Article:
Mello de Matos, B, Brighenti, FL, Do, T et al. (2 more authors) (2017) Acidogenicity of dual-species biofilms of bifidobacteria and Streptococcus mutans. Clinical Oral Investigations, 21 (5). pp. 1769-1776. ISSN 1432-6981

https://doi.org/10.1007/s00784-016-1958-1

Bruno Mello de Matos, Fernanda Lourenção Brighenti, Thuy Do, David Beighton, Cristiane Yumi Koga-Ito

Acidogenicity of dual species biofilms of bifidobacteria and Streptococcus mutans

Bruno Mello de Matos – UNESP, Univ. Estadual Paulista, São José dos Campos-SP; UNIVASF, Univ. Federal do Vale do São Francisco, Paulo Afonso-BA, Brazil
Rua da Alvorada, General Dutra, Paulo Afonso, BA, 48607-190, Brasil
Fernanda Lourenção Brighenti – UNESP, Univ. Estadual Paulista, Araraquara-SP, Brazil
Rua Humaitá, 1680, Centro, Araraquara, SP, 14801-385, Brasil
Thuy Do – School of Dentistry, Faculty of Medicine and Health, University of Leeds, Leeds, UK
Division of Oral Biology, Wellcome Trust Brenner Building, Level 7, St James' University Hospital campus, LS9 7TF, Leeds, UK.
David Beighton – Dental Institute, King's College London, London, UK
Bessemer Rd., Denmark Hill, London SE5 9RW, UK.
Cristiane Yumi Koga-Ito – UNESP, Univ. Estadual Paulista, São José dos Campos-SP, Brasil
Institute of Science and Technology, UNESP - Avenida Engenheiro Francisco José Longo 777, São Dimas, São José dos Campos, SP, 12245-000, Brazil

Corresponding author: Cristiane Yumi Koga Ito, cristiane@fosjc.unesp.br, Telephone: +5512 981253017, Fax: +5512 39479000

Acknowledgements

The authors thank Coordination for the Improvement of Higher Education Personnel– CAPES/Brazil (Grant #3755/10-0) and São Paulo Research Foundation – FAPESP/Brazil (Grant #10/02063-1), and the staff of the Laboratory of Oral Microbiology – Academic Centre for Dentistry Amsterdam (ACTA) – Vrije Universiteit Amsterdam – Netherlands for its contribution (laboratory facilities and

consumables). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Objective: the aim of this study was to evaluate the acidogenicity of dual species biofilms of bifidobacteria and Streptococcus mutans. Materials and Methods: the following strains were tested: Bifidobacterium dentium DSM20436, Parascardovia denticolens DSM10105 and Scardovia inopinata DSM10107. Streptococcus mutans UA159 and Lactobacillus acidophilus ATCC4356 were used as control. Bifidobacteria were studied planktonically as they were not able to form monospecies biofilm, they were grown in biofilms associated with S. mutans. Endogenous polysaccharide reserves of cultures at log phase were depleted. Standardized suspensions of the microorganisms were incubated in growth media supplemented with 10 mM glucose, lactose, raffinose, glucose or xylitol. S. mutans biofilms were grown on glass cover slips for 24 h to which bifidobacteria were added. After 24 h, the dual species biofilms were exposed to the same carbon sources and after 3 h the pH of spent culture media and concentrations of organic acids were measured. Statistical analyses were carried out using ANOVA and Tukey's Test (α=0.05). Results: a higher pH drop was observed when S. mutans was associated with P. denticolens or S. inopinata, in either planktonic and biofilm cultures than from S. mutans alone. Bifidobacteria showed a higher pH drop in the presence of raffinose than S. mutans or L. acidophilus. Conclusions: Dual species biofilms of bifidobacteria and S. mutans produced more acid and a greater pH drops than biofilms of S. mutans alone. Clinical relevance: New insights on the complex process of caries pathogenicity contribute to the establishment of preventive and therapeutic measures, in particular in specific cases, such as in early childhood caries.

Keywords: acidogenicity, bifidobacteria, biofilms, dental caries

Introduction

The etiology of caries is undoubtedly complex. It is generally recognized that microbial, environmental and host factors interact to contribute to dental caries development [1]. Although dental caries is a biofilm-mediated disease, it is unlikely that all members of the oral biofilm participate equally in the caries process. The ecological plaque hypothesis suggests that the cariogenic oral environment will select increased proportions of acidogenic and aciduric microbiota [2]. These microorganisms include lactobacilli, streptococci, Actinomyces spp., yeasts and bifidobacteria [3]. The resultant pH drop may induce dental enamel demineralization under the critical pH of 5.5 [4].

Aas et al. [5] using molecular techniques demonstrated that 10% of subjects with rampant caries in secondary dentition did not have detectable oral levels of S. mutans in intact enamel and white-spot lesions. The authors suggested that at least half of the bacteria associated with dental caries have not yet been cultivated. Thus, there is a considerable body of evidence for the emergence of other taxa, in addition to S. mutans in a cariogenic oral environment or within carious lesions [3].

Many studies have reported the presence of bifidobacteria in the oral cavity of healthy and diseased children and adults. These bacteria were found in saliva, plaque and dental caries [3, 6-7]. Beighton et al. [8] demonstrated that the bifidobacteria levels in adults' saliva were not significantly different from the levels of mutans streptococci. Similar observations were described in caries active children [9].

Bifidobacterium dentium is the most prevalent bifidobacterial species in the oral cavity [3] with Parascardovia denticolens and Scardovia inopinata also frequently isolated [6, 10].

Bifidobacteriaceae consists of seven genera (Aeriscardovia, Alloscardovia, Bi*fi*dobacterium, Falcivibrio, Gardnerella, Parascardovia, Scardovia) and about 36 species, the majority of which have been described and isolated from the intestinal and caecal microbiota. The range of taxa reported to be oral commensal seems primarily restricted to Bifidobacterium dentium, Parascardovia denticolens and Scardovia inopinata [3].

Considering that little is known about the influence of environmental factors - dietary components in particular - on acid production by oral bifidobacteria, and that

acid production from carbon sources is an important cariogenic feature, the aim of this study was to evaluate the acidogenicity of bifidobacteria after exposure to different carbon sources, and determine if bifidobacteria are able to increase the acidogenicity in single and dual species planktonic cultures or biofilms in association with Streptococcus mutans.

Materials and Methods

Strains and incubation conditions

The following type strains of bifidobacteria were used: Bifidobacterium dentium DSM 20436, Parascardovia denticolens DSM 10105 and Scardovia inopinata DSM 10107. Also, Streptococcus mutans UA 159 and Lactobacillus acidophilus ATCC 4356 were included. S. mutans is considered an important species related to dental caries initiation whilst lactobacilli are related to dental caries progression.

Cultures were obtained for each species from two independent frozen stocks. S. mutans and B. dentium were grown in semi-defined medium broth supplemented with yeast extract (SDMY) and 0.2% sucrose [11]. L. acidophilus, P. denticolens and S. inopinata were grown in Lactobacilli MRS Broth (Difco, USA) developed by De Man, Rogosa and Sharpe [12]. All strains were grown to log phase at 37 ºC in anaerobic jars (10% CO_2, 10% H_2, 80% N_2).

Strains were studied planktonically and in biofilms and all experiments were performed in duplicate on two different occasions.

Preparation of planktonic cultures

Bacterial cultures were washed twice in cysteine peptone water (CPW) [5 g/l yeast extract, 1 g/l peptone, 8.5 g/l NaCl, 0.5 g/l L-cysteine-HCl]. Depletion of endogenous carbohydrate reserves in stationary phase cultures was performed by incubating the washed cells for 30 min in water bath at 37 ºC.

Standardized inocula of the microorganisms at OD_{620}=0.7 were prepared in an artificial saliva medium modified by McBain et al., 2005 [13], pH 7.0, for pH drop evaluation.

Pilot tests showed that, even though McBain medium is an artificial saliva medium, it causes undesirable interferences in organic acids analyses by capillary electrophoresis. During those tests, it was verified that the chemical composition of McBain medium produced peaks (observed on Millenium Chromatography Manager Software) that overlap to the acid peaks making them difficult to identify. For this reason, SDM broth (modified version of SDMY broth prepared without yeast extract), supplemented with aqueous solutions of glucose, lactose, raffinose, sucrose or xylitol in final concentrations of 10mM was used to organic acids analyses. Sterile distilled water was used as negative control.

Dual species suspensions were prepared using S. mutans inocula plus bifidobacteria at a 1:1 ratio.

Preparation of biofilms

Pilot studies showed that none of the bifidobacteria species and L. acidophilus was able to form single species biofilms in the model used in the present study. It was therefore not possible to assess the production of acid or utilization of carbohydrates by single species biofilms composed of bifidobacteria or lactobacilli alone. Instead, we assessed the acidogenicity of dual species biofilms formed inoculating either bifidobacteria or lactobacilli onto pre-formed S. mutans biofilms.

To produce the pre-formed S. mutans biofilms, S. mutans cultures were standardized in SDMY (OD_{620}=0.7) and diluted 1:50 in SDMY plus 0.2% sucrose. S. mutans biofilms were grown on glass coverslips (ø12 mm) using an active attachment model [14]. 24-wells plates were filled with 1.5 mL of the diluted inocula per well, and incubated for 24 h. The glass coverslips containing S. mutans biofilms were placed in 24-well plates that were filled with 1.5 mL of either bifidobacteria or L. acidophilus diluted inocula.

The bifidobacteria (B. dentium, P. denticolens or S. inopinata) or L. acidophilus cultures to be added to the S. mutans biofilms were prepared (OD_{620}=0.7) and diluted 1:50. Inocula of B. dentium were prepared in SDMY plus 0.2% sucrose and P. denticolens, S. inopinata and L. acidophilus were prepared in Lactobacilli MRS Broth (Difco, USA).

After 24 h of incubation, dual species biofilms were washed twice in CPW. Depletion of endogenous polysaccharide reserves was performed by incubating the biofilms in CPW for 30 minutes in water bath at 37 ºC.

The depleted biofilms were placed in 1.5 ml McBain medium or SDMY broth supplemented with 10 mM glucose, lactose, raffinose, sucrose or xylitol and incubated at 37 ºC for 3 h.

Assessment of biofilm viability

The presence of S. mutans, lactobacilli and bifidobacterial species in mixed biofilms was evaluated by culture method. Glass coverslips with biofilms were carefully detached from the clamps and placed in 2 mL CPW. Biofilms were dispersed by sonication on ice for 120 times one second at amplitude of 40 W (Vibra Cell™, Sonics and Materials Inc., Newtown, USA) [15]. Serially diluted samples were plated onto SB20 (sucrose bacitracin), Rogosa (Difco, USA) and MMTPY agar plates (modified version of mupirocin trypticase peptone yeast extract) for isolation of S. mutans, lactobacilli and oral bifidobacteria, respectively [8,16]. The plates were incubated anaerobically (as previously described) at 37 ºC for 48 h. Colonies were counted and expressed as colony forming units (CFU). Experiments were performed in sextuplicate on two different occasions.

Assessment of suspensions and biofilms acidogenicity

The pH of the culture medium was measured to estimate biofilm acidogenicity at 0 and 3 h and pH variations calculated. The measurements were performed with the aid of an electrode with a micro-bulb (Hanna, Woonsocket, Rhode Island, USA).

The amount of organic acids was analyzed by capillary electrophoresis (Waters Capillary Ion Analyzer; Milford, MA, USA) in plates with SDM broth. Samples were run in duplicate, and Millenium Chromatography Manager Software, version 3.05 was used for data analysis. Peak identification and peak area integration were manually corrected if necessary. Sodium salts of formic, acetic, propionic, butyric, succinic, and lactic acid were used to prepare single and mixed standard solutions in deionized water,

ranging from 0.05 to 2 mM. Calibration curves were made for each acid separately. As an internal standard, 0.1 mM oxalic acid was included in all samples. Lactic, propionic, acetic, formic, butyric, and succinic acid concentrations were determined [17]. Experiments were performed in quadruplicate on two different occasions.

Data analysis

Initially all data were compared to the appropriate water, no added carbohydrate control. Then for those cultures in which significant changes to pH or to acid levels occurred the results obtained for bifidobacteria species were compared to control microorganisms (S. mutans or L. acidophilus). Statistical analysis were carried out using Graphpad Prism 3 (ANOVA and Tukey's Test, α=0.05).

Results

None of the bacteria in the planktonic phase or in biofilms produced significant changes to the pH of the media or to the concentrations of lactic or acetic acids when incubated with xylitol (data not shown). Table 1 shows the pH drop (ΔpH) after the addition of different carbon sources to single species suspensions. For L. acidophilus, the pH drop was higher than S. mutans when glucose was used. Statistically significant ΔpH were observed, which indicate that the presence of raffinose seems to be better metabolized by the three bifidobacterial species than by S. mutans or L. acidophilus.

Higher pH drop was observed when S. mutans was associated with P. denticolens or S. inopinata, in either planktonic or biofilms cultures (Tables 1 and 2). The association between S. mutans and B. dentium in suspension promoted higher pH drop when lactose, raffinose or sucrose was used in comparison to S. mutans or S. mutans and L. acidophilus (Table 1). However, the co-culture of S. mutans and B. dentium in biofilms promoted lower pH drop than S. mutans single species biofilms or S. mutans and L. acidophilus biofilms.

Final pH for enamel demineralisation was below critical (5.5) for all microorganisms and associations when culture media was supplemented with glucose or sucrose. Co-culture of S. mutans/P. denticolens and S. mutans/S. inopinata led to the highest pH drop in presence of glucose (minimum final pH 4.3), sucrose (minimum

final pH 4.2), and raffinose (minimum final pH 4.6). The pH (5.5) was not reached in the presence of lactose, xylitol or control. Raffinose promoted a pH drop below critical pH in either single species suspensions or associated to other species, both in planktonic and biofilms. For this carbohydrate, final pH from S. mutans or S. mutans and L. acidophilus cultures remained above critical levels (pH 6.2 and 6.3, respectively) (supplementary material).

Table 3 displays the organic acids production of single species and dual species suspensions. Butyric, formic, propionic and succinic acids were below detection limit (0.01 mM, according to Kara et al., 2006 [17]). For B. dentium in single species cultures, more lactate is produced in the presence of raffinose or sucrose. When associated to S. mutans, lactate production was higher in the presence of raffinose. P. denticolens produces more acetate for all carbohydrates.

The same pattern of lactate production is observed for either single species or dual species suspensions of S. inopinata. On the other hand, while acetate production in S. inopinata single species is higher than S. mutans for glucose, raffinose and sucrose, in dual species cultures, significantly higher concentrations of acetate was found for all carbon sources.

In dual-species biofilms, the combination of S. mutans and B. dentium did not produce more acid than S. mutans or S. mutans and L. acidophilus biofilms, except for lactate production in the presence of raffinose. S. mutans and P. denticolens formed more lactate than S. mutans or S. mutans and L. acidophilus biofilms in the presence of glucose and sucrose. S. mutans and S. inopinata biofilms yielded more acetate and lactate in the presence of all carbon sources. When raffinose was added to the culture medium, S. mutans and S. inopinata biofilms produced 14 times more lactate and 48 times more acetate than S. mutans biofilms alone, even though this species participated with 1.46% of the mixed biofilm (Tables 4 and 5).

Discussion

The present study adds important information to the existing evidence in the literature. This is the first time that B. dentium, P. denticolens and S. inopinata are studied alone or in association with S. mutans, in either suspension or biofilms. Bacteria

organized in biofilms are offered a higher antimicrobial resistance not only due its spatial organization – that impairs the penetration of antimicrobial substances – but also due to the low growth rate, phenotypical modifications and also because biofilms are extremely organized communities, in which interaction between cells confers an important resistance mechanism, as previously shown by Kara et al., 2006 [17].

Pilot studies showed that bifidobacteria are not able to form single species biofilms in the model used in the present study (data not shown). This is the reason why bifidobacteria single species biofilms were not evaluated in the present study. This is also a notable finding because it shows the importance of the interaction of bifidobacteria species with other oral microorganisms. More detailed study of bifidobacteria biofilms, including other analyses (i.e., confocal analyses), can generate important data on this interaction and should be conducted in the future.

The ability of bifidobacteria in suspension form to produce acids was already demonstrated in previous studies. However, to the best of our knowledge this is the first report on biofilms of bifidobacteria co-cultured with S. mutans. Haukioja et al., 2008 [18] showed that four different bifidobacteria strains were also able to promote a pH drop below critical pH for enamel demineralisation (5.5) when different carbon sources are used. Moynihan et al., 1998 [19] showed that B. dentium decreases culture medium pH to values lower than enamel critical pH when exposed to glucose or lactose. Nakajo et al., 2010 [20] also demonstrated the ability of bifidobacteria (B. dentium and Bifidobacterium longum) to decrease the pH culture below 5.0 at an initial pH of 5.0-7.0, indicating that these bacteria are able of creating an acidic environment in dental plaque and caries lesions. The acidogenic profile of bifidobacteria reaffirms their role in the acidification of the oral environment, probably contributing in dental caries development.

Carbon sources used in the present study were chosen based on their presence in the diet. Glucose, lactose and sucrose are either naturally present in fruits, vegetables or milk or added at high concentrations to baked products, snacks and sweets [21]. Raffinose is naturally present in beans, cabbage, brussels sprouts, broccoli, asparagus, and whole grains [21]. Xylitol is a natural sweetener that adds texture to foods and is not metabolized by most oral bacteria, including S. mutans [22].

The results of the present study support the evidence that bifidobacteria species present in carious lesions are able to metabolize all carbon sources included in the present study at different rates. Bifidobacteria demonstrated that they are able not only to produce significant amount of acids but also to accentuate biofilm acidogenicity in combination with S. mutans.

A possible explanation for the significant pH drop for the association between S. mutans and bifidobacteria is that S. mutans metabolizes carbon sources at a higher rate, producing acids more quickly than bifidobacteria and lactobacilli. Both lactobacilli and bifidobacteria prefer lower pH to produce acids, so acid production by S. mutans promotes a favorable environment to these species.

Bifidobacteria are able to metabolize raffinose to a higher extent than S. mutans and L. acidophilus, which reflected in a higher pH drop. This is an important finding because bifidobacteria do not require the consumption of snacks or sweets to produce acid, since raffinose is naturally present in healthy foods consumed on a daily basis. This can indicate the cariogenicity of bifidobacteria, which should be clinically evaluated.

Moreover, a synergistic effect between S. mutans and P. denticolens or S. inopinata promoted a higher pH drop than these species alone. These results show that the presence of both species in dental biofilm indicates a higher cariogenic potential than if bifidobacteria are absent. This is of particular interest since some bifidobacteria are used in probiotic foods. The use of probiotics on a daily basis is suggested to modulate oral and intestinal microbiota. However, at the moment there are no clinical trials that proved the beneficial use of bifidobacteria on caries prevention [23]. The results of the present study suggest that the use of these species in probiotics may increase pH drop and acid production in dental biofilm. The clinical outcome of these findings should be further evaluated.

Our results for planktonic cultures demonstrated that S. inopinata was not able to ferment glucose and lactose efficiently, which is in disaccord with the literature [24]. Perhaps it simply ferments these two sugars slowly, in comparison to the rates of fermentation of raffinose and sucrose.

So, further studies on the metabolism of carbon sources should be performed not only on the species investigated in this study, but also on other bifidobacteria such as

Scardovia wiggsiae, which has recently been recognized as a member of the oral microbiota [10,25]. Recently, higher prevalence of S. wiggsiae was found in caries lesions than in controls [26] and this finding reinforce the need of deeper investigation on other species.

Differences in pH drops and acid production observed in the control group might be related to inefficient carbohydrate depletion. So, the production of acids might be explained by the metabolism of residual endogenous polysaccharides.

In this study pH drop was evaluated by measuring the pH at baseline and after 3 h. Multiple measurements of pH over time may also generate interesting results and should be performed in future studies.

Overall, a higher amount of acetate was produced by bifidobacteria when cultured planktonically, which is in agreement with findings reported by Crociani et al., 1996 [24]. This is also the first time that the fermentative profile of bifidobacteria in the presence of lactose, raffinose and sucrose was studied in suspension and biofilms grown in the presence of S. mutans. Although acetate production is beneficial in the intestinal environment, it may have detrimental effects in the oral cavity. Together with other oral species, acetate production may contribute to environmental changes that shift healthy oral microbiota to a more cariogenic one. More importantly, when cultured with S. mutans, bifidobacteria seem to contribute to a rise in lactate production, an important feature in caries etiology. Also, other virulence factors, such as aciduricity, antimicrobial resistance, and metabolic activity should be evaluated in the future.

Based on the findings of the present study, it is concluded that B. dentium, P. denticolens and S. inopinata are as acidogenic as S. mutans. Moreover, dual species biofilms of S. mutans and oral bifidobacteria produced a significantly greater pH drop than those produced by individual species.

Compliance with Ethical Standards

Conflict of Interest

Bruno Mello de Matos declares that he has no conflict of interest. Fernanda Lourenção Brighenti declares that she has no conflict of interest. Thuy Do declares that she has no

conflict of interest. David Beighton declares that he has no conflict of interest. Cristiane Yumi Koga Ito declares that she has no conflict of interest.

Funding

The work was supported by the Coordination for the Improvement of Higher Education Personnel – CAPES/Brazil (Grant #3755/10-0) and São Paulo Research Foundation – FAPESP/Brazil (Grant #2010/02063-1)

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the Declaration of Helsinki (1964) and its later amendments or comparable ethical standards.

References

1. Bretz WA, Corby PM, Hart TC, Costa S, Coelho MQ, Weyant RJ, Robinson M, Schork NJ (2005) Dental caries and microbial acid production in twins. Caries Res 39:168-172.

2. Marsh PD (1994) Microbial ecology of dental plaque and its significance in health and disease. Adv Dent Res 8:263-271.

3. Mantzourani M, Gilbert SC, Sulong HN, Sheehy EC, Tank S, Fenlon M, Beighton D (2009) The isolation of bifidobacteria from occlusal carious lesions in children and adults. Caries Res 43:308-313.

4. Almståhl A, Lingström P, Eliasson L, Carlén A (2013) Fermentation of sugars and sugar alcohols by plaque Lactobacillus strains. Clin Oral Investig 17:1465-1470.

5. Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, Dewhirst FE, Leys EJ, Paster BJ (2008) Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol 46:1407-1417.

6. Modesto M, Biavati B, Mattarelli P (2006) Occurrence of the family Bifidobacteriaceae in human dental caries and plaque. Caries Res 40:271-276.

7. Hojo K, Mizoguchi C, Taketomo N, Ohshima T, Gomi K, Arai T, Maeda N (2007) Distribution of salivary Lactobacillus and Bifidobacterium species in periodontal health and disease. Biosci Biotechnol Biochem 71:152-157.

8. Beighton D, Gilbert SC, Clark D, Mantzourani M, Al-Haboubi M, Ali F, Ransome E, Hodson N, Fenlon M, Zoitopoulos L, Gallagher J (2008) Isolation and identification of Bifidobacteriaceae from human saliva. Appl Environ Microbiol 74:6457-6460.

9. Kaur R, Gilbert SC, Sheehy EC, Beighton D (2013) Salivary levels of bifidobacteria in caries-free and caries-active children. Int J Paediatr Dent 23:32-38.

10. Tanner AC, Mathney JM, Kent RL, Chalmers NI, Hughes CV, Loo CY, Pradhan N, Kanasi E, Hwang J, Dahlan MA, Papadopolou E, Dewhirst FE (2011) Cultivable anaerobic microbiota of severe early childhood caries. J Clin Microbiol 49:1464-1474.

11. Brighenti FL, Luppens SB, Delbem AC, Deng DM, Hoogenkamp MA, Gaetti-Jardim E Jr, Dekker HL, Crielaard W, ten Cate JM (2008) Effect of Psidium cattleianum leaf extract on Streptococcus mutans viability, protein expression and acid production. Caries Res 42:148-154.

12. De Man JC, Rogosa M, Sharpe ME (1960) A medium for the cultivation of lactobacilli. J Bacteriol 23:130-135.

13. McBain AJ, Sissons C, Ledder RG, Sreenivasan PK, De Vizio W, Gilbert P (2005) Development and characterization of a simple perfused oral microcosm. J Appl Microbiol 98:624-634.

14. Exterkate RA, Crielaard W, ten Cate JM (2010) Different response to amine fluoride by Streptococcus mutans and polymicrobial biofilms in a novel high-throughput active attachment model. Caries Res 44:372-379.

15. Silva TC, Pereira AFF, Exterkate RAM, Bagnato VS, Buzalaf MAR, Machado MAAM, ten Cate JM, Crielaard W, Deng DM (2012) Application of an active attachment model as a high-throughput demineralization biofilm model. J Dent 40:41-47.

16. Davey AL, Rogers AH (1984) Multiple types of the bacterium Streptococcus mutans in the human mouth and their intra-family transmission. Arch Oral Biol 29: 453-460.

17. Kara D, Luppens SB, ten Cate JM (2006) Differences between single- and dual-species biofilms of Streptococcus mutans and Veillonella parvula in growth, acidogenicity and susceptibility to chlorhexidine. Eur J Oral Sci 114:58-63.

18. Haukioja A, Söderling E, Tenovuo J (2008) Acid production from sugars and sugar alcohols by probiotic lactobacilli and bifidobacteria in vitro. Caries Res 42:449-453.

19. Moynihan PJ, Ferrier S, Blomley S, Wright WG, Russell RR (1998) Acid production from lactulose by dental plaque bacteria. Lett Appl Microbiol 27:173-177.

20. Nakajo K, Takahashi N, Beighton D (2011) Resistance to acidic environments of caries-associated bacteria: Bifidobacterium dentium and Bifidobacterium longum. Caries Res 44:431-437.

21. Self Nutrition Data. http://nutritiondata.self.com. Accessed 05 May 2016.

22. Fontana M, González-Cabezas C (2012) Are we ready for definitive clinical guidelines on xylitol/polyol use? Adv Dent Res 24:123-128.

23. Twetman S (2012) Are we ready for caries prevention through bacteriotherapy? Braz Oral Res 26 Suppl 1:64-70.

24. Crociani F, Biavati B, Alessandrini A, Chiarini C, Scardovi V (1996) Bifidobacterium inopinatum sp. nov. and Bifidobacterium denticolens sp. nov., two new species isolated from human dental caries. Int J Syst Bacteriol 46:564-571.

25. Downes J, Mantzourani M, Beighton D, Hooper S, Wilson MJ, Nicholson A, Wade WG (2011) Scardovia wiggsiae sp. nov., isolated from the human oral cavity and clinical material, and emended descriptions of the genus Scardovia and Scardovia inopinata. Int J Syst Evol Microbiol 61:25-29.

26. Henne K, Rheinberg A, Melzer-Krick B, Conrads G (2015) Aciduric microbial taxa including Scardovia wiggsiae and Bifidobacterium spp. in caries and caries free subjects. Anaerobe 35(Pt A):60-5.

459 Table 1: pH drop (ΔpH) (average ± sd) for single species and dual-species suspensions (n = 4)

460

	glucose	lactose	raffinose	sucrose	control
S. mutans	2.06 ± 0.01	0.65 ± 0.01	1.06 ± 0.02	2.08 ± 0.01	0.53 ± 0.01
L. acidophilus	2.33 ± 0.03[a]	0.64 ± 0.01	0.94 ± 0.01[a]	1.88 ± 0.03[a]	0.45 ± 0.01
B. dentium	2.18 ± 0.02[a,b]	1.12 ± 0.11[a,b]	2.19 ± 0.02[a,b]	2.21 ± 0.01[a,b]	0.54 ± 0.02
P. denticolens	2.07 ± 0.01[b]	0.87 ± 0.01[a,b]	1.55 ± 0.04[a,b]	1.56 ± 0.01[a,b]	0.68 ± 0.04
S. inopinata	**0.55 ± 0.01**[a,b]	**0.57 ± 0.02**	2.01 ± 0.06[a,b]	2.12 ± 0.06[b]	0.49 ± 0.08
S. mutans + L. acidophilus	2.21 ± 0.01[a]	0.57 ± 0.01	0.75 ± 0.02[a]	2.28 ± 0.04[a]	0.34 ± 0.04
S. mutans + B. dentium	2.11 ± 0.11	0.81 ± 0.04[a,c]	2.04 ± 0.06[a,c]	2.15 ± 0.10[b]	0.48 ± 0.02
S. mutans + P. denticolens	2.42 ± 0.05[a,c]	**1.02 ± 0.02**[a,c]	2.08 ± 0.05[a,c]	2.44 ± 0.04[a,c]	0.93 ± 0.05
S. mutans + S. inopinata	2.40 ± 0.01[a,c]	0.82 ± 0.01[a,c]	2.11 ± 0.02[a,c]	2.45 ± 0.01[a,c]	0.74 ± 0.01

461 Letters: significant differences within the same carbohydrate in relation to S. mutans (a), L. acidophilus (b), S. mutans + L. acidophilus (c); data in bold: no significant
462 differences in the same line in relation to control; ANOVA/Tukey´s test, $p<0.05$

463

464

465

466

467

468

469

470

471

472

473 Table 2: pH drop (ΔpH) (average ± sd) for dual species biofilms (n = 4)

474

	glucose	lactose	raffinose	sucrose	control
S. mutans	1.66 ± 0.08	**0.86 ± 0.05**	1.28 ± 0.10	1.55 ± 0.05	0.76 ± 0.02
S. mutans + L. acidophilus	1.97 ± 0.03[a]	0.93 ± 0.02	1.72 ± 0.06[a]	2.02 ± 0.01[a]	0.80 ± 0.01
S. mutans + B. dentium	1.56 ± 0.03[b]	0.92 ± 0.01	1.30 ± 0.01[b]	1.58 ± 0.08[b]	0.79 ± 0.02
S. mutans + P. denticolens	2.50 ± 0.04[a,b]	**1.17 ± 0.05**[a,b]	1.92 ± 0.08[a,b]	2.37 ± 0.07[a,b]	1.05 ± 0.02
S. mutans + S. inopinata	2.47 ± 0.06[a,b]	**1.11 ± 0.03**[a,b]	1.95 ± 0.10[a,b]	2.35 ± 0.08[a,b]	0.99 ± 0.04

475 Letters: significant differences within the same carbohydrate in relation to S. mutans (a), S. mutans + L. acidophilus (b); data in bold: no significant differences in the same
476 line in relation to control; ANOVA/Tukey´s test, $p<0.05$

Table 3: Lactate and acetate concentration (mM; average ± sd) in suspensions (n = 8)

	glucose		lactose		raffinose		sucrose		control	
	lactate	acetate	lactate	acetate	lactate	acetate	lactate	acetate	lactate	acetate
S. mutans	3.56 ± 0.22	0.36 ± 0.03	**0.00 ± 0.00**	**0.02 ± 0.04**	0.39 ± 0.04	0.32 ± 0.07	3.73 ± 0.30	0.32 ± 0.08	0.00 ± 0.00	0.01 ± 0.02
L. acidophilus	6.34 ± 0.63[a]	0.12 ± 0.03[a]	**0.00 ± 0.00**	**0.02 ± 0.04**	0.64 ± 0.12[a]	0.09 ± 0.04[a]	**0.19 ± 0.04**[a]	**0.02 ± 0.03**[a]	0.00 ± 0.00	0.00 ± 0.00
B. dentium	0.91 ± 0.10[a,b]	2.74 ± 0.13[a,b]	**0.01 ± 0.02**	0.45 ± 0.04[a,b]	1.07 ± 0.06[a,b]	2.75 ± 0.18[a,b]	1.15 ± 0.05[a,b]	3.09 ± 0.16[a,b]	0.01 ± 0.03	0.00 ± 0.00
P. denticolens	0.49 ± 0.05[a,b]	1.74 ± 0.12[a,b]	0.06 ± 0.04[a,b]	**0.52 ± 0.05**[a,b]	0.38 ± 0.07[b]	1.11 ± 0.11[a,b]	0.43 ± 0.06[a]	1.16 ± 0.08[a,b]	0.19 ± 0.03	0.57 ± 0.04
S. inopinata	**0.00 ± 0.00**[a,b]	**0.07 ± 0.04**[a]	**0.00 ± 0.00**	**0.00 ± 0.00**	1.00 ± 0.10[a,b]	2.29 ± 0.17[a,b]	0.95 ± 0.08[a,b]	2.44 ± 0.12[a,b]	0.00 ± 0.00	0.00 ± 0.00
S. mutans + L. acidophilus	4.71 ± 0.36[a]	0.29 ± 0.04	**0.00 ± 0.00**	**0.00 ± 0.00**	0.69 ± 0.08[a]	0.28 ± 0.05	5.25 ± 0.17[a]	0.29 ± 0.05	0.00 ± 0.00	0.00 ± 0.00
S. mutans + B. dentium	3.18 ± 0.49[c]	1.82 ± 0.10[a,c]	**0.00 ± 0.00**	**0.03 ± 0.01**	1.32 ± 0.08[a,c]	2.47 ± 0.22[a,c]	4.21 ± 0.70[c]	2.94 ± 0.09[a,c]	0.00 ± 0.00	0.00 ± 0.00
S. mutans + P. denticolens	5.66 ± 0.29[a,c]	1.97 ± 0.11[a,c]	**0.01 ± 0.02**	**0.47 ± 0.03**[a,c]	1.01 ± 0.09[a,c]	1.46 ± 0.11[a,c]	4.45 ± 0.22[a,c]	1.51 ± 0.13[a,c]	0.01 ± 0.01	0.44 ± 0.05
S. mutans + S. inopinata	4.50 ± 0.55[a]	0.62 ± 0.04[a,c]	**0.00 ± 0.00**	**0.10 ± 0.03**[a,c]	1.56 ± 0.12[a,c]	1.99 ± 0.14[a,c]	4.69 ± 0.65[a,c]	1.85 ± 0.13[a,c]	0.00 ± 0.00	0.21 ± 0.02

Letters: significant differences within the same carbohydrate in relation to S. mutans (a), L. acidophilus (b), S. mutans + L. acidophilus (c); data in bold: no significant differences in the same line in relation to control; ANOVA/Tukey´s test, $p<0.05$

481 Table 4: Lactate and acetate concentration (mM; average ± sd) in biofilms (n = 8)

482

	glucose		lactose		raffinose		sucrose		control	
	lactate	acetate	lactate	acetate	lactate	acetate	lactate	acetate	lactate	acetate
S. mutans	2.96 ± 0.28	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.15 ± 0.13**	0.03 ± 0.04	2.25 ± 0.44	**0.00 ± 0.00**	0.00 ± 0.00	0.00 ± 0.00
S. mutans + L. acidophilus	2.15 ± 0.49[a]	**0.38 ± 0.12**[a]	**0.05 ± 0.05**	**0.22 ± 0.08**[a]	**0.51 ± 0.19**[a]	0.53 ± 0.22[a]	3.23 ± 1.18	**0.22 ± 0.09**[a]	0.05 ± 0.06	0.21 ± 0.20
S. mutans + B. dentium	2.51 ± 0.19	**0.09 ± 0.06**[b]	**0.03 ± 0.07**	**0.06 ± 0.08**	0.40 ± 0.09[a]	0.23 ± 0.05[b]	2.28 ± 0.59	**0.07 ± 0.06**[b]	0.00 ± 0.00	0.03 ± 0.06
S. mutans + P. denticolens	9.50 ± 0.90[a,b]	**0.34 ± 0.05**[a,b]	**0.07 ± 0.04**	**0.26 ± 0.05**[a,b]	1.72 ± 0.21[a,b]	1.24 ± 0.14[a,b]	12.61 ± 1.01[a,b]	**0.34 ± 0.07**[a,b]	0.11 ± 0.06	0.28 ± 0.02
S. mutans + S. inopinata	9.23 ± 0.39[a,b]	0.55 ± 0.02[a,b]	**0.25 ± 0.09**[a,b]	**0.45 ± 0.15**[a,b]	2.16 ± 0.18[a,b]	1.46 ± 0.14[a,b]	11.79 ± 0.72[a,b]	**0.38 ± 0.14**[a,b]	0.09 ± 0.16	0.31 ± 0.17

483 Letters: significant differences within the same carbohydrate in relation to S. mutans (a), S. mutans + L. acidophilus (b); data in bold: no significant differences in the same line in
484 relation to control; ANOVA/Tukey´s test, $p<0.05$

485

486

487

488

Table 5: Colony forming units (CFU/disc; average ± sd) for dual species biofilms and specific species (lactobacilli and bifidobacteria) and percentage of these species in relation to total count (%TM) (n=12)

Biofilm	Total microorganism	Lactobacilli		Bifidobacteria	
		CFU/disc	% TM	CFU/disc	%TM
S. mutans	$2.77 \times 10^7 \pm 1.44 \times 10^7$	-	-	-	-
S. mutans + L. acidophilus	$7.09 \times 10^7 \pm 2.83 \times 10^7$	$4.20 \times 10^5 \pm 2.03 \times 10^5$	0.59	-	-
S. mutans + B. dentium	$5.15 \times 10^6 \pm 2.18 \times 10^6$	-	-	$4.66 \times 10^6 \pm 2.19 \times 10^6$	90.49
S. mutans + P. denticolens	$2.83 \times 10^8 \pm 3.74 \times 10^7$	-	-	$3.84 \times 10^6 \pm 1.67 \times 10^6$	1.36
S. mutans + S. inopinata	$2.52 \times 10^8 \pm 7.12 \times 10^7$	-	-	$3.68 \times 10^6 \pm 1.50 \times 10^6$	1.46