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Full Length Research Paper

Comparison of growth of viable oral bacteria and *Streptococcus mutans* in biofilm models using three different culture media

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Traditional culture-based media can have different ability to recover mutans streptococci from pure culture and oral bacteria from saliva samples after biofilm formation. For this purpose, the aim of this study was to compare three different culture media: Brain heart infusion (BHI), ultrapurified tryptone-yeast extract broth (UTYEB) and defined medium mucin (DMM), in two biofilm models: microcosm and monoculture, over 72 and 96 h incubation periods. Ninety-six bovine teeth were used for each biofilm assay. These specimens were randomly assigned to six different groups (n=8). Fresh stimulated saliva and *Streptococcus mutans* UA159 were used for comparisons among different culture media in microcosms and monoculture assays, respectively. Biofilm was formed on enamel disks for 72 and 96 h and after that, the biomass was collected. The UTYEB was similar to DMM, but significantly different from BHI for bacterial growth in microcosm biofilm. For the monoculture biofilm, DMM was statistically different from BHI and UTYEB. It was concluded that UYTEB is a suitable alternative for microcosm and monoculture assays.

Key words: Brain heart infusion (BHI), ultrapurified tryptone-yeast extract broth (UTYEB), defined medium mucin (DMM), dental plaque microcosm, monoculture.

INTRODUCTION

In vivo biofilm growth experiments are rarely used due to their limitations, such as issues related to the number of participants, non-controlled variables, reproducibility and ethics (Wong and Sissons 2001; Aires et al., 2008). To overcome these limitations, several *in vitro* biofilm growth models have been used (Koo et al., 2003), since they are cheap, fast and easy to reproduce.

Among the *in vitro* models used, monoculture system models are adopted to understand the possible factors that will positively or negatively influence the development of a single species of microorganisms that are isolated in specific conditions. In dentistry, *Streptococcus mutans* monoculture is frequently used in antimicrobial assays. The microcosm system (complex biofilm) is another model, used when higher fidelity in natural behavior is desired to reproduce the relationships of *in vivo* ecosystems (Sissons et al., 1998; Herigstad et al., 2001; Koo et al., 2003; Ccahuana-Vasquez and Cury, 2010;

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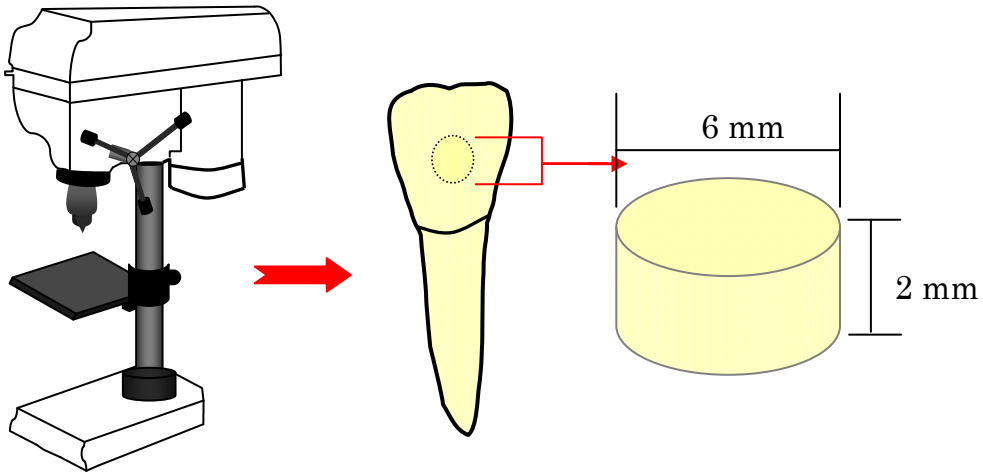

Figure 1. Cylindrical diamond-coated bur (trephine) used for discs enamel/dentin.

Peralta et al., 2013).

As oral fluid is the major source of nutrients for dental biofilm development, defined medium mucin (DMM) is one of the most used media to obtain this *in vitro* fidelity, because it is a "natural saliva" artificial analogue (Wong and Sissons, 2001). Other media are also used for biofilm growth, such as: brain heart infusion (BHI) (Gilbride and Rosendal, 1983; Loesche, 1986; Smith and Beighton, 1986; Bradshaw et al., 1994) and ultrapurified tryptone-yeast extract broth (UTYEB) (Gilbride and Rosendal, 1984). However, each medium has cost and production time peculiarities. Until now, relevant evidence-based findings which warrant the use of each culture medium for evaluation of oral bacteria in biofilm growth is lacking in biofilm models, such as the microcosm or monoculture models.

Therefore, the aim of this study was to quantitatively compare the growth of microorganisms in the microcosms and monoculture model, for 72 and 96 h in three culture media: BHI, UTYEB and DMM. The null hypothesis tested is that the culture media, as well as the time, did not influence cell viability of bacteria.

MATERIALS AND METHODS

In this study, two biofilm system models were used: (1) *S. mutans* monoculture; and (2) dental plaque microcosm, using three culture mediums: BHI, DMM and UTYEB; in periods of 72 and 96 h.

Approval of this research was granted by the Ethics Committee, School of Dentistry, Federal University of Pelotas, Pelotas, RS, Brazil (protocol No. 180/2010).

Culture media

Defined medium mucin

DMM was prepared according to Wong and Sissons (2001). This medium comprises partial purified pig gastric mucin (2.5 g/L^{-1}) (Sigma-Aldrich, St. Louis, USA), urea (1.0 mmol L^{-1}) (Vetec Quimica Fina Ltda, RJ, Brazil), salts (in mmol L^{-1}: $CaCl_2$, 1.0; $MgCl_2$, 0.2; KH_2PO_4, 3.5; K_2HPO_4, 1.5; NaCl, 10.0; KCl, 15.0; NH_4Cl, 2.0) (Vetec Quimica Fina Ltda, RJ, Brazil). The medium also contains a mixture of 21 free amino acids based on human saliva concentrations, in (mmol L^{-1}): arginine (1.30); asparagine (1.73); cysteine (0.05); glutamine (3.03); glycine (1.95); histidine (1.08); isoleucine (2.38); threonine (1.08); tryptophan (0.43); valine (2.38) (Vetec Quimica Fina Ltda, Rio de Janeiro, Brazil) and casein (5.0 g L^{-1}); alanine (1.95); aspartic acid (1.52); glutamic acid (5.41 mmol L^{-1}); leucine (3.68); serine (3.46); tyrosine (2.17) (Labsynth Laboratory Products Ltda., Diadema, SP, Brazil).

Ultrapurified tryptone-yeast extract broth

UTYEB was prepared as previously described (Koo et al., 2003).

Brain heart infusion

The BHI was supplied by Himedia Laboratories Pvt. Ltda, India.

Enamel discs preparation

Ninety-six enamel discs (6.0 mm diameter and 2.0 mm thickness) were obtained from fresh bovine central incisors. A cylindrical diamond-coated bur (trephine) was used perpendicularly to the buccal surface of the teeth (Figure 1). Dentine and enamel surfaces were polished with 400- and 800-grit silicon carbide paper, respectively. Both surfaces were flattened and the dentin was completely removed. All procedures were carried out under constant water cooling. Each disc was individually placed in 1 mL of a solution containing 0.06 mMPi and 0.08 $mMCa^{++}$ and autoclaved. After the sterilization, enamel discs were randomly assigned to 4 groups (n = 8) for the each medium tested. All specimens were vertically suspended on metal devices inside culture plate.

Biofilm accumulation tests

Microcosm

Fresh stimulated whole-saliva was collected from a healthy human

volunteer (male, age 31) who had not been under antibiotic therapy for at least one year. 15 mL of saliva was collected in the morning during fasting, prior to which the volunteer abstained from oral hygiene for 24 h prior to collection. One aliquot of the saliva was taken to determine the baseline microbial composition, measured in colony forming units per unit volume (CFU/mL^{-1}). Aliquots of saliva were plated on blood agar, Rogosa agar, aciduric BHI agar and mitis salivarius agar and incubated at 37°C, under anaerobic conditions for 24 h to verify the absence or presence of the microorganisms evaluated. Colony-forming units (CFU) were calculated using a stereomicroscope.

The prepared enamel discs were transferred into sterile wells (24-well tissue culture plate; TPP-Techno Plastic Products, Trasadingen, SU) and 400 µL of fresh, homogenized saliva was dispensed onto each disc. After 1 h of storage at 37 ± 1°C, the saliva was aspirated, and 1.8 mL of the medium selected (DMM or UTYEB or BHI, supplemented with 1% sucrose) was added for 6 h. Subsequently, the specimens were washed for 10 s with sterile saline and transferred to a new plate with fresh medium for 18 h. This procedure was repeated for 3 and 4 days (72 and 96 h). All plates were incubated at 37°C in an environment of 5-10% CO_2 (Anaerobac-Probac do Brasil products Bacteriological Ltda., Santa Cecília, SP, Brazil) in anaerobic jars (Probac do Brasil Bacteriology Products Ltda). All groups of culture media were incubated at 37°C for 96 h. Results were recorded at 72 and 96 h.

The pH of the contents from each well of culture media was determined after removal of the resin disc (Quimis 50w-Quimis Aparelhos Científicos Ltda., Diadema, SP, Brazil; V621 electrode-Analion, RibeirãoPreto, SP, Brazil).

After the time allowed for biofilm growth, the enamel discs containing the biofilms were washed 3 times with 0.9% NaCl and individually transferred to microcentrifuge tubes containing 1 mL of 0.9% NaCl. The biofilm was detached from the enamel surfaces and solubilized using vortex agitation for 30 s, followed by 30 s of sonication at 30 W (Sonicator DE S500, R2D091109 Brazil). The discs were then carefully removed from the suspension before aliquots of the suspension were used to determine the bacterial viability of the biofilm.

One hundred microliter aliquots of the initial biofilm suspension were diluted in 0.9% NaCl in a series up to 10^{-7}, after which 2 and 20 µL drops of each dilution were inoculated on BHI and blood agar adjusted to pH 7.2 to obtain total microorganism counts. To determine the total aciduric counts, the pH of the BHI was adjusted to 4.8 via the drop wise addition of HCl solution. *Mitis salivarius* agar was supplemented with 0.2 units of bacitracin mL to grow *S. mutans*, while total lactobacilli counts were determined using Rogosa agar (Herigstad et al., 2001). The plates were incubated at 37°C for 48 h under 5-10% CO_2 (Anaerobac-Probac do Brasil Produtos Bacteriológicos Ltda., São Paulo, Brazil) in anaerobic jars (Probac do Brasil Produtos Bacteriológicos Ltda.). The numbers of colony-forming units (CFU) were counted in a stereomicroscope (40x) with external halogen illumination, and the results were expressed as CFU/cm^{-2} of biofilm. Counts for the selective plates were based on colony morphology and verified by Gram-stain and cell appearance using light microscopy.

Streptococcus mutans monoculture

S. mutans UA159 biofilms were formed in bovine enamel discs suspended vertically in UTYEB (10 kDa molecular weight cut-off membrane; Amicon) at 37°C, 10% CO_2 for 3 and 4 days and continuously exposed to 1% sucrose.

S. mutans UA159 colonies were transferred to UTYEB or BHI or DMM (according to the groups), containing 1% sucrose and incubated at 37°C in 10% CO_2 to reactivate the microorganisms. After 18 h of incubation, 100 µL of these reactivated bacteria were transferred to 50 mL of UTYEB containing 1% homogenized sucrose, and 2 mL of this inoculum was transferred to each well of a 24-well culture plate (Koo et al., 2003). The enamel discs were individually positioned in the culture plate containing the inoculum and incubated at 37°C for 24 h under 5-10% CO_2. Daily, the biofilms on enamel disc were washed 3 times in 0.9% NaCl and transferred to a new plate with fresh UTYEB containing 1% sucrose for 24 h. This procedure was repeated for 3 and 4 days (72 and 96 h). All plates were incubated at 37°C in an environment of 5-10% CO_2 (Anaerobac-Probac do Brasil products Bacteriological Ltda., Santa Cecília, SP, Brazil) in anaerobic jars (Probac do Brasil Produtos Bacteriológicos Ltda).

The pH of each well contents after removal of the enamel disc was determined (Quimis 50w-Quimis Aparelhos Científicos Ltda., Diadema, SP, Brazil; V621 electrode-Analion, Ribeirão Preto, SP, Brazil).

Biofilm collection

After 72 or 96 h of biofilm growth, the enamel discs containing the biofilms were washed 3 times in 0.9% NaCl and individually transferred to microcentrifuge tubes containing 1 mL of 0.9% NaCl. The tubes were sonicated at 30W for 30 s (Sonicator DE S500, R2D091109, Brazil) to detach the biofilms formed on the disc (Aires et al., 2008). Aliquots of the suspension were used to determine biofilm bacterial viability. For the bacterial viability, an aliquot of 100 µL of the suspension was serially diluted in 0.9% NaCl in series up to 10^{-7}, and 2 drops of 20 µL of each dilution were inoculated on BHI agar (BD, Sparks, USA) to determine the number of viable microorganisms (Herigstad et al., 2001). The plates were incubated for 72 h at 37°C under 5-10% CO_2 concentration (Anaerobac - Probac do Brasil Produtos Bacteriológicos Ltda., Santa Cecília, SP, Brazil) in anaerobic jars (Probac do Brasil Produtos Bacteriológicos Ltda). CFU were counted and the results were expressed as CFU/cm^2 of biofilm.

Statistical analysis

The equality of the variances and the normal distribution of the errors were checked for all tested response variables. Those that did not satisfy these conditions were submitted to transformations as an attempt to fulfill parametric assumptions. The CFU count data were non-normal and log transformed. Subsequently, statistical analyses were performed with the transformed data. A $\log_{10}$ transformation of each CFU count was performed to normalize the data before statistical evaluation due to the high range of bacterial numbers. Then, to determine viable bacteria counts, statistical analyses were performed using ANOVA on ranks, one-way ANOVA, and the Fisher's least significance difference (LSD) post hoc test for pair-wise means comparisons. Two-way ANOVA (time and media) was used to assess the biofilm monoculture. All statistical testing was performed using Sigma Stat® for Windows Software®, Version 3.5 (Systat Software, Inc., Point Richmond, CA, USA), using a pre-set alpha of 0.05.

RESULTS

The microcosm results are shown in Figure 2. For lactobacilli, there was statistically higher viability in BHI than UTYEB and DMM in 72 h (p=0.001). There were similar results for over 96 h (Figure 2a).

For total aciduric bacteria, the BHI medium showed a lower mean CFU than the two other media (p=0.001). When evaluated over 96 h, bacteria growth was

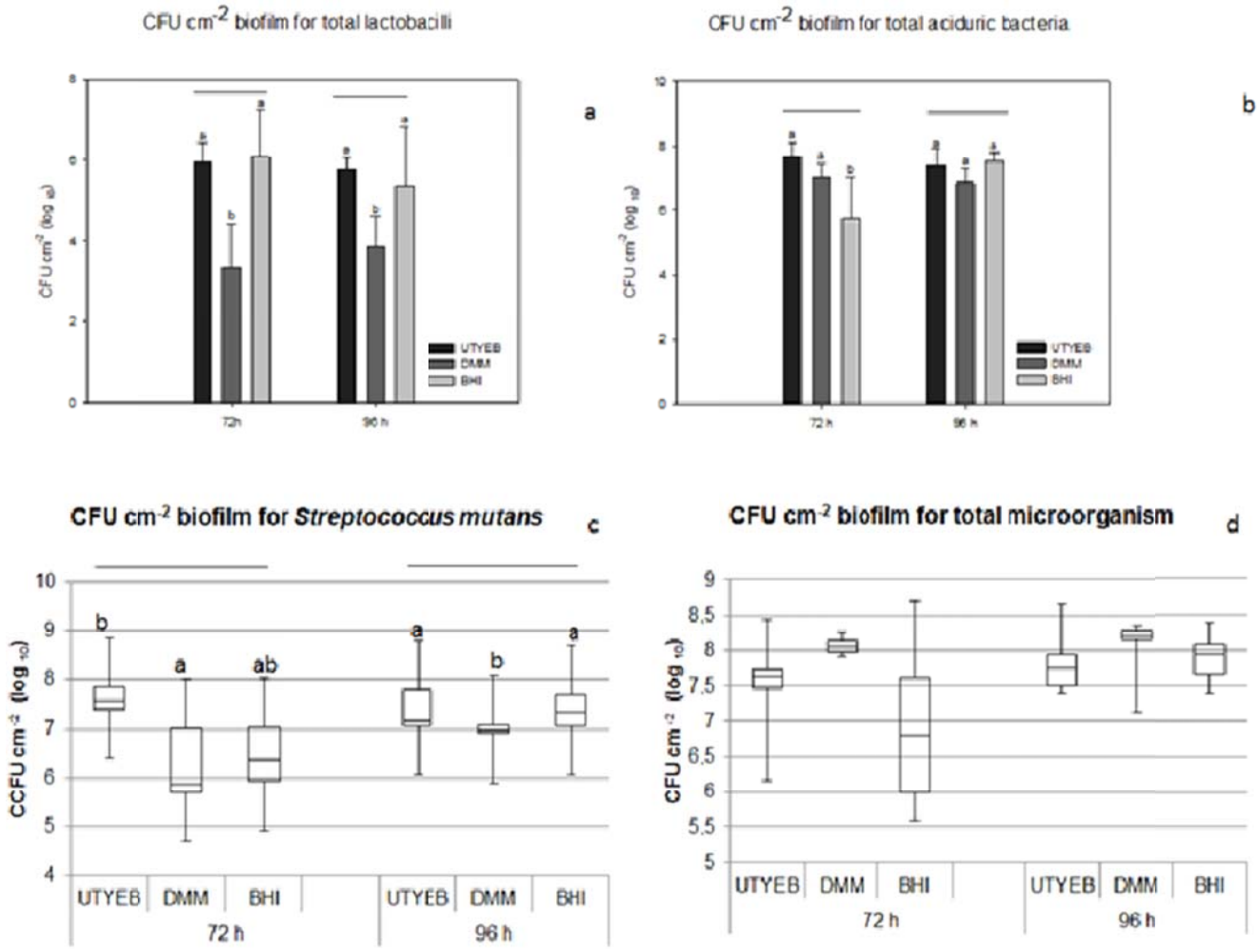

Figure 2. Mean and median of viable bacteria (CFU/cm^{-2} biofilm) in biofilms grown for 72 and 96 h (n = 8). The data were transformed in log10. Within a panel, group values that were identified using similar lower case letters were not significantly different (p>0.05). a = total lactobacilli bacteria; b = total aciduric bacteria; c = *S. mutans*; d = total microorganisms.

statistically similar in all media (p=0.068) (Figure 2b).

Figure 2c shows that UTYEB had the highest growth of *S. mutans*, statistically different from DMM (p=0.013). Over 96 h, the growth of *S. mutans* in UTYEB and BHI were statistically similar, but different in DMM (p=0.029). For total microorganisms, in 72 h the DMM showed the statistically highest CFU for UTYEB and BHI (p=0.013). The three media in 96 h were similar (p=0.360) (Figure 2d).

The monoculture results are shown in Figure 3. It showed a significant difference between media (p<0.001) and incubation periods (p<0.001). Furthermore, interaction was found between the media and incubation periods (p=0.002). In monoculture biofilm, the bacterial growth of *S. mutans* was highest in UTYEB and BHI.

DISCUSSION

The null hypothesis that the culture media, as well as the incubation periods of time, did not influence cell viability of bacteria was partially rejected. It was shown that, in the microcosm model, BHI presented differences in the cell viability of lactobacilli and aciduric bacteria for 72 h. However, the biofilm growth was similar for 96 h. The monoculture model showed lower bacteria cell viability in DMM for both periods.

Biofilm models are important methodologies for assessing antimicrobial effect of the dental materials or other solutions. Those can be formed in different conditions, such as different culture media. Therefore, the conditions for formation of the used biofilm should be as close as possible to the *in vivo* situation (Ccahuana-Vasquez and Cury, 2010). In the present paper, two biofilm models were discussed: microcosm and monoculture.

The microcosm technique is employed as an ecosystem that mimics naturally formed biofilms (Imazato et al., 2006; Cenci et al., 2009; McBain, 2009). In studies focusing on supragingival plaque, a microcosm implies

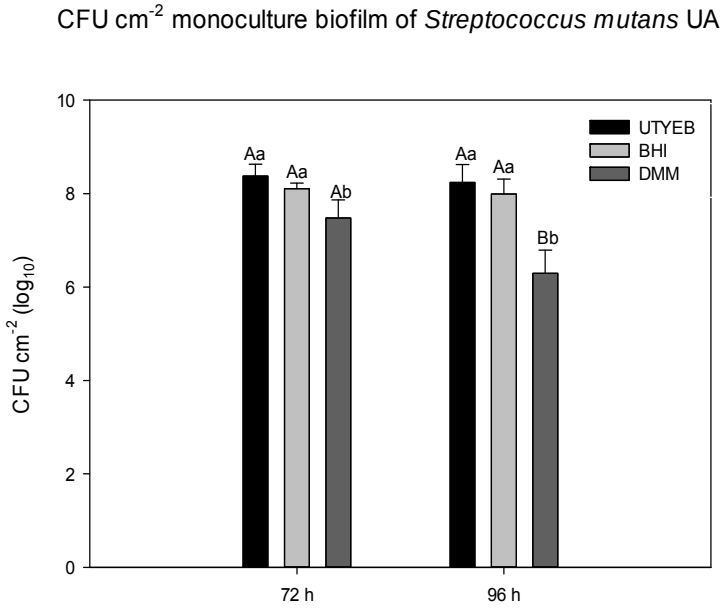

Figure 3. Mean of viable bacteria (CFU/cm^{-2} biofilm) in biofilms grown for 72 and 96 h (n = 8). The data were transformed in $\log_{10}$. Within a panel, group values that were identified using similar lower case letters (media) or capital letters (time) were not significantly different. ($p>0.05$). Abbreviations: UTYEB = ultrapurified tryptone-yeast extract broth; BHI = brain heart infusion; DMM = defined medium mucin.

the use of dental plaque or human saliva as a source of inoculum for an *in vitro* device (Wimpenny, 1997). This study used an *in vitro* microcosm dental biofilm model with a semi-continuous sucrose exposure. The microcosm methodology is more complex and more precise than *in vitro* biofilm assays with a single species because the proportions of species detected in the microcosm community were similar to those observed in supragingival plaque.

The results found in microcosms models can be explained for the continuous exposure of carbohydrate in the BHI and UTYEB broth medium with dextrose and yeast in their composition when compared with the DMM medium. If BHI medium has dextrose in its composition, the biofilm in this medium was in continuing exposure to sucrose. It is known that the lactobacilli have their cell viability significantly increased in the presence of saccharides (Goderska and Czarnecki, 2008). This may explain the increase in cell viability of lactobacilli in the period of 72 h (Figure 2a).

The development of *S. mutans* in different media is in agreement with previous studies (Sissons et al., 1998; Peralta et al., 2013). This may be due not only to their ability to hydrolyze sucrose and other carbohydrates, but also because they are facultative bacteria and have acidogenic and aciduric properties (Loesche, 1986).

It is hypothesis that the nutrition, resource acquisition and pH are important factors in plaque. The media investigated have sufficient nutrients for the growth of oral bacteria. UTYEB has been used in several studies (Gilbride and Rosendal, 1983, 1984; Martin et al., 1985). BHI is a medium with non-salivary proteins. DMM probably showed the highest growth because it has all components of human saliva, being an analog. It has vitamins and growth factors which provide a suitable medium for bacteria development. Moreover, proteins and glycol-proteins in saliva are the major energy source and nutrients for the bacteria (Smith and Beighton, 1986; Bradshaw et al., 1994).

S. mutans showed lower cell viability for DMM in both incubation periods. The other media, BHI and UTYEB, have dextrose, yeast and carbohydrates in their compositions, which might facilitate growth of *S. mutans*. These components are not found in DMM, which might explain the lower cell viability of this bacterium in this medium. Furthermore, *S. mutans* viability rise in the presence of glucose, sucrose or other saccharides and related compounds. Growth also stops or decreases in its absence, and *S. mutans* cannot grow having amino acids as the only energy source (Terleckyj and Shockman, 1975). DMM also comprises mucin, which is unsuited as an energy source for *S. mutans* growth (van der Hoeven et al., 1990) apart from combinations of amino acids (cysteine, arginine, glutamine and glutamic acid), which do not support growth (Mothey et al., 2014).

Conclusions

It was concluded that bacterial growth in BHI had the

worst performance in the microcosm biofilm model. Also, the growth of *S. mutans* in DMM was weak in the monoculture biofilm assay. Overall, the results suggested that UYTEB is a suitable alternative for microcosm and monoculture assays.

Conflict of interests

The authors did not declare any conflict of interest.

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