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ORIGINAL ARTICLE

Correlation between relative bacterial activity and lactate dehydrogenase gene expression of co-cultures in vitro

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Abstract

Objectives The present study aims at correlating the relative bacterial activity with the H^+ concentration and the *ldh* expression of caries-associated bacteria in co-cultures.

Materials and methods Well plates were prepared with BHI medium and cultures of *Lactobacillus paracasei* and *Fusobacterium nucleatum*. Bacterial growth at 37 °C was measured using a microplate-photometer before and after adding sucrose to the samples. Samples of co-cultures ($n = 12$) and single-species cultures ($n = 3$) were taken and pH was assessed. Real-time quantitative PCRs were applied targeting the 16S-gene, the 16S-rRNA, the *ldh*-gene, and the *ldh*-mRNA.

Results For *L. paracasei* with sucrose, an increase in relative bacterial activity (62.8% ± 23.5% [mean, SE]) was observed, while *F. nucleatum* showed a clear decrease in relative bacterial activity (− 35.0% ± 9.6%). Simultaneously, the H^+ concentration increased (1.15E-05 mol*l-1 ± 4.61E-07 mol*l-1). Consequently, a significant positive correlation was found between *L. paracasei's* relative bacterial activity and H^+ concentration (Spearman rank correlation, $r = 0.638$; $p = 0.002$), while *F. nucleatum* exhibited a negative correlation ($r = -0.741$; $p \leq 0.001$). Furthermore *L. paracasei* with sucrose showed a moderate, but significant positive correlation between relative bacterial activity and *ldh*-expression ($r = 0.307$; $p = 0.024$).

Conclusions and clinical relevance The relative bacterial activity after sucrose pulse showed a significant correlation not only to the acid production (H^+ concentration) but also to *ldh* expression of *L. paracasei*. However, further research is required to confirm these findings in a mature biofilm in vivo.

Keywords Dental caries/etiology · Relative bacterial activity · 16S rRNA · Lactobacilli · Fusobacteria · Biofilm · *ldh*-expression

Introduction

Dental caries lesions occur, as changes in the metabolic activity of a bacterial consortium of dental biofilm (dental plaque) provoke a localized chemical dissolution [1]. The "Global Burden of Disease Study 2013" states an incidence of tooth pain due to dental caries lesions with more than 200 million cases in 2013 [2]. It is therefore necessary to improve our understanding of the ecological processes in a caries lesion [3]. Dental caries initiate with a shift towards a more acidogenic potential of the microflora, as, e.g., frequent sugar intake promote acid-induced adaption as well as acid-induced selection of the bacterial microflora [3]. The healthy oral cavity presents a distinctive predominant bacterial flora, which is highly diverse and site and surface specific [4]. Phyla included are Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, Fusobacteria, and TM7 phylum [5, 6]. Dominant species in caries lesions are *Streptococcus mutans*, *Lactobacilli*, and *Bifidobacteria* [7, 8]. Facultative anaerobic and acidogenic

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bacteria often utilize sugar for energy metabolism, and the acidic metabolic byproducts, such as lactic acid, are able to demineralize the tooth surface [9]. Therefore, a high metabolic activity of acidogenic bacteria could be indicative for acid production [10]. However, very little is known about the metabolic activity of caries-associated and non-caries-associated bacteria [4, 11]. Due to technical advances in 16S rRNA-gene analyses, determinations of abundances in microbial communities are improved [12]. Nevertheless, those approaches focusing only on the genome may also amplify DNA from inactive or even dead microorganisms [13]. In contrast, 16S rRNA provides an accurate platform to analyze metabolically active species. Since the ribosome load of a cell rises with its activity [14], the increasing energy metabolism can be displayed with increasing 16S rRNA content. As the 16S-rRNA abundance correlates with the number of ribosomes, while the 16S-gene abundance is a measure for the number of genomes, the quotient of the relative abundances can function as a measure for the relative bacterial activity (Fig. 1) [10]. In contrast to the more comprehensible measure "ribosomes/cell", the relative bacterial activity is a rather robust value, as it is independent from different extraction efficiencies for DNA and RNA [15]. Because both values for the calculation of relative abundances derive from the same sample and the same extraction, both extraction efficiencies are identical and can be removed from the fraction, when calculating the relative abundances. Nevertheless, bacterial metabolism is strongly influenced by the pH of the dental biofilm [16, 17], since it is the key virulence factor for the development of caries lesions and it causes the selection of acidogenic and aciduric species [18].

Most oral acidogenic bacteria produce lactic acid as a result of sugar homofermentation, catalyzed by the enzyme lactate dehydrogenase (LDH). Thus, the production of LDH or—on gene level—the *ldh* expression in acidogenic bacteria directly correlates with their acid production and thereby their cariogenic activity. If increased acid production (measured by *ldh* expression) results in an increased metabolic activity (measured by an increase in ribosomal rRNA), it is likely that the measurement of the relative bacterial activity identifies those bacteria that contribute most to the caries development. However, until now, it has never been shown in literature that the relative bacterial activity correlates with the lactic acid production of caries-associated bacteria. Hypothetically, the

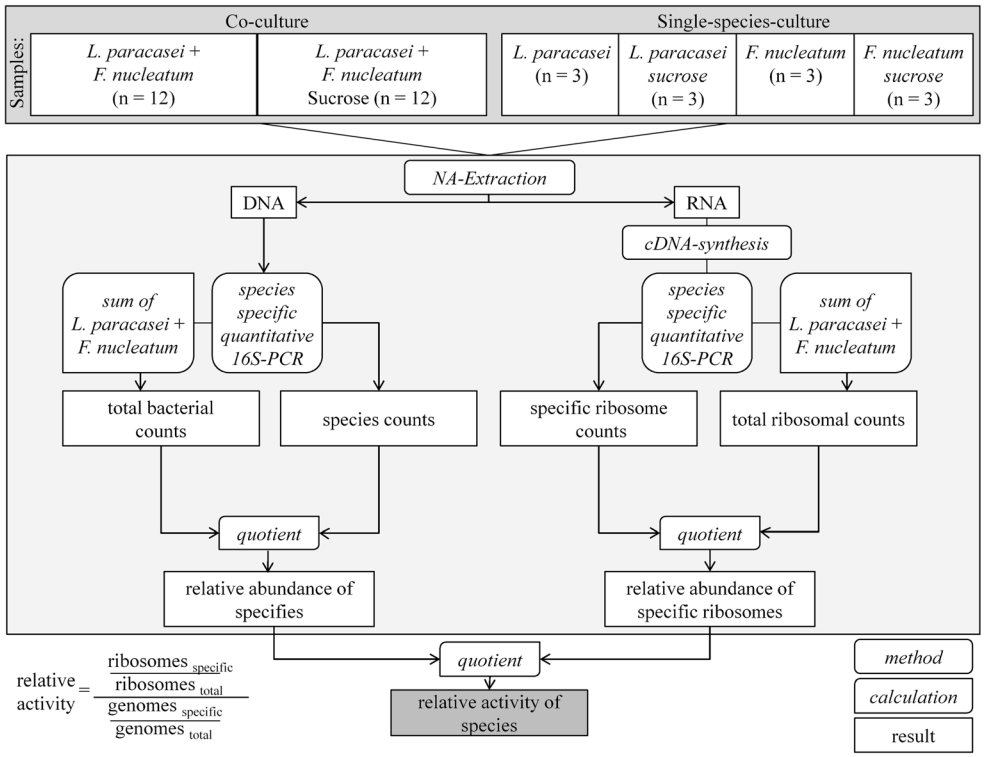


Fig. 1 Flow diagram showing gradual progress from sampling to calculation of relative bacterial activity. Co-cultures of *L. paracasei* and *F. nucleatum* with and without sucrose are shown on the left side. Single-species cultures are presented on the right side. Following the DNA/RNA extraction, specific quantitative 16S-directed PCR reactions were performed. The quotient of species counts and specific ribosome counts represents ribosomes/cell. The quotient of total bacterial counts and species counts represents the relative abundance of species (DNA-level), whereas the quotient of total ribosome counts and specific ribosome counts represents the relative abundance of ribosomes (rRNA-level). The quotient of the relative abundance of a specific ribosome and the relative abundance of the respective genome equals the relative bacterial activity

reason for increased relative bacterial activity besides increased acid production (catabolism) could be also intense bacterial growth (anabolism). In order to answer this question, we developed an in vitro study model with simplified parameters. Instead of working with a complex microbial community in a mature biofilm, we reduced our approach to the 'minimal version' of a planktonic microbial community: We inoculated BHI medium with a caries-associated species (*Lactobacillus paracasei*) and with a non-caries-associated species (*Fusobacterium nucleatum*), to simulate basic shifts and interactions of a cariogenic community when sucrose is supplied. Inclusion criteria for a caries-associated bacterium were: saccharolytic fermentation, high acidogenic/aciduric potential, and a proven association with dental caries lesions [19–21]. Inclusion criteria for non-caries-associated bacteria were: weakly saccharolytic or asaccharolytic fermentation, low acidogenic/moderate aciduric potential, and a proven association with healthy dental surfaces [22, 23].

Therefore, the aim of the present study was to correlate the relative bacterial activity with the H^+ concentration and the *ldh* expression of caries-associated bacteria in co-cultures.

We formulated the following null hypothesis: There is no significant correlation between the relative bacterial activity of *L. paracasei* with the H^+ concentration and the *ldh* expression.

Materials and methods

Preparing bacterial strains

Lactobacillus paracasei E17/91 and *Fusobacterium nucleatum* ATCC 25586 were cultivated on blood agar plates (37 °C for 48 h, under anaerobic conditions). Loopful inoculations of *L. paracasei* and *F. nucleatum* were transferred into 200-ml BHI medium (Brain Heart Infusion CM1135, Oxoid, Hampshire, England [pH = 7.4]) and were finally grown overnight (37 °C for 24 h, under anaerobic conditions). Shortly before preparing the wells, the overnight cultures were vortexed to ensure homogenous suspension of cells and were then used to inoculate the wells without a prior washing step. First 500 μl of modified BHI medium (with addition of L-cysteine and hemine [0.05%]) was loaded into wells (24-Well Multiwell Plates, Falcon, VWR). Wells for single-species culture were further inoculated with 500-μl overnight culture (late stationary growth phase) of the respective organism, whereas wells for co-culture were inoculated with 400-μl overnight culture of *F. nucleatum* and 100-μl overnight culture of *L. paracasei* (Online Resource 2.a). Preliminary growth experiments gave the information that *F. nucleatum* needed a higher start concentration to avoid *L. paracasei* overgrowth (non-published data).

Preparing 24-well plates

Immediately after inoculation of the bacterial suspension into the inner wells, an anaerobic GasPak powder (BD, Sparks, USA) was dispersed in the outer wells. To ensure anaerobic conditions, the indicator of the anaerobic GasPak, which changes its color if it comes in contact with oxygen, was constantly checked (Online Resource 2.a).

Subsequently, a 10-μl drop of a highly viscous 75% sucrose solution was placed under the cover of those wells that were planned for sucrose pulsing. To make sure that the sucrose drop was still attached to the cover and not touching the bacterial suspension, all well plates were visually controlled and finally hermetically sealed with additive free silicone adhesive (Siliconenkit Glas, BISON, EW Goes, Netherlands) to ensure anaerobic growth (Online Resource 2.b). We prepared five identical 24-well plates (each for every time point: 0, 1, 3, 6, and 10 h); because once a well plate was opened for sampling, anaerobic conditions were disturbed and further use of the plate was not possible (Online Resource 2.c).

After 4 h of initial pre-incubation (37 °C with slight shaking), all well plates were centrifuged 1 s at 106 g to ensure that the sucrose drops reached the intended culture, resulting in a final sucrose concentration of 7.43 g/l in each well (Eppendorf, Centrifuge 5804, Wesseling-Berzdorf, Germany). Centrifugation settings were held in so mild conditions that no visual cell settling could be observed.

Growth curves

The bacterial growth [OD_{620nm}] was measured at different time points specified in Online Resource 2.c by the aid of a spectrophotometer (Spectramax i3, Molecular Devices, Biberach an der Riss, Germany; optical density at 620 nm [OD_{620nm}]). During incubation, all well plates were continuously kept at 37 °C on tumbling tables to ensure resuspension throughout the whole experiment. Specific culture conditions are summarized in Table 1.

Sampling for molecular analyses

After 4-h pre-incubation, sucrose pulse was given at 'point zero' ($t = 0$ h). The first well plate out of five was opened and samples were taken for molecular analyses. Each well in co-cultures was sampled four times and immediately transferred into Eppendorf tubes [1.5 mL], resulting in $n = 12$ measurements (Online Resource 2.a). The single-species cultures used as negative controls (for observation of the species behavior alone) were sampled three times (Online Resource 2.a). Immediately after opening the well plate, samples for molecular biological analyses were obtained from all wells (4 × 50 μl per well), centrifuged (5 min/5283 g) and the pellets were stored at − 72 °C.

Table 1 Culture conditions of *Lactobacillus paracasei* and *Fusobacterium nucleatum*

	Lactobacillus paracasei (LP)	*Fusobacterium nucleatum (FN)*
Strain	E17/91	ATCC 25586
overnight culture (OD_{620nm})	0.138 (1:10 dilution with 0.9% NaCl–only for OD meausrement)	0.168 (1:10 dilution with 0.9% NaCl–only for OD meausrement)
Culture condition	37 °C, anaerobic	37 °C, anaerobic
Medium	BHI + 0.1% L-cysteine + 0.05% hemine	BHI + 0.1% L-cysteine + 0.05% hemine
Volume added to well	Co-culture: − 100-µl overnight culture of LP Single-species: − 500-µl overnight culture of LP	Co-culture: − 400-µl overnight culture of FN single-species: − 500-µl overnight culture of FN
Incubation time prior to sucrose pulse	4 h	4 h

pH monitoring and contamination control

Subsequently, the pH was measured in the remaining volume with a MiniTrode (Hamilton, Bonaduz, Switzerland). The electrode tip was immersed in the well at the center of the bacterial suspension until the diaphragm of the tip was covered. Finally, all wells at all time points were checked for cell morphology and contamination by Gram-staining and plating on blood agar (Online Resource 3). The pH monitoring and contamination control were repeated at all subsequent timepoints (1, 3, 6, and 10 h).

Molecular analyses (genome and ribosome count determination)

DNA and RNA were isolated using the extraction kit "innuPREP DNA/RNA Mini Kit" (Analytik Jena, Berlin, Germany). For the initial lysis using lysozyme and mutanolysin (3 mg lysozyme, 100 U mutanolysin, in 200-µl Tris EDTA buffer), the samples were incubated for 10 min at 37 °C. Further isolation was performed according to the manual with elution volumes of 100 µl for both, DNA and RNA. Right after extraction, 10 µl of the RNA was reversely transcribed into cDNA products, using random hexamer primers (60 µM) with the "Transcriptor First Strand cDNA Synthesis Kit" (Roche, Mannheim, Germany) according to the manual. Specific *Lactobacillus*-primer [24] and *Fusobacterium*-primer [25, 26] (Online Resource 1) were used to amplify the 16S rRNA-transcripts or the respective gene in a real-time quantitative polymerase chain reaction (q-PCR). The q-PCRs were performed on a LightCycler 2.0 (Roche) with 1 µl of cDNA after 1:4 dilution in bidistilled water as template.

Molecular analyses (*ldh* expression)

Self-designed L-lactate-dehydrogenase-gene (*ldh*) directed primer with a specificity to the *l-ldh*-variant of all members of the *Lactobacillus casei*-group (Online Resource 1) was used to amplify the *ldh*-gene or the respective cDNA in a q-PCR with 1 µl of DNA or cDNA, the latter again 1:4 diluted in bidistilled water.

Relative bacterial activity

Relative bacterial activity was calculated as described previously [10]. Shortly, total genome counts and total ribosomal count were calculated by the addition of 16S-rRNA-gene counts or 16S-rRNA transcript counts, respectively. The quotient of total bacterial genome counts and specific species genome counts represents the relative genome abundance of the respective bacterium, whereas the quotient of total ribosomal counts and specific species ribosomal counts represents the relative ribosome abundance. The quotient of the relative abundance of a specific ribosome and the relative abundance of the respective genome equals the relative activity. Consequently, values over 100% represent higher ribosome content and thus higher activity of the regarded bacterium compared to the average activity of all bacterial cells; values under 100% represent a lower relative activity (Fig. 1).

Statistical analysis

Data were analyzed using SPSS statistical software (SPSS 23.0; SPSS, Munich, Germany). All the data were not normally distributed; therefore, comparisons between the groups were calculated with the non-parametric, Mann-Whitney *U* test. For correlation analysis with the relative bacterial activity, values of the H^+ concentration were calculated based on the pH values measured. Differences in the H^+ concentration (ΔH^+) were calculated subtracting values from one time point to the previous one (H^+_{1h}-H^+_{0h}; H^+_{3h}-H^+_{1h}). Correlation between ΔH^+ and the difference of relative bacterial activity (Δ RBA) within the first 3 h was tested (RBA_{1h}-RBA_{0h}; RBA_{3h}-RBA_{1h}). Correlation between ΔH^+ and the difference of the relative ribosome abundance (RRA) and the relative genome abundance (RGA) within the first 3 h was also tested (RRA_{1h}-RRA_{0h}; RRA_{3h}-RRA_{1h}/

RGA_{1h}-RGA_{0h}; RGA_{3h}-RGA_{1h}). Finally, correlation between the *ldh* expression and the relative bacterial activity was tested. All correlations were tested with Spearman rank correlation.

Results

Growth curves

Co-cultures without sucrose pulse reached their maximum of 0.32 ± 0.0025 (OD_{620nm}; mean, SE) while completing pre-incubation (Fig. 2a). Co-cultures with sucrose pulse, after reaching a local maximum of 0.37 ± 0.0062 (OD_{620nm}) at the end of pre-incubation, showed a sucrose-induced second growth-phase and reached a new maximum of 0.59 ± 0.0239 (OD_{620nm}) after 6 h (Fig. 2b). Single-species cultures of *F. nucleatum*, which served as a control group, did not show any influence of sucrose pulse resulting in identical growth curves for cultures with and without sucrose. In contrast, *L. paracasei* cultures showed clear differences. While cultures without sucrose pulse remained more or less constant at 0.24 ± 0.0032 (OD_{620nm}), those with sucrose pulse showed an increased growth reaching 0.54 ± 0.0073 (OD_{620nm}).

pH monitoring

All cultures with *L. paracasei* and sucrose showed a clear decrease of pH values (Fig. 2b, dotted lines). *L. paracasei* culture acidity in single-species cultures with sucrose showed the lowest pH of 3.96, whereas *F. nucleatum* in single-species cultures with and without sucrose showed an almost constant pH of 6.3 ± 0.3.

Molecular analyses (genome and ribosome count determination)

During extraction, one replicate of the co-cultures with and without sucrose was lost and could not be further analyzed, reducing the number of measurements to 11. The genome and ribosomal counts of both *L. paracasei* and *F. nucleatum* without addition of sucrose stayed rather constant over 10 h (Fig. 3a). With sucrose pulse, the genome counts for *L. paracasei* were continuously increasing, whereas *F. nucleatum*, after revealing highest genome counts after 1 h ($1.28\ E+07 \pm 1.08E+06$ genomes/µl), steadily decreased. The ribosome counts of *L. paracasei* with sucrose pulse showed a first maximum 1 h after sucrose pulse, while *F. nucleatum* showed overall low ribosome counts over 10 h (Fig. 3b).

Significant positive correlations within the first 3 h after sucrose pulse were found between ΔRRA and ΔH^+ for *L. paracasei* ($r = 0.661$; $p = 0.001$) and a negative correlation for *F. nucleatum* ($r = -0.661$; $p = 0.001$), whereas ΔRGA showed no significant correlation for caries-associated and non-caries-associated bacteria and ΔH^+ (Fig. 6).

Molecular analyses (*ldh* expression)

Without sucrose, *L. paracasei* showed a rather flat curve of *ldh* expression, with a maximum at the beginning, which we

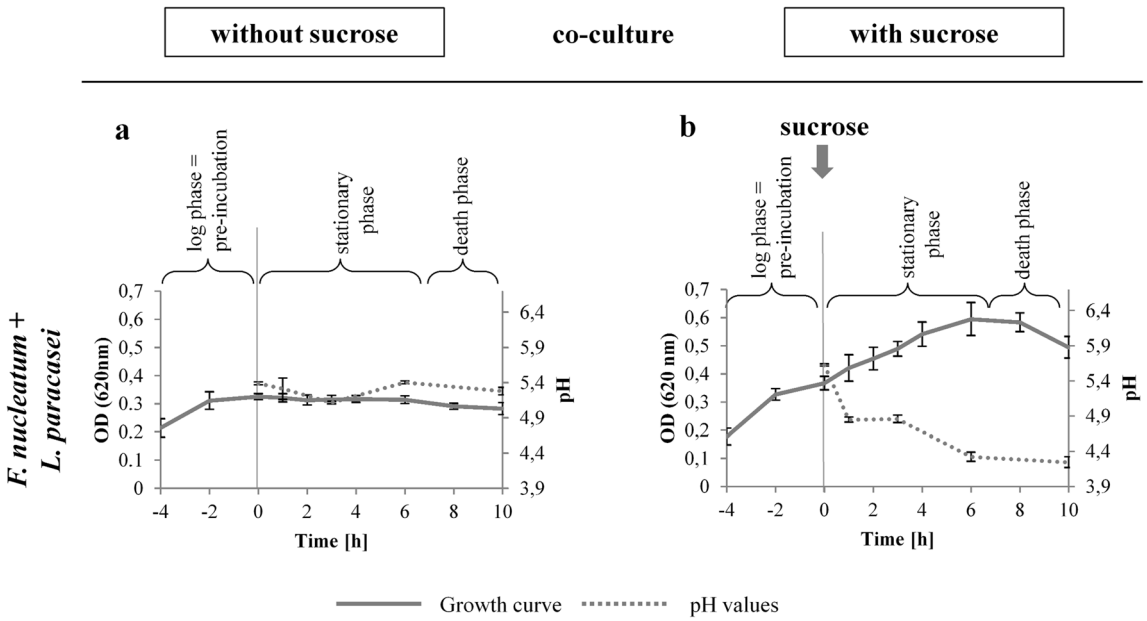


Fig. 2 Growth curve (OD_{620nm}, mean, SE) of *L. paracasei* and *F. nucleatum* as co-culture without (**a**) and with (**b**) sucrose pulse (bold lines) and corresponding pH values (mean, SE) (dotted lines). Co-cultures without sucrose pulse reached their maximum while completing pre-incubation. Co-cultures with sucrose pulse showed a sucrose-induced second growth phase. All cultures with *L. paracasei* and sucrose showed a clear decrease of pH values

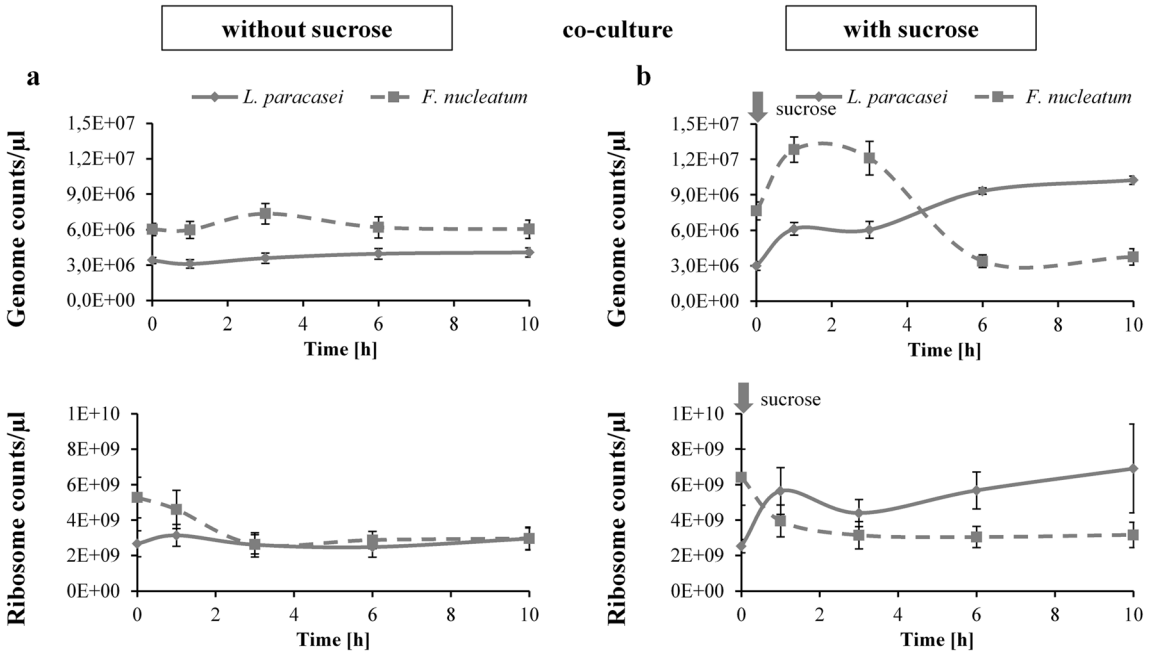


Fig. 3 Genome and ribosome counts (mean, SE) for *L. paracasei* and *F. nucleatum* without (**a**) and with (**b**) sucrose. The genome and ribosomal counts of both *L. paracasei* and *F. nucleatum* without addition of sucrose stayed rather constant. With sucrose pulse, the genome counts for *L. paracasei* were continuously increasing, whereas *F. nucleatum*, after revealing highest genome counts after 1 h, steadily decreased. The ribosome counts of *L. paracasei* with sucrose pulse were continuously increasing, but with a first maximum 1 h after sucrose pulse, while *F. nucleatum* showed overall low ribosome counts

interpret as a residual activity of the pre-culture. With sucrose, *L. paracasei* presented a clear peak (8.17E + 07 ± 2.32E + 07copies/µl) of *ldh* expression 1 h after sucrose pulse, as a prerequisite for intense sucrose hydrolysation and fermentation (Fig. 4).

Interestingly, *L. paracasei* with sucrose showed a significant positive correlation between the relative bacterial activity and the *ldh* expression ($r = 0{,}307$; $p = 0.024$), whereas *L. paracasei* without sucrose showed a negative correlation ($r = -0{,}382$; $p = 0.004$) (Fig. 7b).

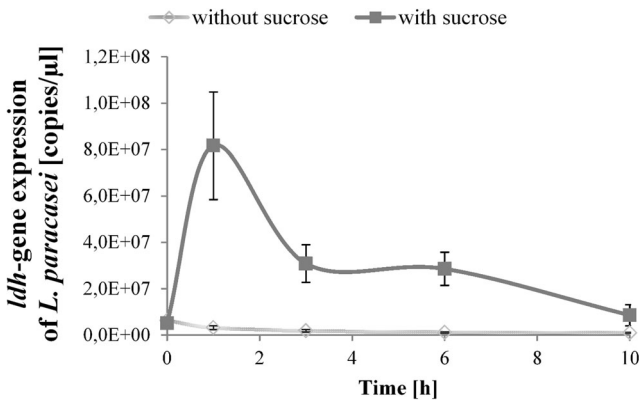


Fig. 4 The *ldh* gene expression of *L. paracasei.* Without sucrose, *L. paracasei* showed a rather flat curve, with the highest expression at the beginning, which we interpret as a residual activity of the pre-culture. With sucrose, *L. paracasei* presented an intense peak of *ldh* gene expression 1 h after sucrose pulse, as a prerequisite for intense sucrose hydrolysation and fermentation

Relative bacterial activity

Without sucrose pulse, both *L. paracasei* and *F. nucleatum* showed nearly constant relative genome abundances over 10 h of around 38% (*L. paracasei*) and 62% (*F. nucleatum*). The relative ribosome abundances behaved rather similar, with equal proportions of ribosomes for both species and a rather flat curve, with a moderate increase for *L. paracasei* after 3 h. This in turn results in a relative bacterial activity of 149.3% (Fig. 5a). With sucrose, the relative genome abundance for *F. nucleatum* decreased abruptly after 3 h from 65.5 to 26.8%, while *L. paracasei* increased from 34.5% to a maximum of 73.2%. The relative ribosome abundance revealed overall higher values for *L. paracasei*, while *F. nucleatum* showed lower values of around 44%. This resulted in a higher relative bacterial activity for *L. paracasei* within the first 3 h in comparison to the growth-competitor *F. nucleatum* with a maximum after 1 h of 188.8% (Fig. 5b).

Significant positive correlation was found between ΔRBA and ΔH^+ for *L. paracasei* ($r = 0.638$; $p = 0.002$), while *F. nucleatum* exhibited a negative correlation ($r = -0.741$; $p \leq 0.001$) (Fig. 7a).

Discussion

To our knowledge, this is the first study, which investigates the relation between acidification of the bacterial environment

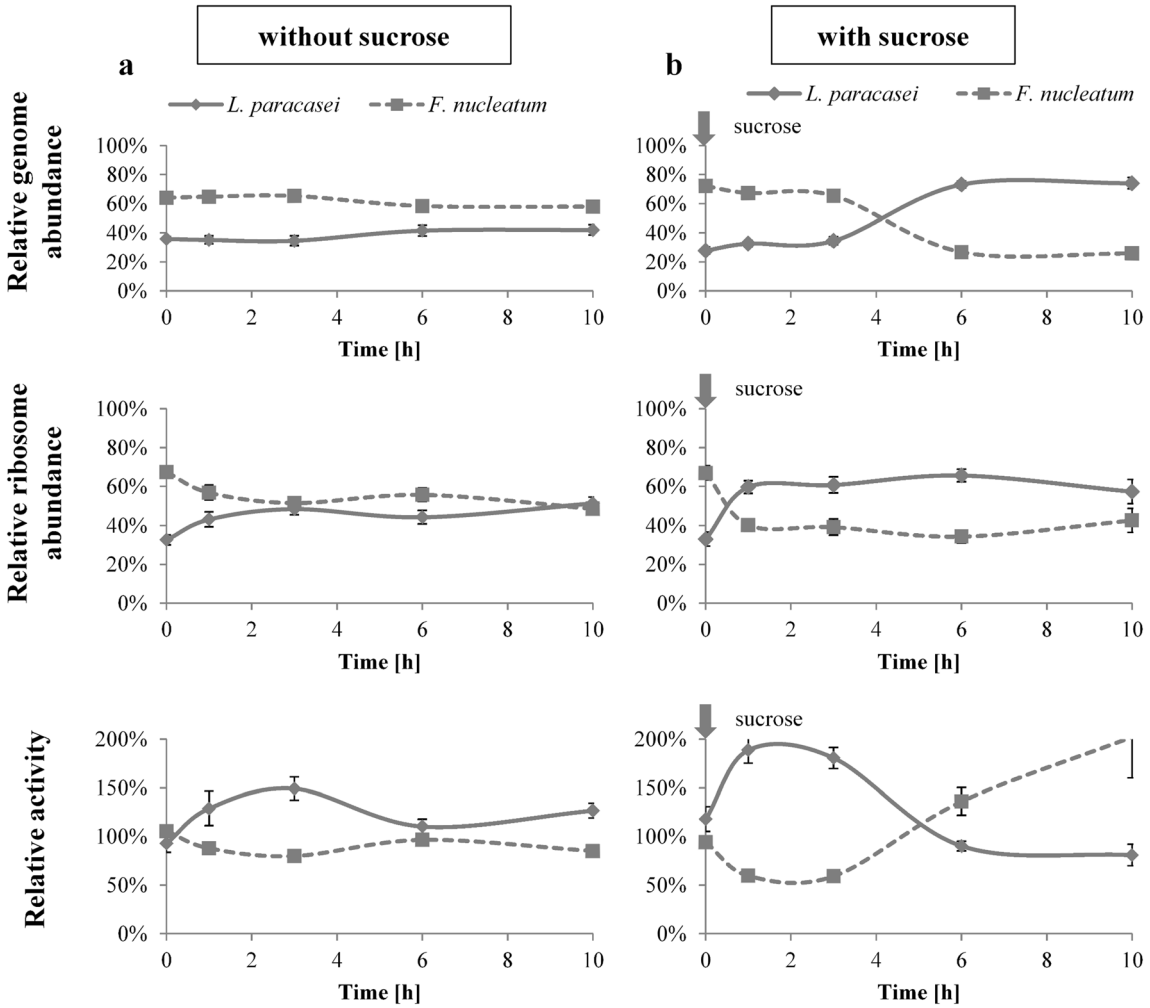


Fig. 5 Relative genome abundance, relative ribosome abundance, and relative bacterial activity (mean, SE) for co-culture without sucrose (**a**) and with sucrose (**b**). The quotient of the relative abundance of a specific ribosome and the relative abundance of the respective genome equals the relative bacterial activity

and the changes in relative bacterial activity of caries-associated and non-caries-associated bacteria.

In our previous in vivo study, we revealed distinct trends of bacterial activity of caries-associated and non-caries-associated bacteria in the biofilm of healthy sound surfaces and cavitated caries lesions. Caries-associated, saccharolytic bacteria, such as *Lactobacillus paracasei*, *Streptococcus mutans*, or *Scardovia wiggsiae*, showed highest relative bacterial activity in caries lesions and lower activities on sound surfaces, whereas weakly saccharolytic *Fusobacterium nucleatum*, as representative for a non-caries-associated species, was most active on healthy sound surfaces [10]. Yet, it was still unclear if the reasons for those metabolic changes in the biofilm were rather caused by the increased growth of the bacteria (anabolism) than increased acid production (catabolism). A variety of organic acids, produced by the complex bacterial consortium within the oral biofilm, can be found in active and arrested lesions. However, active lesions demonstrate a lactate-dominated acid profile [7, 17, 27, 28]. Since the lactate dehydrogenase is an enzyme found in most acidogenic bacteria catalyzing the conversion from pyruvate into lactic acid [29–31], we considered the *ldh*-gene as a suitable parameter to predict acid production in caries-associated bacteria. Therefore, we developed an in vitro study model with simplified parameters, in which the proof of principle was the aim and only two representative species were chosen. A normal plaque consortium consists of multiple species, including several acidogenic and non-acidogenic bacteria, strictly anaerobes and aero-tolerant bacteria, and saccharolytic as well as proteolytic bacteria [5, 32, 33]. Of course, in vitro studies are not able to reflect all complex interactions found in a multi-species biofilm, but they do can demonstrate simple microbe-microbe and microbe-host interactions [34]. Since the aim of this in vitro study was to investigate the relation between the pH drop and the *ldh*-expression with the changes in relative bacterial activity, this 'minimal version' of a planktonic microbial community seems justified. However, further clinical studies should evaluate whether the distinct trends shown in

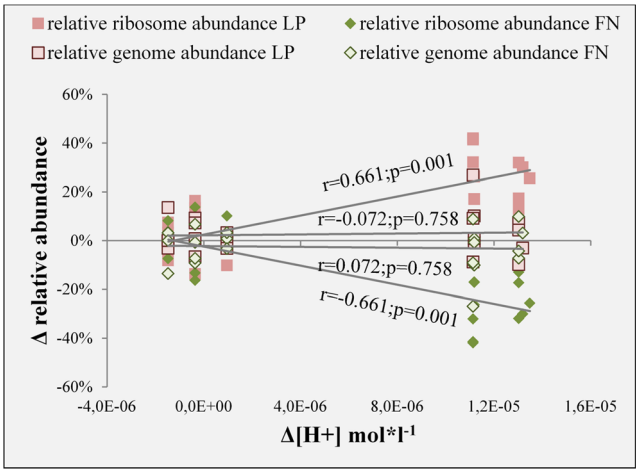


Fig. 6 Scatter plot of significant correlation between ΔRRA/ΔRGA and ΔH^+ of *L. paracasei* (LP) and *F. nucleatum* (FN). The ΔRRA showed a significant correlation for caries-associated and non-caries-associated bacteria [Spearman rank correlation; *L. paracasei* ($r = 0.661$; $p = 0.001$); *F. nucleatum* ($r = -0.661$; $p = 0.001$)], whereas ΔRGA showed no significant correlation for caries-associated and non-caries-associated bacteria and ΔH^+. RRA, relative ribosome abundance; RGA, relative genome abundance

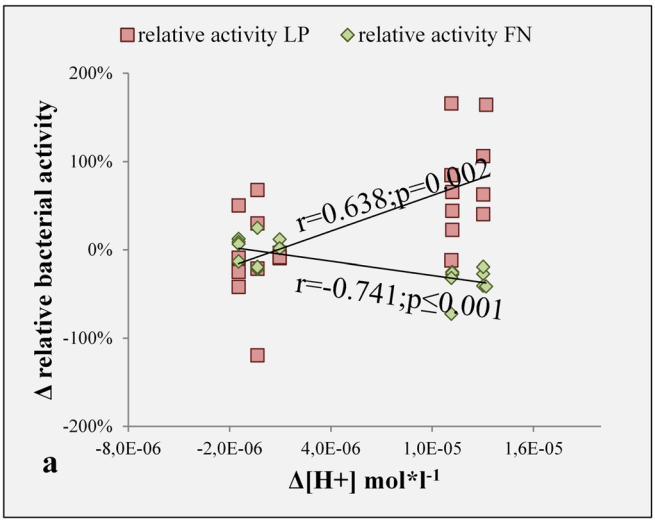
this in vitro study hold when analyzing a multi-species biofilm. Future research should focus on OMICS-based approaches, such as (meta-) genomics, transcriptomics, proteomics, and metabolomics, which can promote important insights into the complex oral ecology [4, 35]. Nevertheless, this in vitro study demonstrated moderate but significant correlation between the H^+ concentration and the *ldh* expression with the relative bacterial activity for *L. paracasei*. Therefore, our null hypothesis was rejected.

pH-monitoring

As expected, single-culture experiments of *L. paracasei* without sucrose pulse did not result in a decrease of initial pH. After sucrose pulse, only *L. paracasei* was able to lower the pH under 4, below the critical pH of 5.5 for enamel demineralization [17, 36], indicating intense sucrose hydrolyzation and fermentation [28]. *F. nucleatum* in single culture, on the other hand, did not change the pH indicating no or very low metabolization of sucrose. However, when both species were co-cultured and supplied with sucrose, again a clear decrease in pH was observed (Fig. 2b). As the reached pH was not as low as in the single-culture experiment of *L. paracasei*, it can be assumed that *F. nucleatum* was able to antagonize to some extent the acidification caused by *L. paracasei*. These observations are in accordance with the findings, that *F. nucleatum* showed high acid-neutralizing activity in vitro [37].

Molecular analyses (genome and ribosome count determination)

Co-cultures without sucrose pulse showed nearly constant genome and ribosome counts for both species, indicating a stable species equilibrium during this experiment (Fig. 3a). However, the equilibrium showed dramatic disturbance when sucrose was added. *L. paracasei* genomes showed a low, but



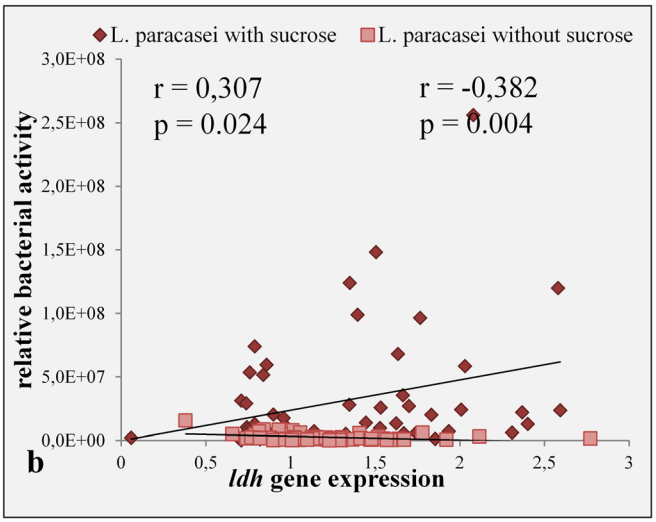


Fig. 7 **a** Scatter plot of significant correlation between ΔRBA and ΔH^+ of *L. paracasei* (LP) and *F. nucleatum* (FN). Caries-associated bacteria showed a positive correlation between relative bacterial activity and acidogenicity (Spearman rank correlation, $r = 0.638$; $p = 0.002$), whereas non-caries-associated bacteria showed a negative correlation between relative bacterial activity and acidogenicity (Spearman rank correlation, $r = -0.741$; $p \leq 0.001$). **b** Scatter diagram of significant correlation between the relative bacterial activity and the *ldh* expression of *L. paracasei*. *L. paracasei* with sucrose showed a significant positive correlation between the relative bacterial activity and the *ldh* expression [Spearman rank correlation ($r = 0{,}307$; $p = 0.024$)], whereas *L. paracasei* without sucrose showed a negative correlation ($r = -0{,}382$; $p = 0.004$). RBA, relative bacterial activity

continuous increase accompanied with a similar rise in ribosome numbers, with a local maximum directly after the sucrose pulse (Fig. 3b). High ribosome numbers for *L. paracasei* in cavitated caries lesions were previously demonstrated in our in vivo study [10]. As the first maximum coincided with the most intense drop in pH, we assume that this peak in ribosome numbers was mainly due to the production of enzymes (such as LDH) necessary for cleavage and further degradation of sucrose resulting in acid production. As Gross et al. already demonstrated, *S. mutans* and *Lactobacillus* species are key players in lesion progression of the young permanent dentition, by fermentation of available carbohydrates to lactic acid [7].

Interestingly and contrary to our expectations, a clear increase in *F. nucleatum* genomes could be observed in the first 3 h after sucrose pulse (Fig. 3b). This increase of *F. nucleatum*, a species known to prefer amino acids and peptides over carbohydrates for energy metabolism [22, 38], was only seen in co-cultures.

One possible explanation for this synchronic behavior within the first few hours could be that *L. paracasei* is able to cleave the disaccharide sucrose into fructose and glucose, which can be weakly utilized by *F. nucleatum* and further be stored as intracellular polysaccharides (IPS) [22, 34, 39, 40]. Indeed, gram-stains of the co-cultures after 3 h indicated shorter *F. nucleatum* cells with more granules compared to those at the beginning, but no increased ribosome numbers could be observed at this time point (Online Resource 3). Thus, we assume that the internalization of carbohydrates, indicated by increased granule numbers [40, 41], might somehow trigger the cell division decoupled from cellular growth, as the low available amino acid resources are mainly used for cell division. After 3 , when the co-cultures were more and more acidic, *F. nucleatum* genomes decreased to a level lower than that without sucrose pulse, which can be explained by an adverse effect of low pH (4.4 versus 5.4 without sucrose).

Relative bacterial activity

The relative genome and ribosome abundance remained rather constant without sucrose pulse, with only a slight decrease of *F. nucleatum* ribosome abundance at the beginning, which we interpret as a residual activity of the pre-culture. Combining both abundance measures resulted in a relative bacterial activity curve with only slight changes (Fig. 5a). The substantial change after 4 h in the genome proportions of the co-cultures with sucrose pulse is the consequence of the growth of the *L. paracasei* population and the simultaneous death of the *F. nucleatum* population (Fig. 5b). This proportional shift took place when the pH dropped below 4.5. This was described before as the critical pH, where acidogenic and aciduric bacteria profit most at the expense of more acid sensitive bacteria [42, 43]. Although the genome proportion of *L. paracasei* increased quite slowly, the respective ribosome proportion increased directly in the first measurement after the sucrose pulse. This in turn resulted in an intense peak of relative bacterial activity until 4 h after sucrose pulse. The genome and ribosome values in this in vitro study illustrate the importance of both, the DNA analysis (providing the genetic repertoire of the microorganism, metagenomics) and the RNA analysis (providing the expression pattern, transcriptomics) [4], as only both measurements combined can point out the changes in relative bacterial activity among caries-associated and non-caries-associated bacteria. Thus, it is plausible to assume that this peak of *L. paracasei's* relative bacterial activity may result from sucrose degradation and acidification. To further clarify this aspect, we also performed correlation analyses with the acid production and the relative ribosome abundance (indicating activity) or the relative genome abundance (indicating growth) (Fig. 6a). Interestingly, the ribosome proportion showed a significant correlation for *L. paracasei* and *F. nucleatum*, but for the genome proportion, no significant correlation was observed. We conclude that the increase in relative bacterial activity measures after sucrose pulse is rather caused by the activation of catabolism (leading to increased acid production) than by the activation of anabolism (leading to bacterial growth and multiplication). Furthermore, the correlation analysis between changes in relative bacterial activity and changes of the H^+ concentration of *L. paracasei* and *F. nucleatum* revealed a significant positive and negative correlation, respectively (Fig. 7a).

Interestingly, and in contrast to the experiment without sucrose pulse, the relative bacterial activity of *F. nucleatum* increased again after 4 h, in spite of its decrease in proportion and cell numbers. Zilm et al. described that a low pH activates the amino acid catabolism, indicating an increased requirement for ATP, supporting the view that energy requiring protective mechanisms are used to maintain internal homeostasis [39].

Conclusions

In conclusion and within the limitation of this study, we showed for the first time that the relative bacterial activity of cariogenic *L. paracasei* after sucrose pulse has a significant correlation not only to the acid production (H^+ concentration) but also to the *ldh* expression in co-cultures. From a cariological perspective, the determination of the relative bacterial activity in health and disease can improve our current understanding of the caries process, as slight changes in bacterial activity can lead to caries lesion progression or regression. However, future clinical studies should evaluate whether

the distinct trends shown in this in vitro study hold when analyzing an in vivo multi-species biofilm.

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Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any authors.

Informed consent For this type of study, formal consent is not required.

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