TITLE

Lactobacillus rhamnosus GG in experimental oral biofilms exposed to different carbohydrate sources

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RUNNING HEAD

LGG in experimental oral biofilms

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Disclosure Statement

The authors declare no conflicts of interests.

Abstract

Probiotic administration may favour caries prevention as recent research has shown. This in vitro study aimed to investigate the growth of *Lactobacillus rhamnosus* GG (LGG) in experimental biofilms exposed to various carbohydrates, and also to assess its cariogenic potential. Multi-species experimental oral biofilms with/without LGG were grown with a sole-carbohydrate source (fructose/glucose/lactose/sorbitol/sucrose). The viable cells of LGG and structure of biofilms were examined after 64.5h of incubation, and pH values of spent media were measured at 16.5h, 40.5h and 64.5h. Fermentation profiles of LGG in biofilm media were assessed with study carbohydrate as the sole energy source. Our results showed that LGG reached higher viable cell numbers with glucose and sucrose in 64.5h multi-species experimental oral biofilms compared to other carbohydrates. When LGG was incorporated in biofilms, no distinct pH changes at all time points were observed under any of the carbohydrates used; the pH values of spent media at each time point were lower when lactose was used, compared to other carbohydrates. The fermentation profiles of LGG in biofilm media were similar to its growth in MRS (no obvious growth with lactose or sucrose). In conclusion, LGG in our in vitro multi-species experimental oral biofilms was capable of surviving and growing well in each carbohydrate source. LGG might not have harmful effects on dental hard tissues. Another finding in our study was that the lowest pH values were observed in the presence of lactose, and the thickest biofilms were in sucrose.

Introduction

Dental caries still remains a global oral health burden worldwide. Caries lesions in enamel and dentin are mainly initiated by the demineralization of the tooth surface through bacterial acid production from sugar [Mayanagi et al., 2017]. Sucrose, fructose, and glucose are considered the most important sugars/carbohydrates in caries development and progression [Marsh, 2003; Selwitz et al., 2007]. Acid-producing bacteria commonly associated with dental caries are *Streptococcus mutans* [Forssten et al., 2010], lactobacilli [Jiang et al., 2015], and *Actinomyces* [Xiao et al., 2016], which are inherent residents of oral biofilms developing on tooth surface. In the last decade, an increasing number of studies have shown great interests in the prevention of caries with the usage of probiotics [Laleman and Teughels, 2015; Jorgensen et al., 2016].

Probiotics are 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' [Hill et al., 2014]. Among the probiotics strains *Lactobacillus rhamnosus* GG (ATCC 53103, LGG) is one of the most documented and widely used probiotic strains in the world. Beneficial effects of LGG in general have been documented in various clinical trials, including studies on diarrhoea, allergy, and liver diseases [Floch et al., 2015].

A fair number of clinical trials also suggest that both short- and long-term intake of probiotic could reduce *S. mutans* counts in saliva and/or plaque [Meurman et al., 1995; Näse et al., 2001; Aminabadi et al., 2011; Laleman et al., 2014; Tehrani et al., 2016]. However, a more pronounced beneficial effect of saliva-derived lactobacilli was observed in subjects without caries experience rather than in individuals with arrested or active caries lesions [Simark-Mattsson et al., 2007]. There is still paucity of evidence to establish relationship between probiotic administration and decayed/missing/filled teeth (DMFt) scores [Simark-Mattsson et al., 2007; Gruner et al., 2016; Tehrani et al., 2016]. In addition, the safety of probiotic use in the oral cavity has been a controversial topic. The genus of *Lactobacillus* is known for their acidophilic properties, which in light of the aetiology of dental caries may impose an inherent risk to dental

hard tissues. In the other hand, probiotics have proven safe both in vitro and in vivo studies [Snydman, 2008]. Pham et al. [2011] have suggested that LGG had no significant effect on cariogenic potential of a complex saliva-derived biofilms. However, Schwendicke et al. [2014a; 2014b] have reported that LGG and *Bifidobacterium* BB12 were found to demineralize both enamel and dentin, and LGG even induced increased demineralization compared to *S. mutans* mono-species biofilm alone. Although there are limited aspects of positive effects for caries prevention and insufficient safety studies, probiotics significantly increased the chance of reducing *S. mutans* [Gruner et al., 2016] and mutans streptococci are major pathogens of dental caries [Takahashi and Nyvad, 2011], which leads the probiotic use in caries prevention as a hot topic. Accordingly, the inhibitory activity of probiotic against common oral pathogens (*S. mutans*, *Candida albicans*, *Streptococcus sanguinis*) has been also tested in vitro [Soderling et al., 2011; Jiang et al., 2015; Wu et al., 2015; Jiang et al., 2016] and its fermentation profiles have been a subject of studies [Hedberg et al., 2008; Douillard et al., 2013]. However, there is limited evidence about the ability of LGG to establish itself in the human mouth and to integrate and interact with oral biofilms. Studies in this regard are needed and would contribute towards understanding the mechanisms behind beneficial effects of probiotics from the oral health perspective.

Our previous results affirmed that probiotic LGG was able to integrate with experimental oral biofilms in vitro and differently affected the growth of tested cariogenic strains [Jiang et al., 2016]. In the present study, a sequel to our previous work, our aim was to investigate the growth of LGG in experimental oral biofilms under various carbohydrate conditions and to evaluate its potential risk for dental hard tissues in terms of pH alterations to growth environment.

Materials and Methods

Strains, growth conditions, and inoculum preparation

LGG, from Valio Ltd., Helsinki, Finland was used as the probiotic strain in our study. Biofilms in control group (5SP) were built with the pool of five species of oral bacterial/yeast strains: *C. albicans* ATCC 10231, *S.*

mutans ATCC 27351, *S. sanguinis* ATCC 10556, *Aggregatibacter actinomycetemcomitans* ATCC 43718, and *Fusobacterium nucleatum* ATCC 25586. Group of 5SP with LGG (5SP+LGG) was the study group. All the strains were maintained as frozen stock at -80^{0}C in 20% skim milk (Difco^{TM}, BD, Becton, Dickinson and Company, Sparks, MD, USA). Before each experiment, strains were cultivated twice on respective agars (details are given in Table 1). Pure colony of each strain was inoculated in 5 mL corresponding cultivation broth, and cultivated anaerobically overnight at 37°C.

Bacterial and yeast strains were harvested by centrifugation for 10 min at 3,000 × *g*, at room temperature, washed three times with 5 mL 0.9% NaCl and re-suspended in biofilm medium base (BMB, biofilm medium sugar free) adapted from Lemos et al. [2010]. The cell suspensions were adjusted to an OD_{490} of 0.130±0.010 (similar to McFarland standard No. 1. The cell concentrations of suspensions were 1.64×10^8 cells/mL for LGG, 3.33×10^7 cells/mL for *C. albicans*, 7.53×10^8 cells/mL for *S. mutans*, 3.31×10^8 cells/mL for *S. sanguinis*, 4.44×10^9 cells/mL for *A. actinomycetemcomitans*, and 1.72×10^8 cells/mL for *F. nucleatum*) by a spectrophotometer (Multisan Plus, Labsystems, Helsinki, Finland, measured by 200 µL each well in 96-well plate). Aliquots of strain suspensions were then pooled for control group (5SP) and study group (5SP+LGG), according to the group setup.

Preparation of biofilms

Biofilms were grown on saliva-coated hydroxyapatite (HA) discs (Clarkson Chromatography Products, Inc., South Williamsport, PA, USA). The discs were 7.0 mm in diameter and 1.8 mm high. The HA discs were placed in a vertical position in disc holders bent from orthodontic wire according to Lemos et al. [2010] with minor modifications. The holders and the HA discs were autoclaved after assembling.

To allow formation of a salivary pellicle, each HA disc was placed in a well of a sterile 24-well polystyrene cell culture plate, fully immersed and incubated with 1.8 mL of processed saliva and by gentle shaking for 4h at room temperature. Whole saliva was collected from 21 healthy

Table 1. Strains and the growth conditions.

Strain	Origin	Agar/Broth	Growth conditions
Lactobacillus rhamnosus GG ATCC 53103 (LGG)	Valio Ltd., Helsinki, Finland	de Man, Rogosa and Sharpe (MRS)	24h, 37°C, 5% CO_2
Candida albicans ATCC 10231	American Type Culture Collection (ATCC)	Sabouraud	24h, 37°C, air
Streptoccus mutans ATCC 27351	ATCC	Brain Heart Infusion (BHI)	24h, 37°C, 5% CO_2
Streptococcus sanguinis ATCC 10556	ATCC	BHI	24h, 37°C, 5% CO_2
Aggregatibacter actinomycetemcomitans ATCC 43718	ATCC	BHI	24h, 37°C, 5% CO_2
Fusobacterium nucleatum ATCC 25586	ATCC	Brucella	48h, 37°C, in anaerobic condition (mixture of 0.2% O_2, 5% CO_2, 9.9% H_2, 84.9% N_2)

volunteers after their informed consent (men, n=10, women, n=11; mean age 35±8). Pregnancy, gingival bleeding, history of antibiotic administration in the past 2 weeks, and eating, drinking and oral hygiene procedures 1.5 hour prior saliva collection were the main exclusion criteria. The processed saliva was prepared and pasteurized according to Guggenheim et al. [2001]. The efficacy of pasteurization was assessed by plating processed saliva samples onto Brucella agar (BBL™, BD, Becton, Dickinson and Company, Sparks, MD, USA, with vitamin K3 10 ug/mL, hemin 5 ug/mL, and 5% defibrinated horse blood from bio TRADING, Mijdrecht, the Netherlands), and cultivated either aerobically or anaerobically for 3 days, until no colonies were observed.

After the saliva-coating step, HA discs were transferred to a new 24-well plate containing 2.5 mL biofilm culture medium and 0.3 mL pooled strains in each well. Six biofilm culture media were used in this study, namely BMB with water (BM-negative), with fructose (BM-fructose), with glucose (BM-glucose), with lactose (BM-lactose), with sorbitol (BM-sorbitol), and with sucrose (BM-sucrose). The concentration of carbohydrate was 3.6 g/L (i.e. 20 mM glucose/fructose/sorbitol or 10 mM lactose/sucrose). Then the plates with HA discs and broth media were incubated anaerobically at 37°C for 64.5 h in dark. Broth media were renewed at 16.5h and 40.5h as the following steps: the discs were first washed by dipping twice into 2.8 mL physiological saline and then transferred to a new 24-well plate containing 2.8 mL fresh broth media per well.

Examination of LGG cells

After 64.5h cultivation and two dip washes in physiological saline, each HA disc was transferred into a sterile 50 mL polypropylene tube containing 5 mL of physiological saline at room temperature, and vortexed (by Vortex-Genie® 2 mixer, Scientific industries, Inc, Bohemia, N.Y., USA) vigorously for 2 min, and sonicated (by Wagner instrusonic, PS-Terä Oy, Lahti, Finland, 90/180 watts) for 5 sec at room temperature. Serial dilutions of the sonicated cells were cultivated on de Man, Rogosa and Sharpe (MRS; Lab M Ltd, Bury, UK) agar plates at 37°C in 5% CO_2

for 72h. Colony forming units (CFU) of LGG were counted based on its colonial morphology on MRS.

Measurement of pH values of spent culture media

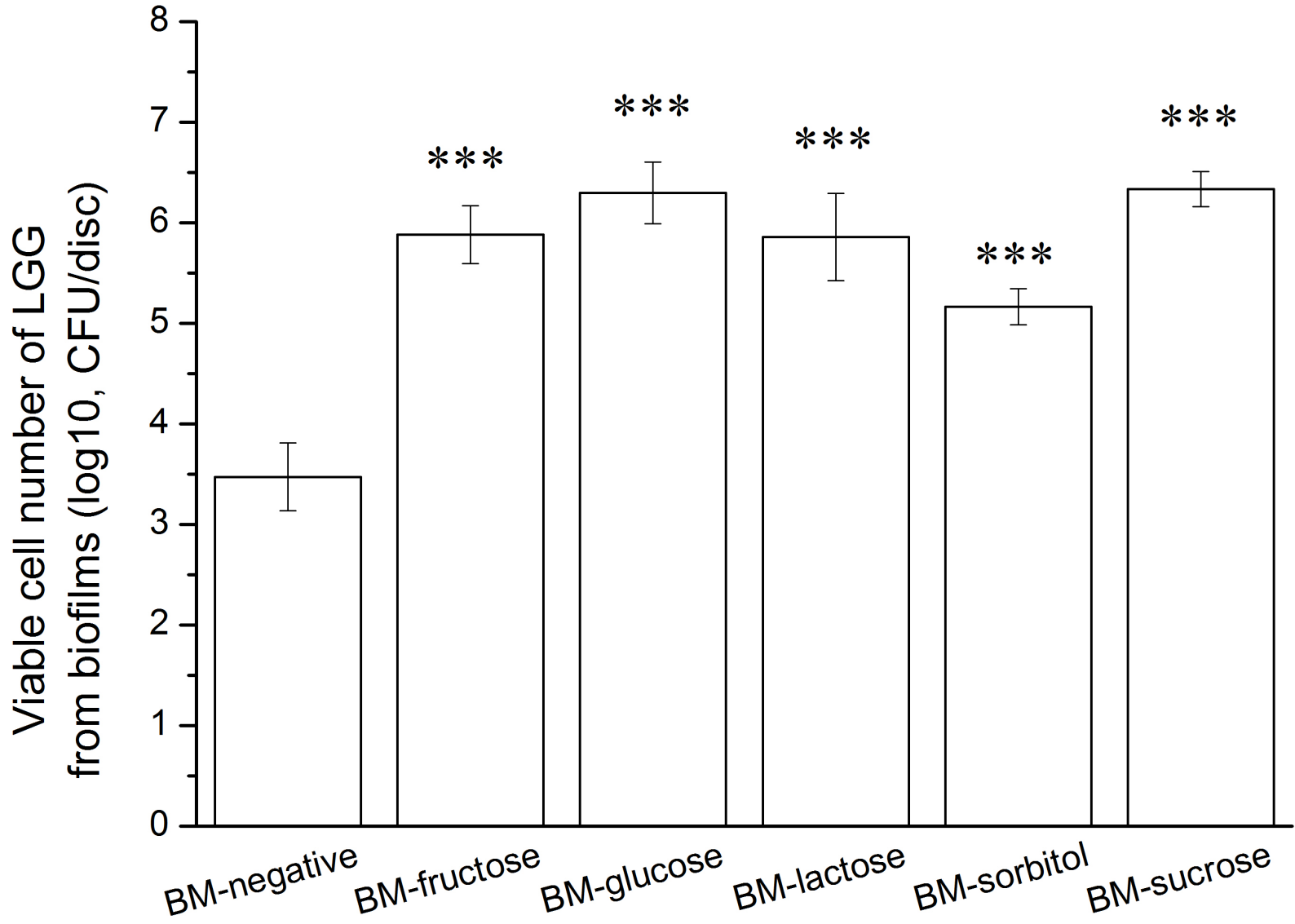
The pH of spent media was measured with a pH meter (pH 1000 L, pHenomenal®, VWR International, Rador, PA, USA) at all three time points, when the HA discs were transferred into fresh media or physiological saline and when the spent media were replaced. The spent media were centrifuged for 10 min, 3,000 × *g* prior to pH measurement from the supernatant.

Structural analysis of biofilms

The biofilm structure was analysed with the method of fluorescence in situ hybridization (FISH).

For FISH analysis, the staining was performed mainly according to the protocol established by Thurnheer et al. [2004]. In short, 64.5h biofilms were fixed immediately with 4% (w/v) paraformaldehyde for 1h at 4°C, permeabilized for 30 min at 37°C by exposure to the mixture (46200 U/ml or 1 mg/ml lysozyme, 98 mM Tris/HCl, 5 mM EDTA, pH 7.5. Extra 100 U/mL mutanolysin was added for Group 5SP+LGG), pre-hybridized in hybridization buffer (0.9M NaCl, 20mM Tris/HCl, 30% Formamide, 0.01% SDS) at 46°C for 15 min, and followed by hybridization for 3h with fluorescently labelled oligonucleotides (Lcas467-Cy3 probe binds LGG: 5´-CCGTCACGCCGACAACAG-3´ [Ardita et al., 2014], and MUT590-Cy5 probe binds *S. mutans*: 5´-ACTCCAGACTTTCCTGAC-3´ [Quevedo et al., 2011]). After hybridization, biofilms were washed twice in wash buffer (20mM Tris/HCl, 5mM EDTA, 102 mM NaCl, 0.01% SDS) and stained with Hoechst (to stain the rest of the strains= *S. sanguinis* + *C. albicans* + *A. actinomycetemcomitans* + *F. nucleatum*) for 5min in dark.

Afterwards all the samples were embedded in Mowiol [Thurnheer et al., 2006] overnight at room temperature and were examined with an inverted Confocal Laser Scanning Microscopy (CLSM) Leica SP8 (Leica Microsystems Gmbh Wetzlar, Germany). CLSM images were obtained with a ×40 water immersion objective. Each biofilm was scanned at randomly selected areas as a series of vertical optical sections, each


Viable cell number of LGG
from biofilms (log10, CFU/disc)
0
1
2
3
4
5
6
7
8
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***
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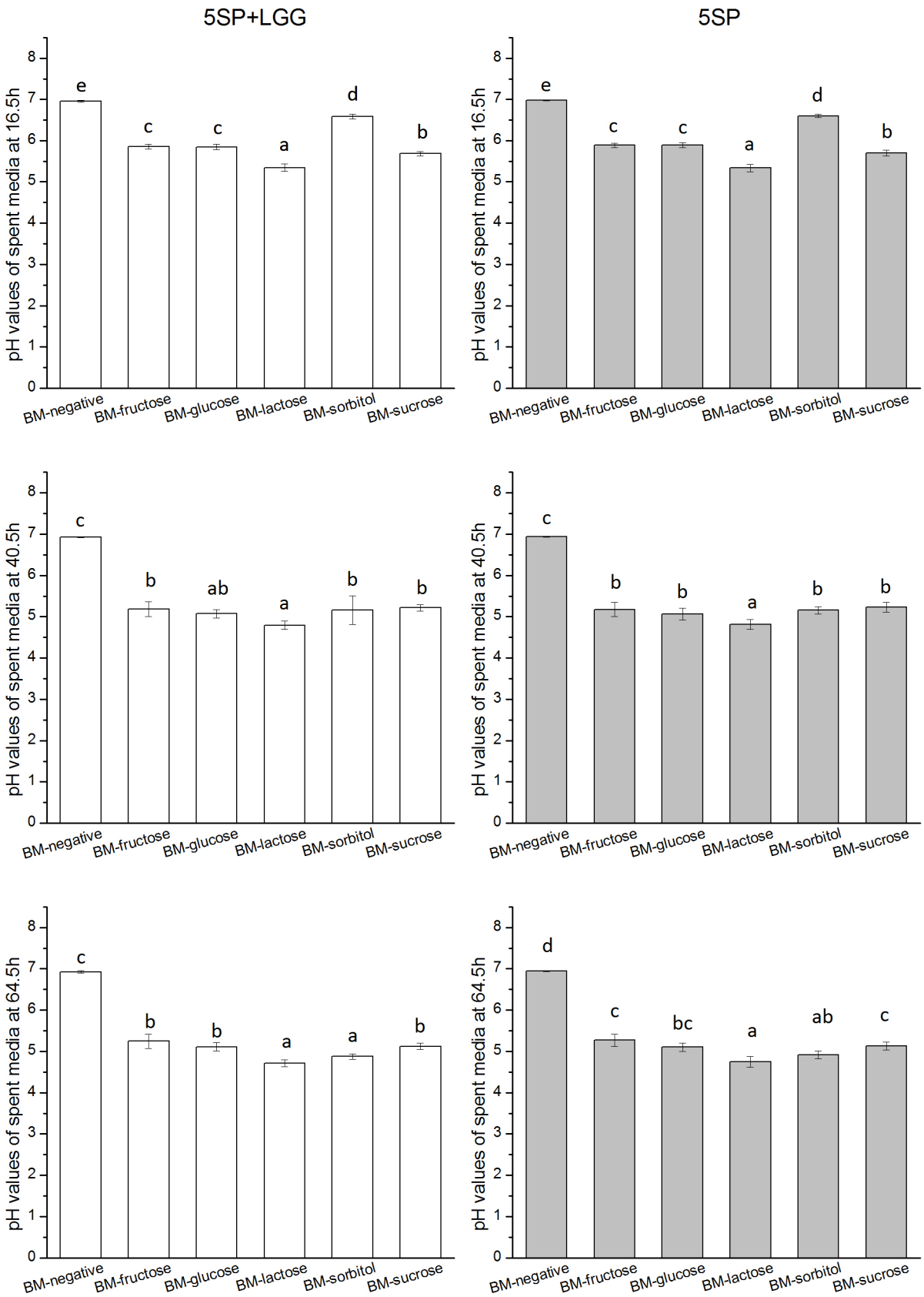
BM-negative
BM-fructose
BM-glucose
BM-lactose
BM-sorbitol
BM-sucrose


5SP+LGG
5SP
pH values of spent media at 16.5h
pH values of spent media at 40.5h
pH values of spent media at 64.5h
0
1
2
3
4
5
6
7
8
e
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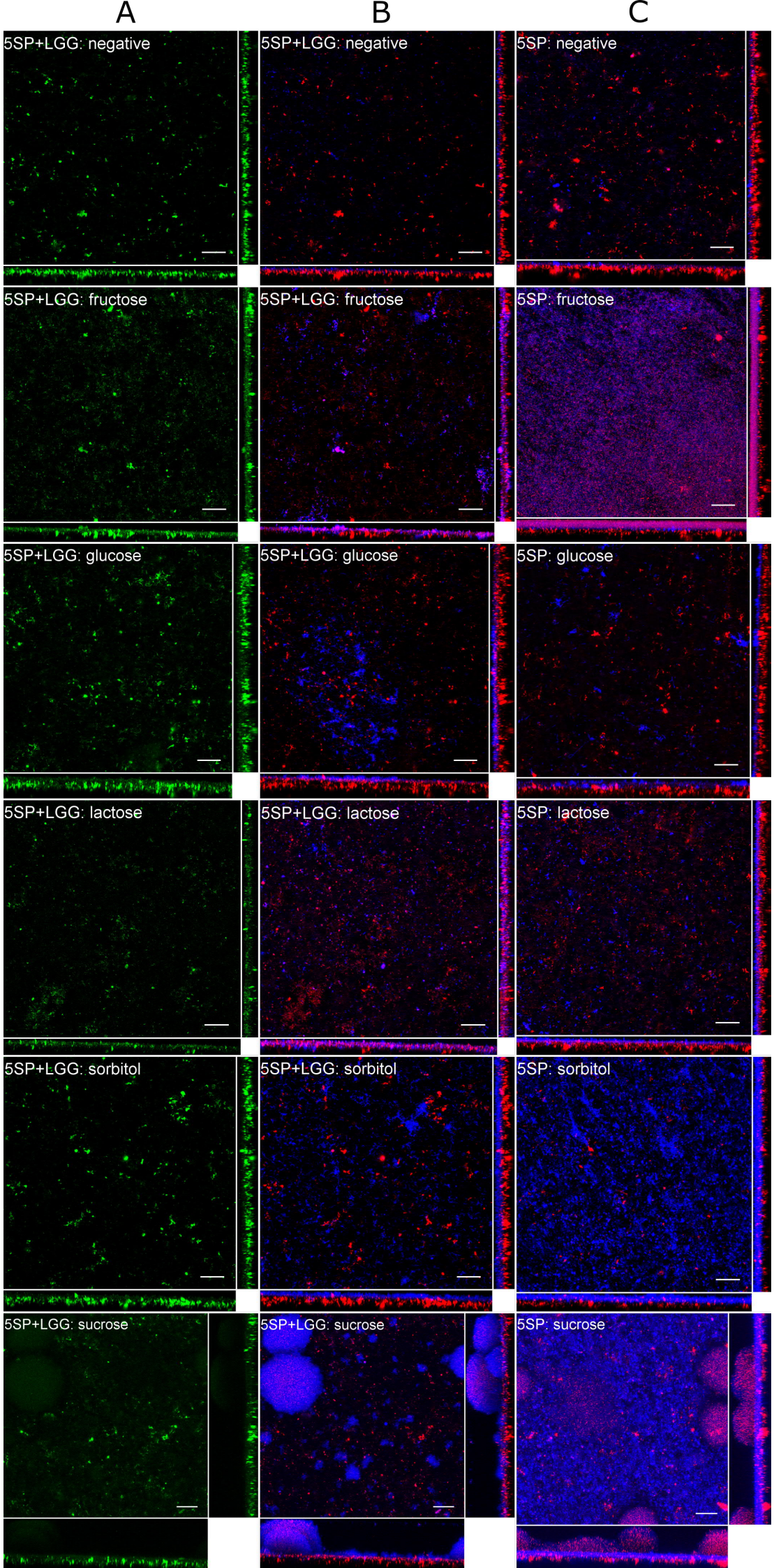
BM-negative
BM-fructose
BM-glucose
BM-lactose
BM-sorbitol
BM-sucrose


A
B
C
5SP+LGG: negative
5SP+LGG: negative
5SP: negative
5SP+LGG: fructose
5SP+LGG: fructose
5SP: fructose
5SP+LGG: glucose
5SP+LGG: glucose
5SP: glucose
5SP+LGG: lactose
5SP+LGG: lactose
5SP: lactose
5SP+LGG: sorbitol
5SP+LGG: sorbitol
5SP: sorbitol
5SP+LGG: sucrose
5SP+LGG: sucrose
5SP: sucrose

section was 0.50 µm thick. Digital images were processed with Fiji [Schneider et al., 2012].

Fermentation profiles of LGG in biofilm medium with sole carbohydrate source.

The overnight cultures of LGG were harvested by centrifugation for 10min at 3,000 × *g*, at room temperature, washed three times with 5 mL 0.9% NaCl and re-suspended in BM-negative medium. The suspensions were adjusted to an optical density at 490 nm (OD_{490}) of 0.360±0.010. The adjusted suspension (200µL) was inoculated into 5mL aliquots of BM-negative, fructose, glucose, lactose, sorbitol, or sucrose media, respectively, and cultivated in 5% CO_2 at 37°C. The growth was measured by observing the changes of OD_{490} at 0, 4, 6, 20, 24 and 48h incubation.

Statistical analysis

Data are shown as means ± standard deviations. Statistical analyses were performed with IBM SPSS Statistics version 22 for Windows. One-way ANOVA and Dunnett's test were used to determine statistical significance in Figure 1, and Duncan's test in Figure 2. A difference was deemed significant at $P<0.05$ or ***$P<0.001$. Log10 transformation of the viable cell numbers was made before the statistical analysis.

Results

Growth of LGG in biofilms

The viable cell numbers of LGG in 64.5h experimental oral biofilms cultured with the five sole-carbohydrate media are presented in Figure 1. LGG was able to use all the supplemented carbohydrates for growth and viable cells of LGG were detected in all the biofilms, including the negative control group. LGG grew to higher number when the carbohydrate source was glucose ($2.33\pm1.60 \times 10^6$ CFU/disc) and sucrose ($2.29\pm0.99 \times 10^6$ CFU/disc) compared with the other carbohydrate sources. These numbers were significantly ($P < 0.001$) higher than that in the negative control group ($3.54\pm2.18 \times 10^3$ CFU/disc). Among the study groups, the lowest viable cell number of LGG was measured when sorbitol was used ($1.55 \pm 0.58 \times 10^5$ CFU/disc). In the presence of lactose

and fructose, the numbers of LGG were $9.67\pm8.12 \times10^5$ CFU/disc and $8.88\pm6.39 \times10^5$ CFU/disc, respectively.

The highest viable cell numbers of LGG in the experimental oral biofilms were observed in the presence of glucose, followed by sucrose, lactose, fructose, sorbitol and negative control.

pH values of spent media

The pH values of spent media (Figure 2) were measured when new broth media were replaced or at the end of cultivation, i.e. at 16.5h, 40.5h and 64.5h, respectively.

The pH values of spent media at 16.5h were above 5. The presence of LGG did not clearly change the pH values of the spent media when comparing the groups of 5SP+LGG and 5SP which had been cultivated with each carbohydrate studied and at each time point.

The pH values in the carbohydrate-supplemented groups were significantly lower than that in the negative group ($P<0.05$).

The lowest pH values in 5SP+LGG and 5SP groups at all time points (respectively at 16.5h: 5.34±0.09 and 5.34±0.09, at 40.5h: 4.79±0.10 and 4.81±0.12, at 64.5h: 4.72±0.09 and 4.75±0.14) were measured from the subgroup BM-lactose.

Biofilms structure

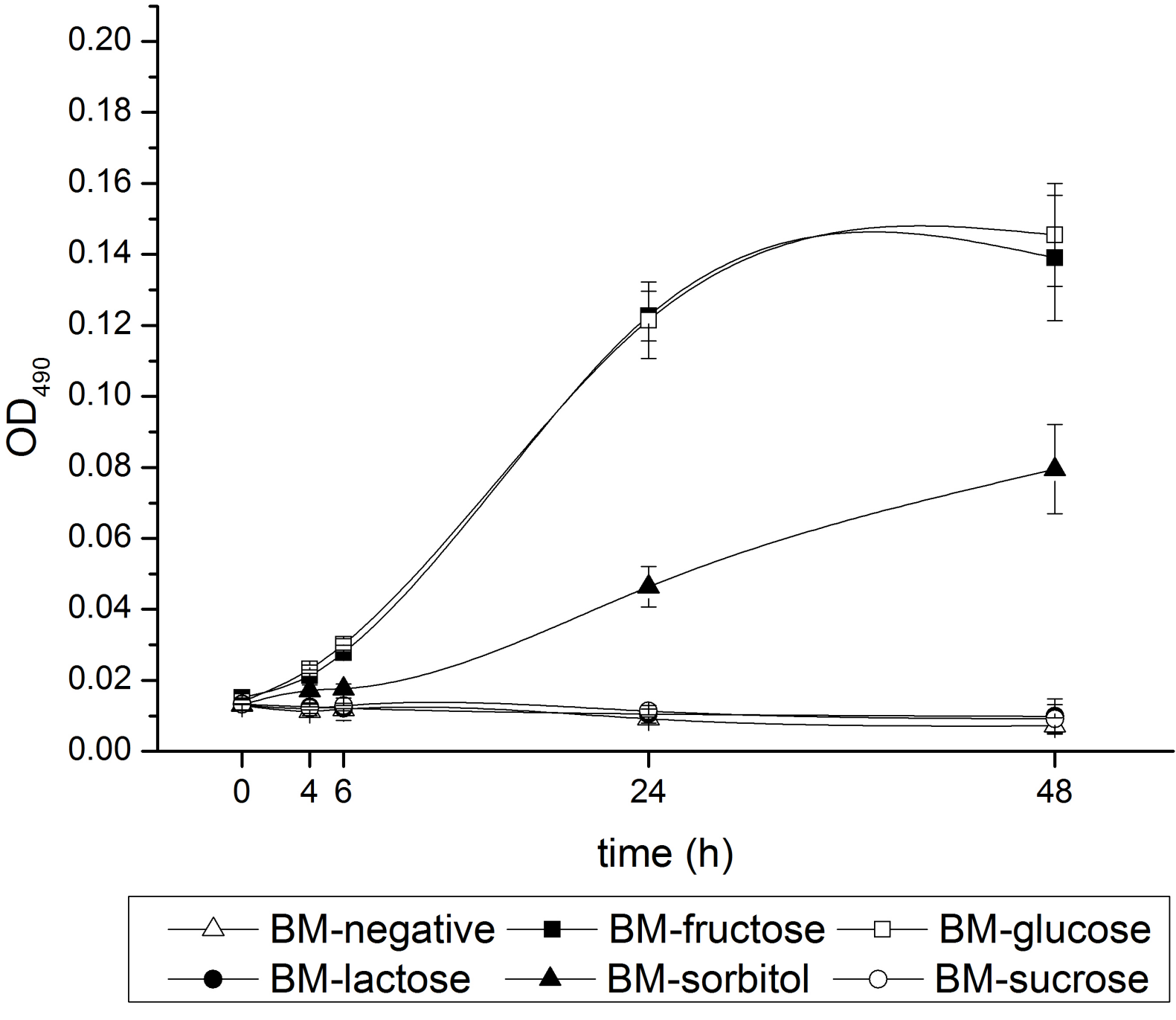
All the microbes in 64.5h biofilms grew out as layer structures (Figure 3), and hemispherical shape structures were observed in the presence of sucrose.

From column A, LGG was able to be detected in 64.5h biofilms of group 5SP+LGG under all the tested carbohydrate conditions.

Comparing columns B and C with each carbohydrate, less microbes (both red and blue channels) were adhered and developed in group 5SP+LGG than in group 5SP. Also the first layer of biofilms of group 5SP was mainly composed of *S. mutans*, but this layer in group 5SP+LGG was mostly mixed up with the rest of the strains.

Planktonic cell growth

In order to compare the growth of LGG in the experimental oral biofilms and as planktonic cells, we also tested the fermentation profiles of LGG in biofilm broth media with the five sole-carbohydrates in 48h. Figure 4


0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
OD490
0
4
6
24
48
time (h)
BM-negative
BM-fructose
BM-glucose
BM-lactose
BM-sorbitol
BM-sucrose

shows that at the end of cultivation LGG grew to highest optical density in subgroups BM-glucose and BM-fructose, and higher in BM-sorbitol. No obvious increases of optical density were found in BM-negative, BM-lactose, and BM-sucrose, respectively.

The highest growth of LGG in the biofilm broth media was in the presence of glucose, followed by fructose, sorbitol, negative control, and lactose, while least growth was observed in the presence of sucrose.

Discussion

This in vitro study aimed to investigate LGG growth in experimental oral biofilms simulating oral conditions and, secondly, to evaluate the potential of this probiotic strain in decreasing pH in its environment in the perspective of dental caries. We built 64.5h multi-species experimental oral biofilms cultivated with fructose, glucose, lactose, sorbitol, and sucrose. Our results demonstrate that LGG can grow to higher viable cell numbers with glucose and sucrose in these multi-species biofilms compared to the other carbohydrates. Furthermore, the addition of LGG did not decrease the pH values in the experimental model systems.

The growth of LGG in the multispecies experimental oral biofilms was different from its growth in mono-species biofilms or as planktonic cells. We found that LGG in our study was able to survive and grow well in a wider spectrum of carbohydrate sources. The growth of LGG as planktonic cells in the biofilm broth media was similar to that in MRS [Jiang et al., 2015], showing low or no capability to utilize sucrose or lactose. But LGG in the multispecies experimental oral biofilms did show better growth in the presence of sucrose or lactose. In the study of Hedberg et al. [2008] LGG was found to ferment sucrose or lactose only after 48h and 72h cultivation. Another possible reason to explain its growth in sucrose and lactose in our experiment is that *S. mutans* [Moye et al., 2014], *S. sangunis* [Tanzer et al., 1971; Yamada et al., 1985] and/or *C. albicans* [Binkley et al., 2014] when present in the biofilms could hydrolyse these two carbohydrates to fructose, glucose, and galactose. Then fructose and glucose could be easily utilised by LGG leading to higher viable cell numbers observed.

One of our important findings is that the growth of LGG in sucrose or lactose with cross-feeding is here demonstrated. Whenever one organism uses metabolites produced by another organism as energy or nutrient sources, it is called cross-feeding [Estrela et al., 2012]. A recent study from Pan et al. [2016] has demonstrated that cooperative cross-feeding between different bacterial species is favoured in structured environments such as bacterial biofilms, suggesting that this type of interactions might be common in natural bacterial communities. Apparently, the nutritional interaction in the present study was beneficial regarding the growth of LGG.

In addition, when glucose was the sole carbohydrate source, the viable cell number of LGG in the multispecies experimental oral biofilms ($2.33\pm1.60 \times 10^6$ CFU/disc) was more than seven times higher than the viable cell number of LGG in its mono-species biofilms ($3.16\pm1.80 \times 10^5$ CFU/disc) [Jiang et al., 2016]. This finding could be explained by the theory that microbial consortia actively attempt to become poly-microbial in order to gain resistance and better survival ability [Wolcott et al., 2013].

In our series LGG showed no cariogenic potential since the pH values of the spent media at 16.5h were not decreased when LGG was co-cultured. These pH values did not drop close to or below the critical levels for dental enamel demineralization (i.e. pH 5.2-5.5) [Dahlén et al., 2012]. This phenomenon in the present study might be explained by three possible ways: 1) The biofilm medium contained 68.5 mmol/L phosphate, which prevented any drastic pH change; 2) the acids produced by LGG were utilized by the other micro-organisms and thus did not affect the environment; and 3) LGG cells comprised only a small part in the biofilms, so the acids generated by them did not decrease the pH in the whole model system.

Commercial probiotic products are now widely available, so safety issues are raising up. Numerous in vitro and in vivo studies have been published to address the consumption of probiotics from various perspectives. For example, Hibberd et al. [2014] have reported that in a 28-day clinical trial, LGG was safe in healthy adults aged 65 years and

older with no serious adverse events. And a two-week consumption of *Lactobacillus reuteri* and LGG appeared not to influence the acidogenicity of plaque of young adults [Marttinen et al., 2012]. Stamatova et al. [2007] have proved that intake of *Lactobacillus bulgaricus* strains is not anticipated to exert any deleterious effects on the regulatory enzymes and structure of the host extracellular matrix. However, some reports do not agree with the above conclusions. Probiotics strains, for example, *Lactobacillus salivarius* strains, LGG, BB12 have been reported to show ability to induce caries and mineral loss in vivo and in vitro [Matsumoto et al., 2005; Pham et al., 2009; Schwendicke et al., 2014a; Schwendicke et al., 2014b]. These contradictory reports urge more relevant future studies to clarify the safety issue. Meanwhile the effects of probiotics are strain-dependant, it is crucial to select no cariogenic risk strains as oral probiotics.

In the present study, it was interesting to find out that the pH values of the spent media were lowest in the presence of lactose. Lactose is one of the major sugars in dairy products and its fermentation can potentially demineralize dental hard tissues. Traditionally, sucrose is regarded the most cariogenic sugar [Boonyanit et al., 2011]. In our study sucrose in the growth medium indeed resulted in low pH values of the spent media but not as low pH values as lactose. This finding might be used to advise consumers to choose lactose-free probiotic products. However, it should be kept in mind that milk, for example, has a strong buffering capacity [Salaun et al., 2005]. Thus, studies in clinical setting are called for before drawing any further conclusion in this respect.

Although lactose led to a lowest pH, sucrose resulted in thicker biofilms, which agrees with and proofs that sucrose is the most cariogenic sugar [Gupta et al., 2013]. And the biofilms' structure implies that *S. mutans* colonized the saliva-coated HA surface earlier than the rest of the strains and the addition of LGG affected the adherence of *S. mutans*, which are consistent with previous observations [Li et al., 2004; Jiang et al., 2016]. These results all prove that this biofilm model is effective and repeatable. One of the strengths of this study is to involve multi-species to build biofilms to mimic a complex ecosystem, but it is also a limitation.

Because the dynamic oral cavity contains far more species to form various microbial communities, and there are great inter-individual variations [Sato et al., 2015]. Also the tested strains are all reference strains. Hence the findings in this study need to be further confirmed and ideally in a clinical setting.

Within the limitations of this study, LGG in our in vitro multi-species experimental oral biofilms was capable of surviving and growing well with each of the studied carbohydrate sources. The lowest pH values were observed in the presence of lactose.

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Author Contributions

All authors conceived and designed the experiments. QJ performed the experiments, analysed the data and drafted the manuscript. VK, RK, IS, JHM revised the manuscript. All authors read and approved the final manuscript.

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Legends

Table 1. Strains and the growth conditions.

Figure 1. Viable cell number of LGG from 64.5h multi-species experimental oral biofilms. Biofilms (5SP+LGG) were cultured with fructose (BM-fructose), glucose (BM-glucose), lactose (BM-lactose), sorbitol (BM-sorbitol), sucrose (BM-sucrose), and with carbohydrate free (BM-negative) culture media. Three independent experiments were conducted, each experiment contained two parallels. Every two parallels generates an average. Three averages were involved in the statistical analyse. Each average was based on log10 transformation, and analysed with one-way ANOVA and Dunnett's test were compared with BM-negative. Data represent the means ± SDs, ***P<0.001.

Figure 2. The pH of spent culture media for multi-species experimental oral biofilms with (5SP+LGG) and without (5SP) LGG. pH was measured at 16.5, 40.5, and 64.5h. Three independent experiments were performed, each experiment contained two parallels. Two parallels generated an average. Three averages were involved in the statistical analysis. One-way ANOVA with Duncan's test were done, different small letters stand for a significant difference (P<0.05). Data represent the means ± SDs.

Figure 3. FISH staining of fixed 64.5h biofilms of group 5SP+LGG and 5SP cultivated in different carbohydrates. The groups and tested carbohydrates are labelled to the top left corner of each image. Green (Lcas467-Cy3): LGG, red (MUT590-Cy5): *S. mutans*, blue (Hoechst): the rest of the strains=*S. sanguinis* + *C. albicans* + *A. actinomycetemcomitans* + *F. nucleatum*, pink/purple: co-localization of red and blue, black: non-cells area. Column A: Group 5SP+LGG with only green, column B: Group 5SP+LGG with only red and blue, column C: Group 5SP+LGG with red and blue. Each image includes the maximum intensity projections of xy- (top left), yz- (top right, rightmost is closer to HA discs), and xz-planes (bottom, bottom end is closer to HA discs). The scale bar is 30 μm.

Figure 4. Growth curves of LGG cultivated in biofilm broth media with a sole carbohydrate for 48h. The biofilm culture medium was biofilm medium with fructose (BM-fructose), glucose (BM-glucose), lactose (BM-lactose), sorbitol (BM-sorbitol), sucrose (BM-sucrose), or with carbohydrate free (BM-negative). Two independent experiments were performed, each experiment contained three parallels. Data represent the means ± SDs of all six values.