Reducing the variability between constant-depth film fermenter experiments when modelling oral biofilm

Running Title: Dual CDFF model

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Abstract

Aims: The inherent instabilities associated with the development of multispecies biofilm communities within the constant-depth film fermenter (CDFF) and other microcosm systems can yield unacceptable variability between experiments which could limit their potential applications in oral microbiology. The extent of this variability needs to be determined and a protocol developed which minimises it.

Methods and Results: Two, custom-made CDFFs were supplied concurrently with the same inoculation culture, begat from an aliquot of a saliva pool and artificial saliva growth medium via a dual-channel pump. Transformed $\log_{10}$ data of the viable counts at fixed time points were analysed using the Bland-Altman approach to test for levels of agreement between two CDFFs running concurrently and those CDFFs run in series. The *c*oefficients$_{95\%}$ of agreement were lower (i.e. less variable) in the concurrent model than when run in series for total counts of bacteria (1.238 vs 2.124), *Lactobacillus* spp. (0.517 vs 1.431) and Mutans streptococci (2.817 vs 3.864). Other measures of variability showed a similar trend.

Conclusions: Operating CDFFs concurrently minimises the degree of difference and variability between them.

Significance and Impact of Study: Operating CDFFs concurrently will improve the sensitivity for experiments which seek to determine the effects of a variable, such as a nutritional supplement or antimicrobial agent, and a control.

Introduction

Laboratory models of bacterial communities should be capable of replicating the heterogeneity of the microenvironment, this is especially important in biofilm (Wimpenny, 1981). The constant-depth film fermenter (CDFF) has been used for over two decades to model the growth of microbial biofilms (Peters and Wimpenny, 1988) and had been employed to model dental plaque (Kinniment et al., 1996a). The CDFF consists of a rotating stainless steel turntable which holds polytetrafluoroethylene (PTFE) sample pans housed in a sealed glass unit enclosed by two stainless steel end-plates. The upper end-plate possesses ports for sample removal and gas exchange along with three liquid input ports which are typically used for the inoculation culture, growth medium and spare which can be used to introduce an adjunct agent. The lower end-plate has an outlet drain for the removal of liquid waste. Each sample pan has five cylindrical plugs on which the substrata of interest can be placed and accurately recessed to a known depth. During operation, the turntable rotates and two spring-loaded PTFE blades smear incoming growth medium over the sample pans whilst removing excess cellular material to ensure that the biofilm grows at a constant depth. The sample pans can be independently removed from the CDFF to assess the biological parameters of the growing biofilm or study chemical changes in the substratum. The operating methodology of this model have been more comprehensively described elsewhere (Pratten, 2007).

There are three basic methods of inoculating the CDFF when modelling oral biofilm; single-species (Pratten et al., 1998b), defined multispecies (Kinniment et al., 1996a) and microcosm (i.e. an aliquot of a saliva pool or plaque sample) (Pratten et al., 2003, Hope et al., 2003, Hope and Wilson, 2006). Whilst single-species cultures allow experiments to be focused on a particular organism of interest, the lack of

interaction between members of the wider microbial community means that this remains an unrealistic model of oral biofilm. Defined multispecies cultures can be used to study specific interactions between different members of the oral community. The number of different bacterial species that have been incorporated into such pseudo oral microbiota range from two (Dalwai et al., 2006) to ten members (Bradshaw et al., 1996b). Defined microbial communities have an advantage in that they can be used to replicate, albeit to a limited extent, the complex interactions and interdependencies which may occur in the oral ecosystem. The limited number of members and defined nature of such models allows these interactions to be studied in detail and has proven a very useful tool for modelling the oral microbial ecosystem (McKee et al., 1985, Bradshaw et al., 1989, Kinniment et al., 1996b).

The most representative model of the commensal oral microbiota comes from a salivary or plaque inoculum containing a microcosm of the many hundreds of different culturable microorganisms. However, an inherent variation between different samples remains, even when these are obtained from the same individual at different times. This variation can be somewhat reduced by collecting saliva or plaque samples from a number of donors, combining them to form a ‘saliva pool’, before splitting the pool into a series of (essentially) identical aliquots. Such a pool serves to be representative of ‘average saliva’. While there is no doubt that a saliva pool will contain a greater diversity of microbial constituents than a single saliva sample from an ‘average person’, pooling should ensure a degree of conformity between aliquots and ensures that they are not missing any ‘important’ members of the oral microbiota. However; aggregations of bacteria in the form of plaque debris are often visible to the naked eye in the saliva pool aliquots, suggesting that such pools are not entirely homogeneous. Whilst additional processing would obviate this

problem, subjecting the bacteria to increased fluid shear forces risks damaging some of the fragile organisms found in the oral cavity, particularly filamentous anaerobes such as *Actinomyces spp*. (Hope and Wilson, 2003) and cause oxidative stress in strict anaerobes such as *Prevotella* spp (Bradshaw et al., 1996a). Microcosm biofilm cultures grown in the CDFF have been shown to respond to the addition of adjunct agents such as sucrose (Hope et al., 2005), supplementary feed containing starch (McBain et al., 2003), chlorhexidine (Pratten et al., 1998b) and fluoride (Deng et al., 2005).

Although *in vitro* models have an inherent reproducibility when compared to *in vivo* experiments, in that all parameters are controllable, minor variations in the bacterial composition between different aliquots of the saliva pool inocula can lead to corresponding differences in the resulting microcosm community (Ledder et al., 2006). These perturbations could be magnified by the growth conditions of the inoculation culture and initial phase of biofilm formation.

The aim of this study was to develop a novel, concurrent CDFF protocol using two smaller custom-made CDFFs in which experiments were carried out concurrently using the same inoculum and liquid medium supply whilst located within the same incubator. The aim was to investigate the microbiological variability between CDFFs run either in series or in parallel.

Materials and Methods

Experimental Protocol

To make the proposed concurrent CDFF approach feasible, smaller versions of the device were commissioned (J. Abbott, West Kirby, Merseyside) to fit side-by-side within a standard laboratory incubator (IP250-U, LTE Scientific Limited, Oldham, UK)

(Figure 1, inset). The width of the 'compact constant-depth film fermenters' used throughout this study were 140 mm. Each turntable had a diameter of 100 mm which could accommodate eight PTFE sample pans, yielding a total of forty individual ~5 mm substrata discs capable of supporting aliquots of the biofilm. For comparison, the 'standard', 15 sample pan CDFF is 230 mm wide, has a turntable diameter of 150 mm and can hold a total of seventy-five substrata discs (Figure 2).

A saliva pool was constructed by collecting whole, unstimulated saliva samples from local research staff volunteers which comprised a mixture of smokers and non-smokers, none of whom had been prescribed antibiotics two months prior to their donation (n = 23) (Ethical Approval: University of Liverpool Research Governance Office, Physical Interventions Sub-committee, Ref: RETH000377). 3 ml aliquots were taken from each saliva sample, combined together and homogenised by magnetic stirring. An equal volume of sterile skim milk powder (Oxoid, Basingstoke, UK) mixture was added to the saliva pool to give a final concentration of 10% w/v, this acted as a cryoprotectant (Cody et al., 2008). The pool was then split into a number of 1.8 ml aliquots and stored at -80ºC until required. The saliva pool preparations were undertaken as quickly as possible in order to minimise the cytotoxic effects of oxygen on the strict anaerobic microorganisms.

Two CDFFs were fully loaded with forty, 4.8 mm hydroxyapatite (HA) discs (Clarkson Chromatography Products, South Williamsport, PA, USA) recessed to a depth of 200 µm. The CDFFs were sterilised by dry-heat at 140ºC for 3 hours. This method of sterilising had been previously validated (data not shown). All other glassware and media components were sterilised by autoclaving at 121ºC for 15 minutes.

The circuit diagram of the concurrent CDFF set-up is shown in Figure 1. An inoculation flask was prepared with one litre of a mucin containing artificial saliva growth medium (Pratten et al., 1998c) comprising of Lab-lemco 1 g l^{-1}, yeast extract 2 g l^{-1}, proteose peptone 5 g l^{-1} (all Oxoid), type III hog gastric mucin 2.5 g l^{-1}, sodium chloride 0.2 g l^{-1}, potassium chloride 0.2 g l^{-1}, calcium chloride 0.3 g l^{-1} (all Sigma-Aldrich, Poole, UK). The flask was then located in an incubator at 37ºC before an aliquot of the saliva pool was thawed and added whilst the contents were magnetically stirred. A multichannel peristaltic pump (323 Du pump with 318MC head; Watson Marlow, Falmouth, UK) was used to introduce the inoculum into both CDFFs at a flow rate of 0.72 ml min^{-1}. The inoculum was exhausted after approximately 12 hours at which point a second input of sterile artificial saliva medium was initiated at a flow rate of 0.38 ml min^{-1} per CDFF. Two CDFF experiments were carried out concurrent followed by two carried out in series.

Sample pans were removed from both CDFFs regularly throughout the course of the experiment, on days 4, 6, 8, 12 and 14. All five, biofilm laden HA discs were extracted from the sample pans using sterile forceps and vortex mixed for 30 seconds in 900 µl of phosphate-buffered saline (PBS) to disrupt the biofilm. The resulting cell suspensions were then serially diluted up to 10^6 in additional PBS. These dilutions were cultured on Blood Base No. 2 Agar (BA) (Bioconnections, Leeds, UK) containing 5% defibrinated horse blood (TCS Biosciences, Buckingham, UK) to enumerate viable bacteria in terms of total colony forming units (CFU) present per mm^2 of HA disc. Additional solid selective growth media were used to isolate and enumerate particular organisms of interest; *Streptococcus spp.* on *Mitis Salivarius* agar (MSA) (BD, Oxford, UK) supplemented with 1 ml^{-1} tellurite solution; total mutans streptococci group on Tryptone Yeast Cysteine agar (Lab M, Bury, UK)

supplemented with sucrose (Sigma) and bacitracin (Sigma); *Lactobacillus spp. o*n Rogosa agar (RA) (Oxoid, Basingstoke, UK). All plates were incubated at 37°C for 72 hours under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2).

Statistical Analysis

The level of agreement between CDFFs exposed to the same growth conditions was examined in the constituent bacterial populations associated with dental caries (Streptococcus spp. and Lactobacillus spp.) and in the bacterial population as a whole (total viable counts). All the analyses described were undertaken to examine the levels of agreement between CDFFs operating as a concurrent system (i.e. run concurrently) and then for agreement between CDFFs operating in series (i.e. run on separate occasions). Statistical analyses were implemented using SPSS for Windows Release 16.0.2 (SPSS UK Limited, Woking, UK). Bland-Altman plots (also known as a Tukey mean-difference plots) (Altman, 1991, Bland and Altman, 1986, Bland and Altman, 1999), with limits of agreement were used to assess agreement between counts in paired samples. The Bland-Altman test is typically used to analyse the level of agreement between two methods of measurement. Bias was tested using paired t-tests. Analysis included the calculation of average difference and limits of agreement as well as a graphical display of datasets as scatter plots. In order to apply parametric tests (where it is assumed that data is normally distributed) viable counts per population were subjected to $\log_{10}$ transformations to obtain data with normal distribution and this was checked by the generation of simple histograms.

Results

Biofilm formation and growth

The plaque biofilm community reached a pseudo-steady state after four days growth (Figure 3). In relation the total counts of bacteria on non-selective blood agar, the proportions of *Streptococcus* spp. ranged from 1.9 to 14%, Mutans streptococci from 0.003 to 0.004% and *Lactobacillus* spp. from 0.65 to 1%.

Explorative analysis of agreement between experiments in series and concurrently

Figure 4 shows the Bland-Altman plots for total count for each microbial group. These plots the show differences between $\log_{10}$ viable microbial counts from pairs of samples, plotted against their respective averages. There was no evidence of systematic bias between pairs of samples run concurrently for any of the microbial groups. For pairs of samples run in series, there were statistically significant differences between pairs for *Streptococcus spp*., Mutan streptococci and *Lactobacillus spp.* suggesting systematic bias between runs (Table 1). Coefficients$_{95\%}$ of agreement were lower (i.e. less variable) in the concurrent model than when run in series for total counts of bacteria (1.238 vs 2.124), *Lactobacillus* spp. (0.517 vs 1.431) and Mutans streptococci (2.817 vs 3.864). A similar trend was observed in the limits of agreement$_{95\%}$ (Table 1).

Discussion

Significant variation between CDFF runs has been previously reported within a nine-species defined biofilm model (Kinniment et al., 1996a) and similar problems have been observed using microcosm inocula despite being derived from the same source (Pratten et al., 1998a). Such variations were attributed to slight unavoidable

differences between aspects of CDFF operation which could be exacerbated by heterogeneity in the inoculum (Ledder et al., 2006) and the presence of unculturable microorganisms in the inoculums (Pratten et al., 2003). Despite these reported limitations, the CDFF remains a versatile method for producing reproducible aliquots of oral biofilm (Pratten and Wilson, 1999). This variability between CDFF runs has implications for when separate experiments are conducted to determine the effects of a variable upon the microbial composition of the biofilm. Reducing the variability within an experiment will improve the detection limits when testing the effects of adjunct agents or other changes in environmental conditions. The physical separation of the two CDFFs precluded the possibility of any cross-over of bacteria or materials between the two devices. Other than sharing an effluent bottle for convenience, the only physical connection between the two CDFFs is at their points of medium ingress, which are protected by grow-back traps.

The most likely source of variation between two experiments which use polymicrobial, microcosm inocula is differences between the two aliquots of inocula used, even if the material has been pooled and split. In the case of the CDFF, differences between said inocula have the opportunity to become magnified in the inevitable enrichment process that occurs both in the liquid phase of the inoculation culture and also in the biofilm modality inside the fermenter.

An elegant approach to avoid these inherent minor variations between experiments is to use a ‘split CDFF’ design (Deng et al., 2005). This involved modifying a standard fifteen pan CDFF (University of Wales, Cardiff, UK) which was allowed to operate as normal during the inoculation and biofilm growth phase, by rotating the turntable through 360º. The CDFF was then operated in ‘split mode’ where two different adjunct agents could be added via separate input ports to distinct

halves of the biofilm samples. 'Split mode' was achieved by removing four sample pans, at opposite sides of the turntable, and engaging a reversing circuit connected to the motor power supply via micro-switches. This modality enabled the turntable to oscillate through a fixed arc of 180º, but unfortunately leaves only eleven sample pans in the split CDFF, five in one half and six in the other. Whilst no carry-over of bacteria or agents between the two sides of the 'split CDFF' was reported, a more rigorous approach would be to operate two CDFF's concurrently under identical conditions. However; the physical dimensions, weight and cost of the CDFF limit the practical implementation of this concurrent approach. The primary benefit of a concurrent, concurrent CDFF system for studying oral biofilm would be the ability to operate one CDFF as a control whilst a second unit is subjected to an external influence, such as the addition of an adjunct agent.

The flow rate of artificial saliva into a standard CDFF was nominally set at 0.5 – 0.72 ml min^{-1} (Pratten, 2007) in order to mimic the mean flow rate of saliva in man (Guyton et al., 1997, Lamb et al., 1991). To keep this same relative flow of growth medium over the sample pans in the smaller CDFF, the artificial saliva flow was proportionally adjusted to accommodate eight rather than fifteen sample pans (0.72 ml min^{-1} × $^{8}/_{15}$ = 0.38 ml min^{-1}). The flow rate of the inoculum into the two CDFFs was kept at 0.72 ml min^{-1} since it was the growth kinetics within the inoculation vessel that was considered important and not the flow rate into the CDFF *per se.* Having fewer sample pans in the compact CDFF inevitably means that fewer time points or shorter time course experiments have to be undertake when compared to the standard CDFF.

Although the mean differences in viable counts of bacteria provide a good description of the difference between biofilms produced within CDFFs, the most

objective measure is to deduce the degree of variability observed in each type of model – in this case either concurrent or in series CDFFs. A certain degree of variability, or lack of agreement, is inevitable when comparing two microbiological growth experiments in a CDFF. This is due in part to the inherently complexity in their operation, but more importantly to the dynamic multispecies ecosystem that develops into a pseudo steady-state microbial biofilm ecosystem which undergoes periodic sloughing events. When running CDFFs on in series occasions, standard deviation was 1.391 $\log_{10}$ CFU mm^{-2} whereas it was only 0.619 $\log_{10}$ CFU mm^{-2} in CDFFs run concurrently within the concurrent CDFF design.

Operating CDFFs concurrently, in the 'concurrent' modality minimises both the degree of difference and variability, and reduce the likelihood of systematic bias, between CDFF experiments designed to compare the effects of potential variables. This novel method will therefore increase the sensitivity of CDFF based experiments to ascertain the effects of an external influence such as a nutrient supplement, antimicrobial agent, probiotic culture or environmental stimulus upon the oral microbiota.

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References

Altman, D. G. 1991. Statistical analysis of comparison between laboratory methods. *J Clin Pathol,* 44**,** 700-701.

Bland, J. M. and Altman, D. G. 1986. Statistical Methods For Assessing Agreement Between Two Methods Of Clinical Measurement. *Lancet,* 327**,** 307-310.

Bland, J. M. and Altman, D. G. 1999. Measuring agreement in method comparison studies. *Stat Methods Med Res,* 8**,** 135-160.

Bradshaw, D. J., Marsh, P. D., Allison, C. and Schilling, K. M. 1996a. Effect of oxygen, inoculum composition and flow rate on development of mixed-culture oral biofilms. *Microbiology,* 142**,** 623-629.

Bradshaw, D. J., Marsh, P. D., Schilling, K. M. and Cummins, D. 1996b. A modified chemostat system to study the ecology of oral biofilms. *J Appl Bacteriol,* 80**,** 124-130.

Bradshaw, D. J., Mckee, A. S. and Marsh, P. D. 1989. Effects of carbohydrate pulses and pH on population shifts within oral microbial communities in vitro. *J Dent Res,* 68**,** 1298-1302.

Cody, W. L., Wilson, J. W., Hendrixson, D. R., Mciver, K. S., Hagman, K. E., Ott, C. M., Nickerson, C. A. and Schurr, M. J. 2008. Skim milk enhances the preservation of thawed -80ºC bacterial stocks. *J Microbiol Meth,* 75**,** 135-138.

Dalwai, F., Spratt, D. A. and Pratten, J. 2006. Modeling shifts in microbial populations associated with health or disease. *Appl Environ Microb,* 72**,** 3678-3684.

Deng, D. M., Van Loveren, C. and Ten Cate, J. M. 2005. Caries-preventive agents induce remineralization of dentin in a biofilm model. *Caries Res,* 39**,** 216-23.

Guyton, A. C., Hall, J. E. and Schmitt, W. 1997. Secretary functions of the alimentary tract. *Human physiology and mechanisms of disease.* Philadelphia: Saunders

Hope, C. K., Petrie, A. and Wilson, M. 2003. In vitro assessment of the plaque-removing ability of hydrodynamic shear forces produced beyond the bristles by 2 electric toothbrushes. *J Periodontol,* 74**,** 1017-1022.

Hope, C. K., Petrie, A. and Wilson, M. 2005. Efficacy of removal of sucrose-supplemented interproximal plaque by electric toothbrushes in an in vitro model. *Appl Environ Microb,* 71**,** 1114-1116.

Hope, C. K. and Wilson, M. 2003. Effects of dynamic fluid activity from an electric toothbrush on in vitro oral biofilms. *J Clin Periodontol,* 30**,** 624-629.

Hope, C. K. and Wilson, M. 2006. Biofilm structure and cell vitality in a laboratory model of subgingival plaque. *J Microbiol Meth*, 66, 390-398

KINNIMENT, S. L., WIMPENNY, J. W., ADAMS, D. and MARSH, P. D. 1996a. Development of a steady-state oral microbial biofilm community using the constant-depth film fermenter. *Microbiology,* 142 Pt 3**,** 631-8.

Kinniment, S. L., Wimpenny, J. W., Adams, D. and Marsh, P. D. 1996b. The effect of chlorhexidine on defined, mixed culture oral biofilms grown in a novel model system. *J Appl Bacteriol,* 81**,** 120-125.

Lamb, J. F., Ingram, C. G., Johnston, I. A. and Pitman, R. M. 1991. Gastrointestinal system and nutrition. *Essentials of physiology.* Oxford: Blackwell Scientific Publications.

Ledder, R. G., Gilbert, P., Pluen, A., Sreenivasan, P. K., De Vizio, W. and Mcbain, A. J. 2006. Individual microflora beget unique oral microcosms. *J Appl Microbiol,* 100**,** 1123-31.

Mcbain, A. J., Bartolo, R. G., Catrenich, C. E., Charbonneau, D., Ledder, R. G. and Gilbert, P. 2003. Growth and molecular characterization of dental plaque microcosms. *J Appl Microbiol,* 94**,** 655-664.

Mckee, A. S., Mcdermid, A. S., Ellwood, D. C. and Marsh, P. D. 1985. The establishment of reproducible, complex communities of oral bacteria in the chemostat using defined inocula. *J Appl Bacteriol,* 59**,** 263-275.

Peters, A. C. and Wimpenny, J. W. 1988. A constant depth laboratory film fermenter. *Biotechnol Bioeng.* 32, 263-270.

Pratten, J. 2007. Growing Oral Biofilms in a Constant Depth Film Fermentor (CDFF). *Current Protocols in Microbiology***,** 1B.5.1-1B.5.17.

Pratten, J., Barnett, P. and Wilson, M. 1998a. Composition and susceptibility to chlorhexidine of multispecies biofilms of oral bacteria. *Appl Environ Microb,* 64**,** 3515-3519.

Pratten, J., Smith, A. W. and Wilson, M. 1998b. Response of single species biofilms and microcosm dental plaques to pulsing with chlorhexidine. *J Antimicrob Chemoth,* 42**,** 453-459.

Pratten, J., Wills, K., Barnett, P. and Wilson, M. 1998c. In vitro studies of the effect of antiseptic-containing mouthwashes on the formation and viability of Streptococcus sanguis biofilms. *J Appl Microbiol,* 84**,** 1149-1155.

Pratten, J. and Wilson, M. 1999. Antimicrobial Susceptibility and Composition of Microcosm Dental Plaques Supplemented with Sucrose. *Antimicrob Agents Ch.,* 43**,** 1595-1599.

Pratten, J., Wilson, M. and Spratt, D. A. 2003. Characterization of in vitro oral bacterial biofilms by traditional and molecular methods. *Oral Microbiol Immun,* 18**,** 45-49.

Wimpenny, J. W. T. 1981. Spatial order in microbial ecosystems. *Biol Rev,* 56**,** 48.

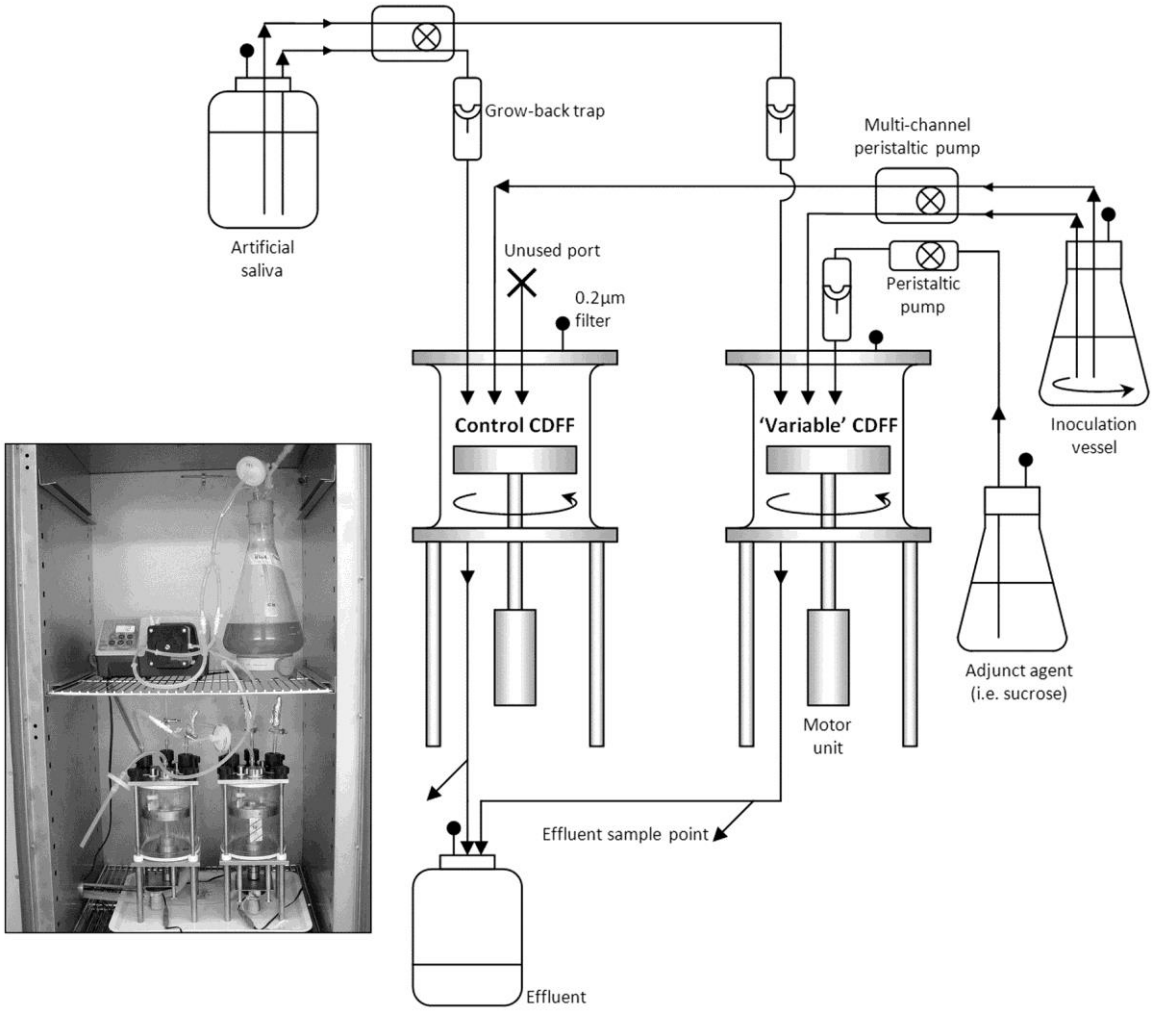

Figure 1 Circuit diagram of the concurrent compact constant-depth film fermenter. Inset: Operational photograph with inoculation vessel *in situ*.

Figure 2 The ‘standard’ CDFF with 15 sample pans (left; University of Wales, Cardiff) compared to the compact CDFF with 8 sample pans (right; J. Abbott, West Kirby, Merseyside).

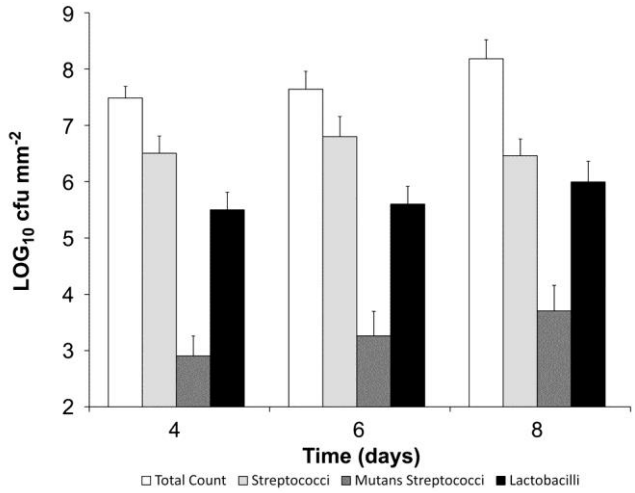

Figure 3 Viable counts of bacteria growing as 200 µm thick oral biofilm within a CDFF as determined by growth on selective and non-selective solid growth media. Error bars give standard deviations (n=16)

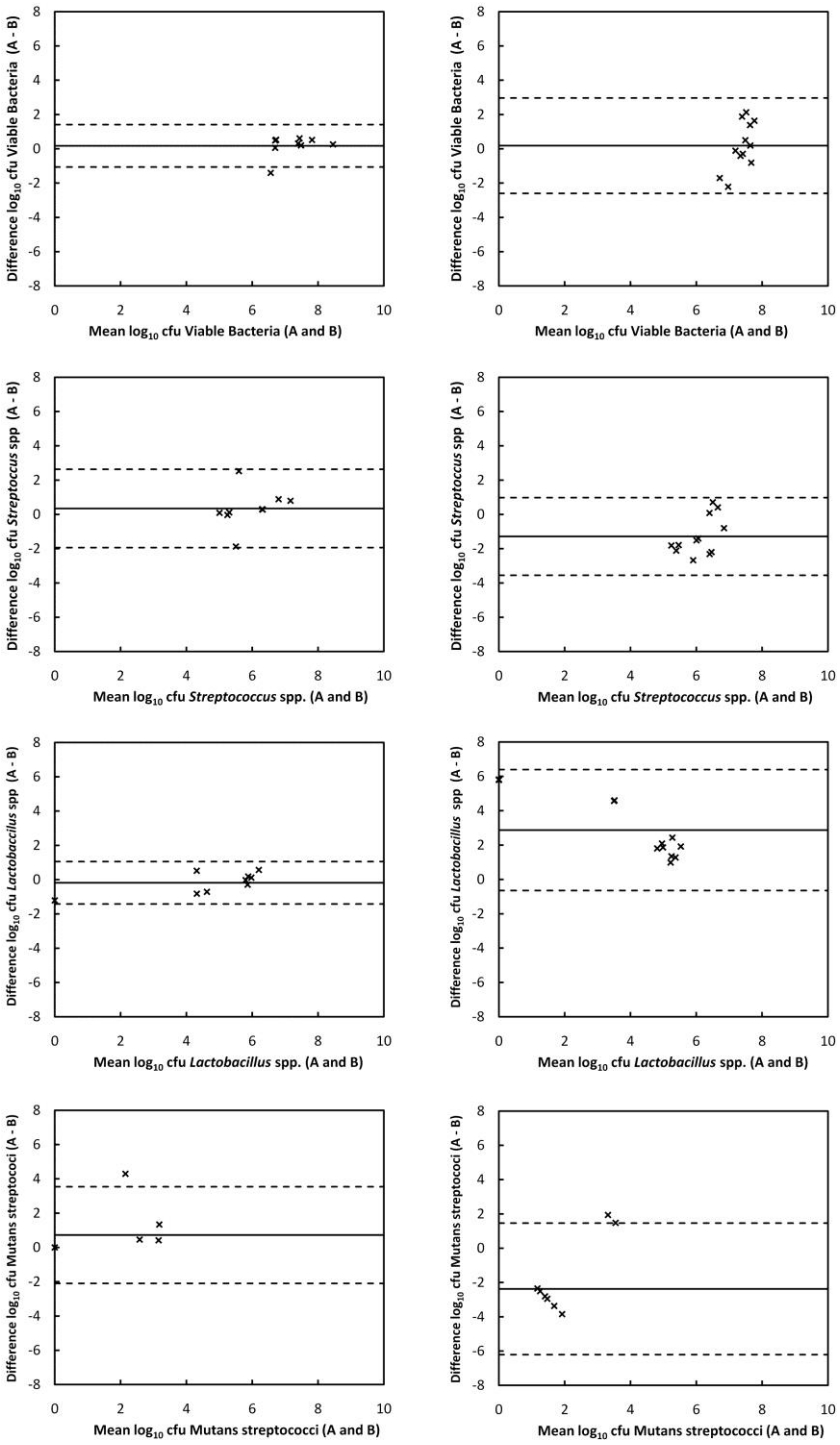

Figure 4 Bland-Altman plots of the differences between microbial counts in two constant-depth film fermenters operated concurrently (left column) or in series (right column). The mean differences (solid line) as well as upper and lower 95% limits of agreement (mean ± 2SD) are given (dashed line). The closer the 95% limits of agreement are to the mean difference and the tighter the clusters of data point, the better the agreement is between the measurements.

Table 1 Analysis of agreement between viable counts of bacteria growing as oral biofilm in constant depth film fermenters either operated concurrently (Conc.) or in series. All counts were $\log_{10}$ transformed prior to analysis.

	Total Count		**Total Streptococci**		**Total Lactobacilli**		**Mutans streptococci**	
	Conc. (n=9)	In series (n=12)	Conc. (n=9)	In series (n=12)	Conc. (n=9)	In series (n=12)	Conc. (n=9)	In series (n=12)
Minimum difference	0.058	-0.11	-0.035	0.085	-0.029	0.985	0.000	-1.937
Maximum difference	-1.406	-2.21	2.522	-2.676	-0.819	4.601	4.290	-3.844
Mean difference	0.173	0.181	0.342	-1.287	-0.058	2.288	0.722	-2.372
Standard deviation (SD)	0.619	1.391	1.142	1.131	0.517	1.431	1.409	1.917
$\text{Coefficient}_{95\%}$ of agreement	1.238	2.124	2.285	2.262	1.035	2.861	2.817	3.864
$\text{Limits}_{95\%}$ of agreement	-1.065, +1.410	-2.600, +2.963	-1.942, 2.627	-3.549, +0.975	-1.092, +0.977	-0.573, +5.149	-2.095, +3.539	-6.206, +1.462
p-value (paired t-test)	0.426	0.660	0.396	0.002	0.162	0.002	0.389	<0.001