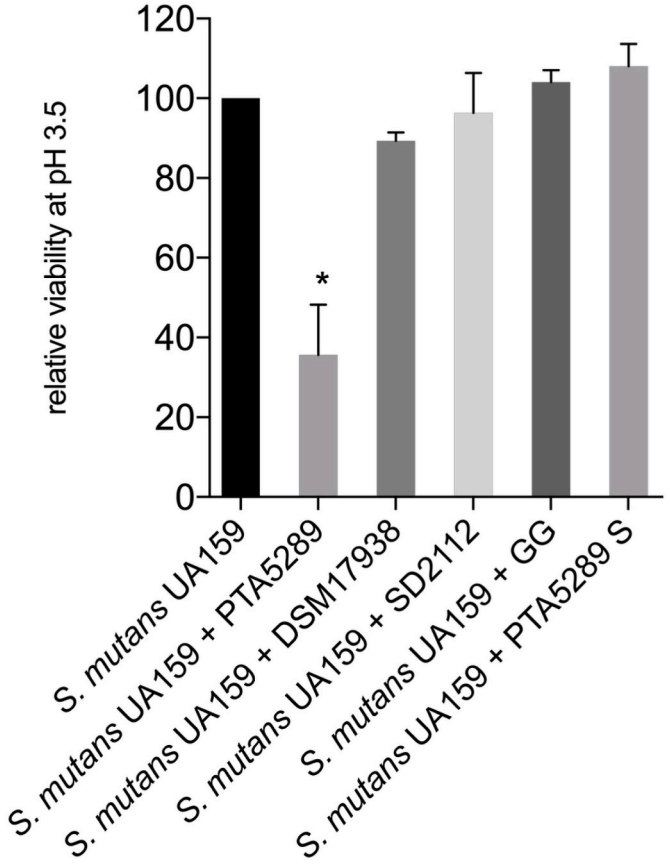

Fig. 3. Viability of *S. mutans* ATCC UA159 in the initial stages of biofilm formation with *L. reuteri* strains: ATCC PTA5289 (+PTA5289), DSM17938 (+DSM17938) and SD2112 (+SD2112), *L. rhamnosus* strain GG (+GG) or *L. reuteri* ATCC PTA5289-derived supernatant (+PTA5289 S). Percent viability of *S. mutans* cells when grown together with the probiotic strains or supernatant is presented as relative to the viability of single-species initial biofilms of *S. mutans* ATCC UA159. The cells were exposed to pH 5.5 for 2 h followed by an acid challenge at pH 3.5 for 30 min. Viability was assessed with LIVE/DEAD® BacLight™ staining and CLSM. Percentage viability of cells was evaluated by counting manually and graphs show the mean and standard deviation of three independent biological replicates, *P < 0.05.

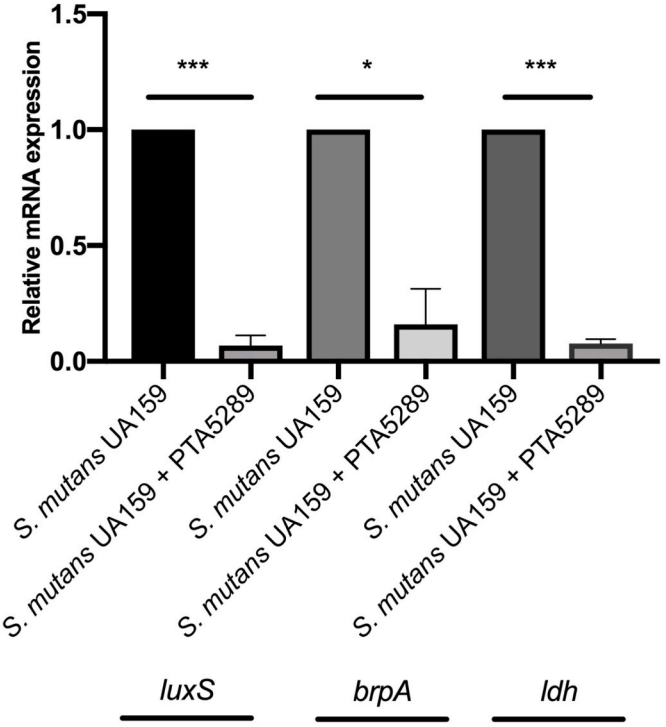

Fig. 4. qPCR analysis of *luxS, brpA* and *ldh* expression in *S. mutans* ATCC UA159 during the initial stages of biofilm formation in the presence or absence of *L. reuteri* ATCC PTA5289. The effect of *L. reuteri* is presented as relative to *S. mutans* alone in the same experiment (n = 3, * = p < 0.05, *** = p < 0.001).

modification, propose that changes in the biofilm environment cause enrichment of aciduric and acid tolerant bacterial species, leading to a low pH environment which pushes the re-/demineralization balance at the hard tissue surface towards net demineralization [4,24]. We have shown that several bacterial species abundant in the healthy oral biofilm induce an ATR when exposed to a moderate change in environmental pH [30] and that the effect occurs rapidly following attachment to a surface [35]. Therefore, in this study we investigated whether probiotic bacteria could inhibit ATR induction during the initial stages of biofilm formation. The main finding was that *L. reuteri* ATCC PTA5289 had a significant inhibitory effect in all the bacteria that induced an ATR, while one strain of *S. oralis* which was already highly acid tolerant and did not induce an ATR, was unaffected. This is in keeping with previous studies of this strain of *S. oralis* (CW), which showed a high level of inherent acid tolerance which was not increased further by acid adaptation [30]. The inhibition was most prominent for the two *S. mutans* strains tested, but was also seen in *S. gordonii* and *A. naeslundii*. The phenotypic changes accompanying ATR development include an increased ability to survive and metabolize at low pH [25], resulting in an increased capacity for acid production even when the biofilm pH is low. Data from other *in vitro* studies show that *L. reuteri* ATCC PTA5289 can reduce lactic acid production in both single-species *S. mutans* biofilms and biofilms containing both *S. mutans* and *A. naeslundii* [21,37] and a recent clinical study revealed that daily intake of *L. reuteri* strains ATCC PTA5289 and DSM 17938 for three weeks led to a significant reduction in plaque acidogenicity in response to a 1-min mouth rinse with 10% sucrose [38]. Although not investigated here, our results suggest that inhibition of ATR development in common oral bacteria in the presence of *L. reuteri* ATCC PTA5289 could contribute to this effect.

To shed light on possible mechanisms by which *L. reuteri* PTA5289 exerted an inhibitory effect on ATR development, we investigated the expression of three key genes known to be involved in acid tolerance in *S. mutans* (*luxS*, a global regulator of cellular function [26], *brpA* [39], and *ldh* [27]) during ATR induction in the presence and absence of *L. reuteri* ATCC PTA5289. The results showed a significant down-regulation of all three genes compared to when an ATR was induced in *S. mutans* alone. This indicates that *L. reuteri* ATCC PTA5289 exerts an effect on acid tolerance through pathways involved in maintenance of intracellular pH and acid stress adaptation in *S. mutans*.

The effect of *L. reuteri* ATCC PTA5289 seen in this study was associated with the presence of live bacteria and could not be reproduced using supernatants even when the number of *L. reuteri* cells was doubled. Supernatants from L. *reuteri* ATCC PTA5289 have been shown to inhibit growth of *S. mutans*, possibly through production of H_2O_2 [37]. However, our findings are consistent with other studies showing that the physical presence of *L. reuteri* had an effect on biofilm pH and enamel demineralization that was not obtained using culture supernatants [21] and that a whole culture of *L. reuteri* ATCC 23272 had greater inhibitory effect on growth of *S. mutans* than spent medium [38].

Since the inhibitory effect on ATR development by *L. reuteri* PTA5289 was greatest in this strain, *S. mutans* UA159 was used to test the effects of other probiotic bacteria. *L. rhamnosus* GG, isolated from the human gastrointestinal tract, is one of the most widely used probiotic bacteria and has shown health effects, including prevention and treatment of gastro-intestinal infections and diarrhea, and stimulation of immune responses during vaccination [40]. However, in keeping a clinical investigation showing that short-term consumption of

L. rhamnosus GG had no effect on plaque acid production [41], no effect on ATR development was seen in this study. This strain has, however, been demonstrated to reduce caries experience after long-term consumption and lower mutans streptococci counts in plaque and saliva *in vivo* [42] as well as inhibiting *S. mutans* biofilm formation *in vitro*; an effect that has been ascribed to a decrease in expression of glucosyltransferase and consequent reduction in glucan production [43,44].

Interestingly, the breast milk-derived *L. reuteri* strains; SD2112 and DSM 17938 (a plasmid-cured version of SD2112), which have similar attributes [45], also lacked the effect on ATR development in *S. mutans* UA159 seen for strain ATCC PTA5289. Since previous studies have demonstrated that strains ATCC PTA5289 and DSM17938 are similar in their capacity to generate a number of substances associated with probiotic effects, including H_2O_2 [46] and ammonia [47] these substances may not underlie the differential effect seen here. However, further investigations using for instance a peroxidase to quench H_2O_2 and a buffer to neutralize ammonia would be required to exclude these mechanisms. Multilocus sequence analysis (MLSA) has revealed that human-derived *L. reuteri* strains belong to two different multilocus sequence types [48] with strain ATCC PTA5289 (under its original designation, FJ1) assigned to clade II and strains SD2112 and DSM 17938 to clade VI [49]. Comparison of other probiotic features of these two clades reveal significant differences, for instance, in the production of the antimicrobial substance, reuterin [49]. However, glycerol was not present in the growth medium in the studies and during stationary phase growth (which can be related to biofilm growth) SD2112 and DSM 17938 produce significantly *more* reuterin than ATCC PTA5289 [50]. In addition, since an antimicrobial substance such as reuterin would be expected to affect viability of the oral strains independent of pH, and in this study all the oral strains showed a high viability at pH 7.5 and pH 5.5, a major role for this substance in the effect appears unlikely. This is in agreement with a recent investigation which revealed that *L. reuteri* ATCC PTA5289 exerts probiotic effects on streptococci that are independent of reuterin [22].

5. Conclusion

The results of this study thus show that the presence of *L. reuteri* ATCC PTA 5289 can inhibit the development of an ATR in the initial stages of biofilm formation in common oral bacteria. The effect is strain-specific and involves down-regulation of key genes involved in maintenance of intracellular pH and acid stress adaptation in streptococci. Specific strains of *L. reuteri* may thus have a role to play in caries prevention by inhibiting development of an acid tolerant biofilm microbiota.

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Availability of data and materials

All data analyzed in this study are included in this published article in the form of tables and figures.

Competing interests

The authors declare that they have no competing interests.

Credit Author Statement

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Declaration of competing interest

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Data availability

Data will be made available on request.

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References

[1] Verma D, Garg PK, Dubey AK. Insights into the human oral microbiome. Arch Microbiol 2018;200:525–40.
[2] Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, Lakshmanan A, Wade WG. The human oral microbiome. J Bacteriol 2010;192:5002–17.
[3] Sanz M, Beighton D, Curtis MA, Cury JA, Dige I, Dommisch H, Ellwood R, Giacaman RA, Herrera D, Herzberg MC, Könönen E, Marsh PD, Meyle J, Mira A, Molina A, Mombelli A, Quirynen M, Reynolds EC, Shapira L, Zaura E. Role of microbial biofilms in the maintenance of oral health and in the development of dental caries and periodontal diseases. Consensus report of group 1 of the Joint EFP/ORCA workshop on the boundaries between caries and periodontal disease. J Clin Periodontol 2017;44:5–11.
[4] Caselli E, Fabbri C, D'Accolti M, et al. Defining the oral microbiome by whole-genome sequencing and resistome analysis: the complexity of the healthy picture. BMC Microbiol 2020;20(1):120.
[5] Marsh PD. Are dental diseases example of ecological catastrophes? Microbiol 2003; 149:279–94.
[6] Takahashi N, Nyvad B. Caries ecology revisited: microbial dynamics and the caries process. Caries Res 2008;42:409–18.
[7] Hill C, Guarner F, Reid G, et al. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 2014;11(8):506–14. https://doi.org/10.1038/nrgastro.2014.66.
[8] Quigley EM. Prebiotics and probiotics: their role in the management of gastrointestinal disorders in adults. Nutr Clin Pract 2012;27(2):195–200.
[9] Lebeer S, Vanderleyden J, De Keersmaecker SC. Genes and molecules of lactobacilli supporting probiotic action. Microbiol Mol Biol Rev 2007;2:728–64.
[10] Rondanelli M, Faliva MA, Perna S, Giacosa A, Peroni G, Castellazzi AM. Using probiotics in clinical practice: where are we now? A review of existing meta-analyses. Gut Microb 2017;8:521–43.
[11] Gruner D, Paris S, Schwendicke F. Probiotics for managing caries and periodontitis: systematic review and meta-analysis. J Dent 2016;48:16–25.
[12] Stensson M, Koch G, Coric S, Abrahamsson TR, Jenmalm MC, Birkhed D, Wendt LK. Oral administration of *Lactobacillus reuteri* during the first year of life reduces caries prevalence in the primary dentition at 9 years of age. Caries Res 2014;48:111–7.
[13] Hedayati-Hajikand T, Lundberg U, Eldh C, Twetman S. Effect of probiotic chewing tablets on early childhood caries: a randomized controlled trial. BMC Oral Health 2015;15:112.
[14] Laleman I, Detailleur V, Slot DE, Slomka V, Quirynen M, Teughels W. Probiotics reduce mutans streptococci counts in humans: a systematic review and meta-analysis. Clin Oral Invest 2014;18:1539–52.
[15] Alforaidi S, Bresin A, Almosa N, Lehrkinder A, Lingström P. Oral colonisation after the administration of drops containing *Lactobacillus reuteri*. Oral Health Prev Dent 2020;18:1017–23.
[16] Romani Vestman N, Hasslöf P, Keller MK, Granström E, Roos S, Twetman S, Stecksén-Blicks C. *Lactobacillus reuteri* influences regrowth of mutans streptococci after full-mouth disinfection: a double-blind, randomised controlled trial. Caries Res 2013;47:338–45.
[17] Marttinen A, Haukioja AL, Keskin M, Söderling EM. Effects of *Lactobacillus reuteri* PTA5289 and *L. paracasei* DSMZ16671 on adhesion and biofilm formation of *Streptococcus mutans*. Curr Microbiol 2013;67:193–9.
[18] Twetman L, Larsen U, Fiehn NE, Stecksen-Blicks C, Twetman S. Coaggregation between probiotic bacteria and caries-associated strains: an *in vitro* study. Acta Odontol Scand 2009;67:284–8.
[19] Hasslöf P, Hedberg M, Twetman S, Stecksen-Blicks C. Growth inhibition of oral mutans streptococci and Candida by commercial probiotic lactobacilli–an *in vitro* study. BMC Oral Health 2010 Jul 2;10:18.
[20] Keller MK, Hasslof P, Stecksen-Blicks C, Twetman S. Co-aggregation and growth inhibition of probiotic lactobacilli and clinical isolates of mutans streptococci: an *in vitro* study. Acta Odontol Scand 2011;69:263–8.

[21] Chen Z, Schlafer S, Göstemeyer G, Schwendicke F. Probiotic effects on multispecies biofilm composition, architecture, and caries activity *in vitro*. Microorganisms 2020 Aug 21;8:1272.
[22] Van Holm W, Verspecht T, Carvalho R, et al. Glycerol strengthens probiotic effect of Limosilactobacillus reuteri in oral biofilms: a synergistic synbiotic approach. Mol Oral Microbiol 2022;37(6):266–75.
[23] Carlsson J, Hamilton IR. Metabolic activities of oral bacteria. In: Thylstrup A, Fejerskov O, editors. Textbook of clinical cariology. second ed. Copenhagen, Denmark: Munksgaard; 1994. p. 71–110.
[24] Fejerskov O, Nyvad B, Kidd EAM. Pathology of dental caries. In: Fejerskov O, Kidd E, editors. Dental caries. second ed. Oxford, United Kingdom: Blackwell Munksgaard; 2008. p. 19–48.
[25] Svensäter G, Larsson UB, Greif ECG, Cvitkovitch DG, Hamilton IR. Acid tolerance response and survival by oral bacteria. Oral Microbiol Immunol 1997;12:266–73.
[26] Wen ZT, Burne RA. LuxS mediated signaling in *Streptococcus mutans* is involved in regulation of acid and oxidative stress tolerance and biofilm formation. J Bacteriol 2004;186:2682–91.
[27] Welin J, Wilkins JC, Beighton D, et al. Effect of acid shock on protein expression by biofilm cells of *Streptococcus mutans*. FEMS Microbiol Lett 2003;227(2):287–93.
[28] McNeill K, Hamilton IR. Effect of acid stress on the physiology of biofilm cells of *Streptococcus mutans*. Microbiology 2004;150:735–42.
[29] Welin-Neilands J, Svensater G. Acid tolerance of biofilm cells of Streptococcus mutans. Appl Environ Microbiol 2007;73(17):5633–8.
[30] Boisen G, Davies JR, Neilands J. Acid tolerance in early colonizers of oral biofilms. BMC Microbiol 2021;21:45.
[31] Beighton D, Hardie JM, Whiley RA. A scheme for the identification of viridans streptococci. J Med Microbiol 1991;35:367–72.
[32] Whiley RA, Beighton D. Current classification of the oral streptococci. Oral Microbiol Immunol 1998;13:195–216.
[33] Sarkonen N, Könönen E, Summanen P, Könönen M, Jousimies-Somer H. Phenotypic identification of Actinomyces and related species isolated from human sources. J Clin Microbiol 2001:3955–61.
[34] Hamilton IR, Svensater G. Acid-regulated proteins induced by *Streptococcus mutans* and other oral bacteria during acid shock. Oral Microbiol Immunol 1998;13:292–300.
[35] Welin-Neilands J, Svensater G. Acid tolerance of biofilm cells of *Streptococcus mutans*. Appl Environ Microbiol 2007;73:5633–8.
[36] Wen ZT, Yates D, Ahn SJ, Burne RA. Biofilm formation and virulence expression by *Streptococcus mutans* are altered when grown in dual-species model. BMC Microbiol 2010;10:111.
[37] Yu H, Ganas P, Schwendicke F. Environment-specific probiotic supernatants modify the metabolic activity and survival of *Streptococcus mutans in vitro*. Front Microbiol 2020 Jun 26;11:1447.
[38] Alforaidi S, Bresin A, Almosa N, Lehrkinder A, Lingström P. Effect of drops containing *Lactobacillus reuteri* (DSM 17938 and ATCC PTA5289) on plaque acidogenicity and other caries-related variables in orthodontic patients. BMC Microbiol 2021 Oct 6;21(1):271.
[39] Wen ZT, Baker HV, Burne RA. Influence of BrpA on critical virulence attributes of *Streptococcus mutans*. J Bacteriol 2006;188(8):2983–92.
[40] Segers ME, Lebeer S. Towards a better understanding of *Lactobacillus rhamnosus* GG – host interactions. Microb Cell Factories 2014;13(Suppl 1):S7.
[41] Marttinen A, Haukioja A, Karjalainen S, Nylund L, Satokari R, Öhman C, Holgersson P, Twetman S, Söderling E. Short-term consumption of probiotic lactobacilli has no effect on acid production of supragingival plaque. Clin Oral Invest 2012;16:797–803.
[42] Näse L, Hatakka K, Savilahti E, Saxelin M, Pönkä A, Poussa T, Korpela R, Meurman JH. Effect of long-term consumption of a probiotic bacterium, *Lactobacillus rhamnosus* GG, in milk on dental caries and caries risk in children. Caries Res 2001;35:412–20.
[43] Lee SH, Kim YJ. A comparative study of the effect of probiotics on cariogenic biofilm model for preventing dental caries. Arch Microbiol 2014;196:601–9.
[44] Söderling EM, Marttinen AM, Haukioja AL. Probiotic lactobacilli interfere with *Streptococcus mutans* biofilm formation *in vitro*. Curr Microbiol 2011;62:618–22.
[45] Rosander A, Connolly E, Roos S. Removal of antibiotic resistance gene-carrying plasmids from *Lactobacillus reuteri* ATCC 55730 and characterization of the resulting daughter strain, *L. reuteri* DSM 17938. Appl Environ Microbiol 2008;74:6032–40.
[46] Jorgensen MR, Kragelund C, Jensen PO, Keller MK, Twetman S. Probiotic *Lactobacillus reuteri* has antifungal effects on oral Candida species *in vitro*. J Oral Microbiol 2017;9(1):1274582.
[47] Jalasvuori H, Haukioja A, Tenovuo J. Probiotic *Lactobacillus reuteri* strains ATCC PTA5289 and ATCC 55730 differ in their cariogenic properties *in vitro*. Arch Oral Biol 2012;57:1633–8.
[48] Oh PK, Benson AK, Peterson DA, Patil PB, Moriyama EN, Roos S, Walter J. Diversification of the gut symbiont *Lactobacillus reuteri* as a result of host-driven evolution. ISME J 2010;4:377–87.
[49] Spinler J, Sontakke A, Hollister EB, Venable SF, Oh PL, Balderas MA, Saulnier DMA, Mistretta T-A, Devaraj S, Walter J, Versalovic J, Highlander SK. From prediction to function using evolutionary genomics: human-specific ecotypes of *Lactobacillus reuteri* have diverse probiotic functions. Genome Biol Evol 2014;6:1772–89.
[50] Spinler JK, Taweechotipatr M, Rognerud CL, Ou CN, Tumwasorn S, Versalovic J. Human-derived probiotic *Lactobacillus reuteri* demonstrate antimicrobial activities targeting diverse enteric bacterial pathogens. Anaerobe 2008;14:166–71.