Archives of Oral Biology (2004) **49**, 789–798

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Archives of Oral Biology

www.intl.elsevierhealth.com/journals/arob

Biofilm formation of *Candida albicans* is variably affected by saliva and dietary sugars

Ye Jin*, Lakshman P. Samaranayake, Yuthika Samaranayake, Hak Kong Yip

Faculty of Dentistry, The University of Hong Kong, Oral Biosciences, 5/F, Prince Philip Dental Hospital 34, Hong Kong, Hong Kong

Accepted 23 April 2004

KEYWORDS
Candida albicans;
Adhesion;
Biofilm;
XTT reduction assay;
ATP bioluminescence

Summary The pathogenesis of both superficial and systemic candidiasis is closely dictated by properties of the yeast biofilms. Despite extensive investigations on bacterial biofilms, the characteristics of candidal biofilms, and various factors affecting this process remain to be determined. Therefore we examined the effect of human whole saliva and dietary sugars, glucose and galactose on the adhesion and biofilm formation of *Candida albicans*. Biofilms of *C. albicans* isolate 192 887 g were developed on polystyrene, flat-bottomed 96-well microtiter plates and monitored using ATP bioluminescence and tetrazolium (XTT) reduction assays as well as the conventional colony forming unit (CFU) evaluation. Our data showed that both the ATP and the XTT assays strongly correlated with the CFU assay (ATP versus CFU: $r = 0.994$, $P = 0.006$; XTT versus CFU: $r = 0.985$, $P = 0.015$). Compared with a glucose—supplemented (100 mM) medium, galactose containing (500 mM) medium generated consistently lower levels of both candidal adhesion and biofilm formation (all $P < 0.05$), but a higher pace of biofilm development over time (96 h). Whist the presence of an immobilised saliva coating had little effect on either the candidal adhesion or biofilm formation, the addition of saliva to the incubation medium quantitatively affected biofilm formation especially on day 3 and 4, without any significant effect on yeast adhesion. To conclude, biofilm formation of *C. albicans* within the oral milieu appears to be modulated to varying extents by dietary and salivary factors and, further investigations are required to elucidate these complex interactions.

Introduction

Candida species are considered important opportunistic pathogens due to the increasing frequency of infections they cause in compromised patient groups such as those on cancer chemotherapy, broad-spectrum antibiotics, and HIV-infected individuals. Of the many pathogenic *Candida* species, *Candida albicans* is the major fungal pathogen of humans.[1] In the oral cavity, a niche they frequently inhabit as commensals, these yeasts exist predominantly within biofilms, which are spatially organised heterogeneous communities of fungal cells encased in a matrix of extracelluar polymeric substances (EPS).[2,3] *Candida* biofilms can also develop on surfaces of prosthesis and medical devices, and exhibit resistance to both antifungals and host defenses compared with their free-living planktonic counterparts. This is likely to be the cause of recalcitrant persistence of *Candida* on inert, inserted surfaces or, superficial mucosae.[3]

Once a prosthesis is placed in the oral cavity, it will be instantaneously coated with a pellicle of

*Corresponding author. Tel.: +852 2859 0480; fax: +852 2547 6133.
E-mail address: lakshman@hku.hk (Y. Jin).

0003-9969/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.archoralbio.2004.04.011

saliva. Additionally, the developing biofilms are constantly exposed and bathed in saliva. However, the effect of saliva on *Candida* adhesion and subsequent biofilm formation remains obscure. Although efforts have been made to evaluate the role of salivary pellicle on yeast adhesion and biofilm formation, the data available at present are inconsistent and confusing. For instance, some have reported a heightened degree of candidal adhesion when the substrata were coated with submandibular-sublingual saliva.[4] Likewise, increased biofilm activity of *C. albicans* was observed in the presence of a pellicle derived from unstimulated whole saliva.[5] Others, in contrast, have observed inhibition of candidal adhesion consequential to coating a substratum with unstimulated whole saliva.[6,7,32] San Millán et al.[8] found paradoxically that the presence of a salivary pellicle (unstimulated whole saliva) initially enhanced yeast adhesion and then, after 60 min of incubation, inhibited the adhesion. These conflicting results warrant further research into this issue. Moreover, there is no information on the possible changes in candidal adhesion or biofilm formation when a medium supplemented with saliva is applied to *Candida* suspensions.

The effect of dietary sugars on *Candida* biofilm formation has also been studied from an oral point of view as dietary carbohydrates modulate microbial colonization of the latter niche.[9] Two monosaccharides, glucose and galactose, have been extensively investigated for their effects on candidal adhesion. Galactose, which is present in free form in a wide range of foods,[10,11] has been reported to be able to alter the components of outermost surfaces of fungal cell wall and consequently modify candidal adhesion.[7,12] Similarly free glucose, a monosaccharide in fruits and used in the manufacture of candy, chewing gum, table syrups, and for many other purposes, has also been found to affect candidal adhesion.[5,13,14] Data, however, are limited on the effect of these sugars on *Candida* biofilm formation.

A simple, reliable quantitative assessment method is a prerequisite for investigating the nature of biofilm formation. Traditionally, evaluation of colony forming unit (CFU) was used for such work despite the laborious work involved and, difficulties in disrupting cell aggregates without affecting viability. Recently, a few novel assays, which are based on metabolic activity rather than viability, have been developed for biofilm quantification. Of these, tetrazolium salt 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay[15–18] is a colorimetric method that quantifies the number of living cells in a biofilm whilst in the ATP bioluminescence assay, cell quantification is based on a reaction between the intracellular ATP and the enzymatic system luciferin–luciferase. The light emission from the latter reaction is proportional to the number of viable cells within the sample as long as they are metabolically active and, can be accurately quantified.[19]

We therefore characterised the adhesion and biofilm formation of *C. albicans* under a variety of environmental conditions that are likely to be operational intra-orally. Biofilm quantification was performed using XTT and ATP assays as well as CFU estimation in order to standardise these three methods. The parameters that were evaluated included the effect of a mixed salivary coating (a pellicle), and saliva and glucose or galacatose supplements of the growth medium, on *C. albicans* biofilm formation on plastic surfaces.

Materials and methods

Organisms and growth conditions

C. albicans 192 887 g, an oral isolate found to be a good biofilm producer in our previous studies,[16] was used in all experiments. The isolation and confirmation of the identity of this isolate has been described elsewhere.[20] Before the assays, the isolate 192 887 g was sub-cultured from a thawed suspension of the pure isolate stored in the Oral Bioscience Laboratory at the Prince Philip Dental Hospital of the University of Hong Kong. The identity of the isolate was reconfirmed using the germ tube test and API20C-AUX method.

The *C. albicans* isolate 192 887 g was first cultured at 37 °C for 18 h in Sabouraud's dextrose agar (SDA). Then, a loopful of the yeasts was inoculated in yeast nitrogen base (YNB) medium supplemented with 100 mM glucose. After overnight broth culture, the yeasts were harvested in the late exponential growth phase. Before use in adhesion and biofilm experiments, the yeasts were washed twice with 5 ml of phosphate buffered saline (PBS; pH 7.2; Ca^{2+}- and Mg^{2+}-free). Then the washed cells were resuspended in growth media (adhesion experiments) or PBS (biofilm experiments), and adjusted to an optical density of 0.38 at 520 nm (10^7 cells/ml).

Comparison of growth rates of planktonic yeasts in glucose and galactose

To assess growth rates of *C. albicans* grown in glucose and galactose media, we prepared cell suspensions (10^6 cells/ml) of the isolate 192 887 g in YNB containing 100 mM glucose or 500 mM galac-

tose. Each suspension was inoculated in triplicate wells of a microtiter plate and incubated overnight at 37 °C in a microtiter plate reader (SpectraMAX 340 Tunable Microplate Reader, Molecular Devices Ltd., Sunnyvale, CA, US). The optical density at 520 nm of each well was measured at 2 h intervals in an automated fashion to generate the growth curves.

Adhesion assay

Candidal adhesion assays were performed in wells of microtiter plates (IWAKI, Tokyo, Japan) (Fig. 1). 100 μl of cell suspension (10^7 cells/ml) in YNB containing glucose (100 mM) or galactose (500 mM) was added to each well of a pre-sterilised, polystyrene, 96-well microtiter plate and incubated for 90 min at 37 °C in a shaker at 75 rpm. We chose the incubation during of 90 min for the adhesion assay since previous work has demonstrated that adherent populations on the substratum do not form a structurally differentiated community (biofilm) during this period of time.[13] Afterwards, the cell suspensions were aspirated, and each well washed twice with 150 μl of PBS to remove loosely adherent cells.

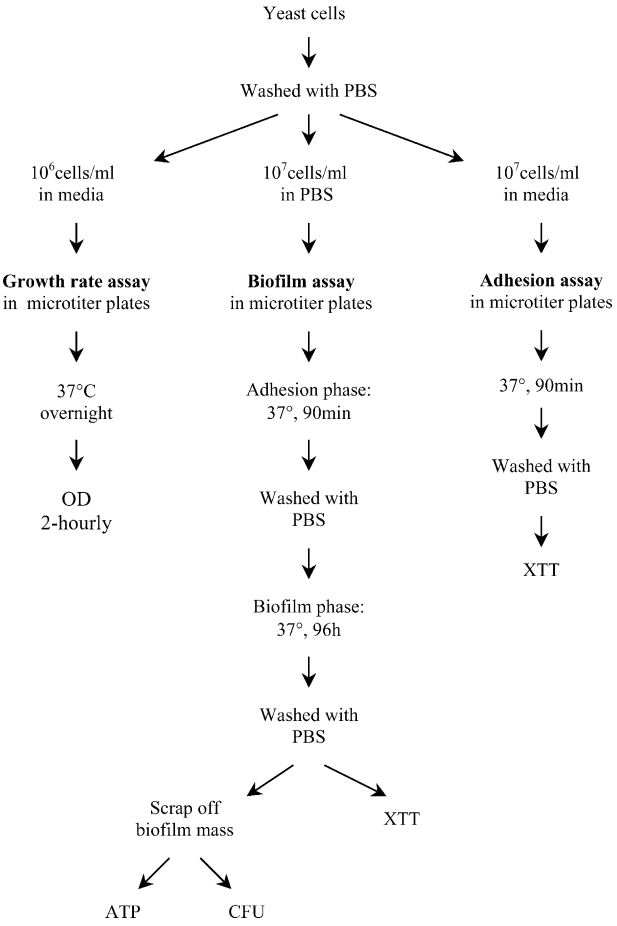

Figure 1 A flow chart illustrating the experimental steps of the study. OD: optical density measurement; PBS: phosphate buffered saline; XTT: tetrazolium salt 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay; ATP: adenosine triphosphate ATP bioluminescence assay; CFU: colony forming unit.

Biofilm formation

Biofilms of *C. albicans* were permitted to grow in wells of microtiter plates (IWAKI, Tokyo, Japan) as previously described (Fig. 1).[16] The adhesion phase of candidal biofilm formation was performed in the same fashion as the adhesion assays described above except that the yeast suspension (10^7 cells/ml) was in PBS instead of YNB containing either glucose (100 mM) or galactose (500 mM). Following the 90 min adhesion phase, each well was washed twice with 150 μl of PBS to remove loosely adherent cells. 100 μl of YNB containing glucose (100 mM) or galactose (500 mM) was then pippetted into each of the washed wells and the plates incubated at 37 °C in a shaker at 75 rpm for 96 h. The growth medium was replenished daily. It should be noted that ''adhesion'' in the following text refers to that described in the adhesion assay instead of the adhesion phase of candidal biofilm formation.

Saliva collection

Whole unstimulated saliva was collected from five healthy adult volunteers who were asked not to eat before 2 h prior to collection. The saliva donors were also asked to rinse their mouths gently with water before sampling to decrease bacterial contamination. Saliva was collected on ice for 10 min, pooled and centrifuged at 12 000 × *g* for 15 min at 4 °C. The resulting supernatant was immediately stored at −70 °C until used.[5,21] Saliva samples collected using an analogous protocol have been demonstrated to contain most salivary contents associated with microbial adhesion and biofilm formation, such as proline-rich proteins, mucins, and a variety of enzymes.[21]

Saliva assay

The effect of saliva on candidal adhesion and biofilm formation was investigated in two different ways, by coating the substrata with saliva or by, exposing the yeast biofilms to a saliva-supplemented growth medium. To investigate the effect of a saliva coating on candidal adhesion and biofilm formation, the wells of the microtiter plates were pretreated with

unstimulated whole saliva. Each well was coated with 50 μl of thawed saliva and then incubated for 4 h at 37 °C (coating phase). Afterwards, the saliva was removed and each well washed twice with 200 μl of PBS. 100 μl of standardised cell suspension (1×10^7 cells/ml) was then inoculated into each well and the whole assembly handled as described above. In experiments examining the development of biofilms exposed to a saliva-supplemented medium, the growth medium in each well comprised 80 μl of glucose-supplemented YNB and, 20 μl of saliva (test group) or PBS (controls). All assays were carried out on three different occasions in triplicate.

Adenosine triphosphate (ATP) bioluminescence assay

After biofilm formation, each well was washed four times with PBS. Then, 200 μl of PBS was pipetted into each well and the biofilm mass was meticulously scraped off the well wall using a sterile scalpel. The resultant suspension (200 μl) containing the detached biofilm cells was transferred to a sterile 6 ml tube. This manoeuvre was repeated on four occasions yielding a total of 800 μl of cell suspensions for each well, which were then vortexed for 3 min and read using the ATP assay.

A commercially available ATP analyser was employed for quantification of ATP in viable yeasts within biofilms. In brief, the ATP analyser was calibrated before ATP measurement to minimise the inter-experiment variability, according to the manufacture's instruction (AF-100, DKK.TOA Corp., Tokyo, Japan). Afterwards, 100 μl each of sample (cell suspension) and the extractant supplied by the manufacturer was added into a new container which was immediately inserted into the ATP analyser for 30 s for extraction of intracellular ATP. Afterwards, 100 μl of luminescent reagent was added to this mixture and the result was recorded as ATP concentration (mol/l). The ATP assays were carried out on three independent occasions, with three samples on each occasion.

Correlation between ATP readings and numbers of planktonic yeasts

A standard curve was constructed in order to correlate the ATP concentrations and the yeast numbers. For this purpose, a series of cell suspensions with concentrations ranging from 10^1 to 10^5 cells/ml of the isolate 192 887 g were prepared using a hemocytometer. Afterwards, the ATP yield of each suspension was evaluated as described above and the standard curve constructed.

XTT reduction assay

XTT-reduction assay was performed as described previously.[16] Briefly, XTT (Sigma, MO, USA) was dissolved in PBS at a final concentration of 1 mg/ml. The solution was filter sterilised using a 0.22 μm-pore-size filter and stored at −70 °C until required. Menadione (Sigma, MO, USA) solution (0.4 mM) was also prepared and filtered immediately before each assay. Prior to each assay, XTT solution was thawed and mixed with menadione solution at a volume ratio of 20−1.

Either the adherent yeasts (adhesion assay) or the biofilms (biofilm assay) were washed four times with 200 μl of PBS to remove loosely adherent cells. Afterwards, 158 μl of PBS, 40 μl of XTT, and 2 μl of menadione were inoculated to each of the pre-washed wells. After incubation in the dark for 2 h at 37 °C, 100 μl of the solution was transferred to a new well and a colorimetric change in the solution measured using a microtiter plate reader (SpectraMAX 340 Tunable Microplate Reader, Molecular Devices Ltd., Sunnyvale, CA, US) at 490 nm.

Colony forming unit (CFU) of resuspended biofilm cells

Biofilm cells were scraped off the well walls as described above and the suspensions obtained gently sonicated for 3 min to destroy the aggregates. The suspensions were then inoculated on SDA, according to the pour plating method, and the resultant CFU quantified.[22]

Statistical analyses

The statistical analyses were performed using SPSS 11.0 for windows. One-way ANOVA was performed to analyse the differences among multiple means. According to the tests of homogeneity of variances, either Bonferroni or Dunnett T3-test was carried out for post-hoc multiple comparisons. *P*-values of <0.05 were considered statistically significant.

Results

Evaluation of quantitative methods used in this study

To be able to predict cell concentration using the ATP index, a standard curve was generated for these two parameters using *C. albicans* 192 887 g (Fig. 2). On regression analysis a coefficient of correlation of 0.999 and $P < 0.0001$ were noted, indicating that the yeast cell numbers in suspension can be con-

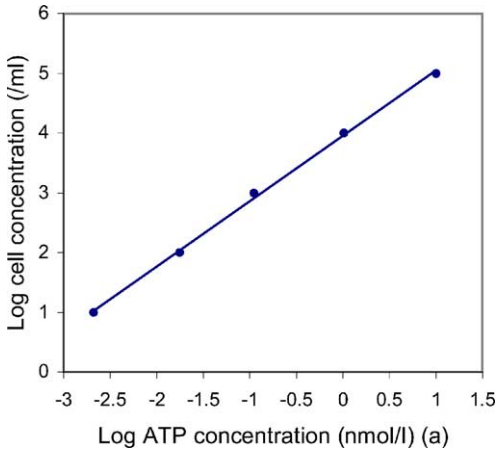

Figure 2 The relationship between ATP bioluminescence and cell concentrations of *C. albicans* determined by a hemocytometer. Linear regression analysis revealed perfect linearity between the $\log_{10}$ ATP concentration and $\log_{10}$ cell number ($r = 0.999$; $P < 0.0001$). Regression formula was as follows: lg Cell concentration = 1.0947 × lg ATP concentration + 3.9567. The isolate used was 192 887 g.

fidently predicted using the ATP method in combination with the generated standard curve.

Another quantitative technique used in the present study was the XTT reduction assay which has been extensively used for biofilm quantification.[16,23,24] Since both the ATP and the XTT assays are based on metabolic activity which may vary in planktonic and biofilm phase cells,[25] we further compared these two methods with the CFU values of the resuspended biofilm cells, as the latter is unlikely to be significantly affected by the metabolic state of the cells. For this purpose, *Candida* biofilms were formed on either nude or saliva-coated surfaces in glucose- or galactose-supplemented media and the cell mass quantified either using the XTT, ATP or the CFU assays. As shown in Fig. 3a–c, there were no significant variations in cell mass when quantified by any of the three assays. Additionally, on regression analyses significant correlations were also noted between the three methods (XTT versus ATP $r = 0.980$; $P = 0.02$; XTT versus CFU $r = 0.985$; $P = 0.015$; CFU versus ATP $r = 0.994$; $P = 0.006$) (Fig. 4).

Effects of dietary sugars and saliva on C. albicans adhesion

The major advantage of the XTT assay is that manual cell detachment is not necessary and the attendant loss in cell numbers leading to high signal to noise ratio does not occur with this technique. This is especially true in the early adherent phase of the biofilm where the cell numbers are relatively small. We, therefore, used the XTT assay exclusively for the adhesion experiments in the first phase of the study.

When the effect of glucose and galactose on cell adhesion was compared, culture of yeasts in YNB containing glucose (100 mM) led to a significantly higher degree of adhesion than cells grown in YNB supplemented with galactose (500 mM), regardless of the saliva coating of the wells (In the presence of saliva coating: $P = 0.009$; in the absence of saliva coating: $P < 0.0001$). In addition to the adhesion ability per se, another possible reason for the varying degrees of yeast adhesion in glucose and galactose may be the relative differences in growth rates of planktonic yeast cells in these nutrient media. We therefore compared their growth profiles by monitoring turbidity (OD520) of cell suspensions containing the two sugars for a period of 16 h. As seen in Fig. 5, *Candida* cells continuously grew at a high rate in 100 mM glucose while the cell growth in 500 mM galactose was slow and reached a plateau after 10 h.

On examining the effect of a mixed salivary pellicle, no significant difference in adhesion was noted between nude and saliva-coated surfaces, independent of the nature of the dietary sugar (In glucose: $P = 1.000$; in galactose: $P = 0.560$). Similarly, yeasts grown in YNB supplemented with or without mixed saliva demonstrated comparable levels of adhesion ($P = 0.223$) (Fig. 6).

Effects of dietary sugars and saliva on biofilm formation of C. albicans

As shown by the three different quantitative methods, the candidal biofilm development under different conditions shared similar patterns, i.e. all the biofilms first grew rapidly in the first few days and then reached a plateau (Fig. 3).

Our data, comparing biofilm formation in different sugars, indicated a higher degree of biofilm formation by fungal cells grown in glucose compared with galactose either on nude or saliva-coated surfaces (Fig. 3) (all $P < 0.05$). Apart from the biofilm quantity, the growth rates of biofilms were also influenced by the nature of the dietary sugar (Fig. 3). Initially, biofilm growth in glucose was more rapid than that in galactose despite the higher molarity of the latter (Fig. 5). After day 1, however, the biofilm growth in glucose slowed down relative to galactose and for both sugars, the biofilm growth reached a plateau after day 3. Overall, the nature of the sugars in the growth medium influences not only the quantity but also the growth rate of *Candida* biofilms.

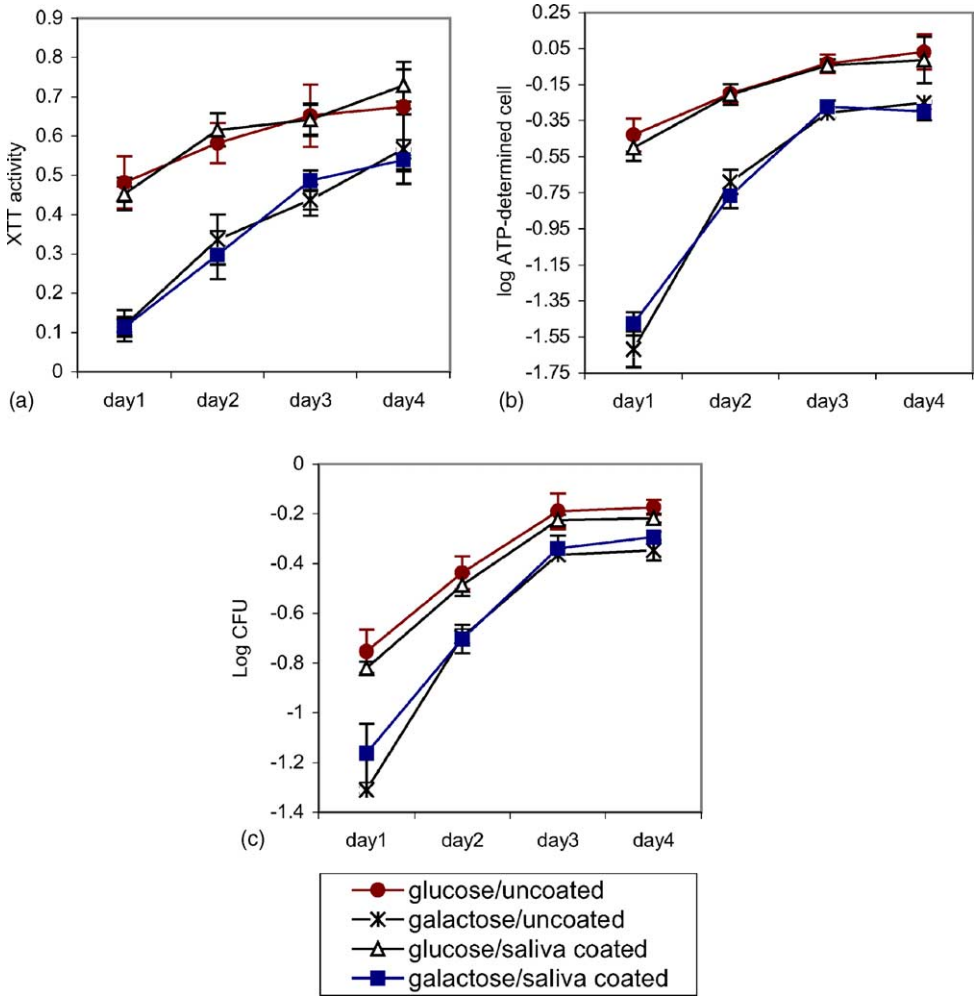

Figure 3 Biofilm formations of *C. albicans* 192 887 g in a medium containing either 100 mM glucose or 500 mM galactose and in the absence or the presence of a mixed saliva coating. The methods used for biofilm quantification were XTT reduction assay (a), ATP bioluminescence assay (b) and CFU evaluation (c). Data are means ± standard deviations of three independent experiments performed in triplicate.

All the tissue and inert surfaces in the oral cavity are inevitably exposed to saliva and, it is pertinent to elucidate the effect of saliva on *Candida* biofilm formation. As seen in Fig. 3, biofilm formation was not appreciably affected by the presence of a saliva coating ($P > 0.05$). However, yeasts grown in a saliva-supplemented medium produced a significantly lower biofilm mass than controls especially on days 3 and 4 ($P < 0.0001$ for both days) although no such a difference was noted in the first two days of incubation (day 1, $P = 0.356$; day 2, $P = 0.183$) (Fig. 6).

Discussion

A variety of methods have been recently used in quantification of *Candida* biofilms on different substrata. These include XTT, ATP, crystal violet absorbance, dry weight and CFU assays. However, to our knowledge, standardization of these techniques has not been undertaken to date. In order to properly correlate the biofilm data emanating from various centers it is essential that a normative assessment of the methods is performed. Hence a primary aim of the current study was to compare three different methods, XTT assay, ATP bioluminescence assay and CFU quantification in evaluating biofilm growth of *Candida*.

ATP bioluminescence assay is based on the oxidative decarboxylation of luciferin in the presence of ATP and Mg^{2+}. The light emission from the reaction is proportional to the number of viable cells within a sample.[19] The ATP method has been widely applied to quantify somatic cells,[26] microbes,[27] and bacterial[19]and *Candida* biofilm formation.[5]

In the present study, we observed excellent linearity between ATP concentration and number of

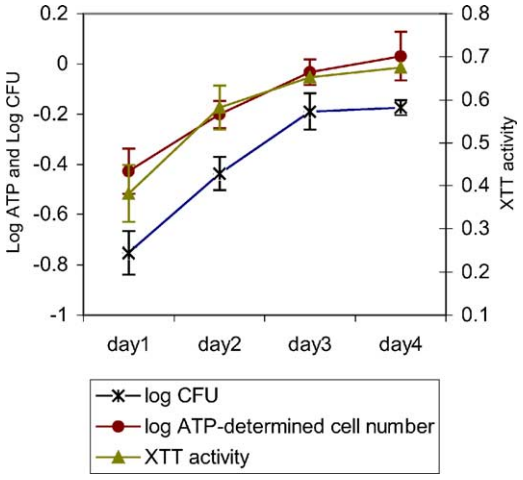

Figure 4 Biofilm formation of *Candida albicans* 192 887 g in a medium containing 100 mM glucose in the absence of a mixed saliva coating. Quantification was performed by XTT reduction assay, ATP bioluminescence assay and CFU evaluation. Data are means ± standard deviations of three independent experiments performed in triplicate.

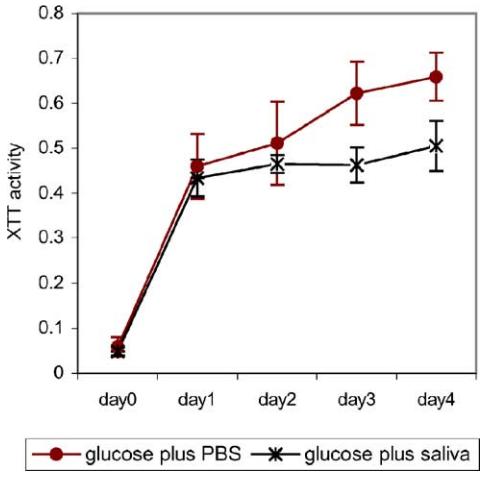

Figure 6 Biofilm formation of *Candida albicans* 192 887 g in a medium containing either 100 mM glucose and PBS or glucose and saliva. Biofilm formation was evaluated using XTT reduction assay. Data are means ± standard deviations of three independent experiments performed in triplicate.

planktonic yeasts ($r = 0.999$, $P < 0.0001$), concurring with the findings of others.[28] Our previous studies have also demonstrated that XTT activity is linearly associated with cell numbers and hence a reliable method for biofilm quantification.[16] It is however noteworthy that biofilm cells, which are encased within an EPS matrix, may possibly have limited access to nutrients and oxygen, resulting in possible alterations in their metabolic activity. If this were the case, neither the ATP nor the XTT assay, which are based on the metabolic activity of the cells, would correctly reflect the total cell numbers. To exclude this possibility, we quantified *Candida* biofilm formation using the traditional CFU method in addition to the ATP and XTT assays. Although quantification of CFU of resuspended biofilm cells is time-consuming and laborious, it directly enumerates the cell numbers, not being overtly influenced by their metabolic status. Indeed, our data showed that the CFU assay correlated well with the ATP and XTT readings, suggesting that the yeast cells encased within the biofilm matrix are metabolically active. Therefore, we propose that any one of the three methods evaluated can be reliably used for quantification of *Candida* biofilm mass although in our hands the XTT assay proved to be the simplest.

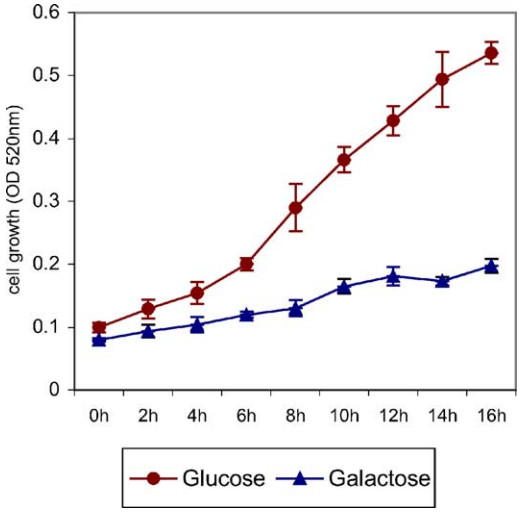

Figure 5 Growth profiles of planktonic cells of *Candida albicans* 192 887 g grown in 100 mM glucose- and 500 mM galactose-containing YNB. The suspensions in wells of a microtiter plate were incubated in a microtiter plate reader for 18 h at 37 °C. During the incubation, the OD at 520 nm was measured at 2 h intervals to generate growth curves. Data are means ± standard deviations of three independent experiments performed in triplicate.

We observed some common features of *Candida* biofilms irrespective of the growth conditions. Thus all biofilms progressively developed for up to 3–4 days and then reached a plateau even though the medium was replenished daily. This growth pattern implies that the cell numbers of a mature *Candida* biofilm attain a steady state at a critical point.

To investigate the effect of carbohydrates on candidal adhesion or biofilm formation, previous workers have pre-cultured yeast cells with sugar-supplemented media for up to 18 h.[12,14] The subsequent adhesion or biofilm studies were conducted in sugar-free media as the carbon source might affect the growth rate of yeasts during the assays

and confound the resultant data. Yet, in a biological milieu, such as the oral cavity, sugars are always present and their effect on both the cell adhesion and subsequent biofilm formation need to be evaluated. Therefore, media with sugar supplementation were used throughout our adhesion and biofilm assays (except for the adhesion phase of the biofilm experiments).

Our data showed that galactose (500 mM)-grown yeasts exhibited significantly lower degrees of both adhesion and biofilm formation than glucose (100 mM)-grown fungal cells, in agreement with the previous work using the same experimental protocol.[5] Yet, some early studies have shown that galactose-grown yeasts are far more adherent than glucose grown counterparts.[13,14] However, it is noteworthy that the fungal cells were pre-cultured up to 18 h in different sugars prior to adhesion or biofilm assays in the latter investigations. In addition, these studies were performed in static media which were not replenished as in the current study and, depletion of nutrients may have modified biofilm growth. Growth profiles of planktonic yeast cells in glucose and galactose were also compared in the present study, and former elicited a higher growth rate. The differential growth rate of glucose- and galactose-grown yeasts as well as the prolonged experimental period compared with previous studies are likely to be reasons for the observed higher degree of adhesion and biofilm production in glucose-supplemented media.

On the first day of biofilm formation, glucose-grown biofilms developed more rapidly than the counterparts grown in galactose. After day 1 and, until day 3 the growth rate in galactose was faster than that of glucose. Previous studies have demonstrated that galactose may alter the outermost surface components of yeast wall,[7,12] resulting in increased synthesis of fibrillar mannoprotein adhesins.[29,30] These additional adhesins are thought to facilitate fungal adhesion and subsequent biofilm formation.[30,31] In addition, compared with cells grown in other sugars, galactose-grown yeasts appear to show the highest degree of hydrophobicity and therefore more likely to aggregate in aqueous media.[33] These may be reasons for the observed rapid development of biofilms in galactose compared with glucose.

Any material inserted into the oral cavity is instantaneously coated by a saliva pellicle. Our results revealed that the presence of a saliva coating (unstimulated whole saliva) did not significantly influence the degree of either *C. albicans* adhesion ($P > 0.05$) or biofilm growth ($P > 0.05$) (Fig. 3), regardless of the dietary sugars supplements in the media. In contrast to these findings, previous workers have demonstrated that coating the substrata with unstimulated whole saliva significantly inhibited adhesion[6,7,32] but resulted in increased biofilm formation by *C. albicans*.[5]

It is well known that some salivary proteins, such as human fibronectin[34] and members of the basic proline-rich protein family [35], can act as receptors for *C. albicans*. On one hand, these proteins, when immobilised on a surface, may possibly promote *Candida* adhesion and subsequent biofilm formation by acting as receptors for free-living planktonic yeasts whilst on the other, they may simultaneously block binding sites originally present on the substratum. In addition, the presence of salivary antimicrobial proteins has been demonstrated,[8,20,36] which further complicates this issue.

Due to these conflicting observations, further research is needed to clarify the role of saliva in early and late biofilm development. However, it is worth emphasizing that the quality of saliva needs to be standardised as vast differences exist between mixed and glandular saliva, pooled and individual saliva, stimulated and unstimulated saliva and finally, saliva collected at different times of the day. Previous work has shown clearly the importance of quality control of saliva in studies of this nature.[37]

Apart from the effect of an acquired pellicle on candidal adhesion and biofilm formation, salivary components are likely to influence these processes when suspended in the liquid phase of the experimental medium. To explore this possibility, we compared *Candida* adhesion in YNB medium with and without mixed saliva supplements. Our data indicate that, compared with saliva-free controls, the presence of saliva in the YNB medium does not significantly affect candidal adhesion ($P = 0.223$) (Fig. 6) although it distinctly reduces the magnitude of biofilm development after 48 h incubation ($P < 0.05$) (Fig. 6). The decreased biofilm formation in the presence of saliva in the YNB medium is likely to be, at least partially, due to the salivary antimicrobial proteins, such as sIgA,[8] histatin 5,[36] lysozyme, and lactoferrin,[20] which may inhibit the growth of the yeast populations in candidal biofilms.

A variety of adhesion/biofilm models have been used in previous studies discussed above such as polypropylene tubes containing acrylic beads,[4] multi-well plates with acrylic sheets within the wells,[5–7,32] and petri dishes or multi-well plates per se as substrata.[8] The difference in the selection of the adhesion/biofilm models may possibly partially contribute to inconsistent results noted in the literature. Of the various adhesion/biofilm model systems, the multi-well plate system, which permits rapid processing of large numbers of samples, was

used in the present study. The well walls of the commercially available plates are identical in surface roughness and surface free energy and therefore, yield more comparable and controllable biofilms compared with other model systems which use self-prepared acrylic strips or beads as substrata.

In summary, our data indicate that environmental factors operational within the oral milieu, such as dietary sugars and saliva could modify candidal adhesion and biofilm formation to varying extents. The modulation of the growth rate of yeast cells and cell wall components provide some clues for the role of dietary sugars in this process. The effect of unstimulated whole saliva on candidal adhesion and biofilm formation varies depending on whether this biological fluid is in the suspended or the immobilised phase. Complexity of the composition and, the subject-to-subject variations in the quality of human saliva are likely to further complicate these complex phenomena.

Acknowledgements

This work described in this paper was partially supported by a Grant from the Research Grants Council of the Hong Kong Special Administrative Region of China (HKU7013/99M) and a grant from the Committee of Research and Conference Grants (a/c 10203775) of the University of Hong Kong.

References

1. Samaranayake LP, Lamb AB, Lamey PJ, MacFarlane TW. Oral carriage of *Candida* species and coliforms in patients with burning mouth syndrome. *J Oral Pathol Med* 1989;**18**:233–5.
2. Branchini ML, Pfaller MA, Rhine-Chalberg J, Frempong T, Isenberg HD. Genotypic variation and slime production among blood and catheter isolates of *Candida parapsilosis*. *J Clin Microbiol* 1994;**32**:452–6.
3. Samaranayake LP, Cheung LK, Samaranayake YH. Candidiasis and other fungal diseases of the mouth. *Dermatol Ther* 2002;**15**:252–70.
4. Edgerton M, Scannapieco FA, Reddy MS, Levine MJ. Human submandibular-sublingual saliva promotes adhesion of *Candida albicans* to polymethylmethacrylate. *Infect Immunol* 1993;**61**:2644–52.
5. Nikawa H, Nishimura H, Hamada T, Kumagai H, Samaranayake LP. Effects of dietary sugars and, saliva and serum on *Candida* biofilm formation on acrylic surfaces. *Mycopathologia* 1997;**139**:87–91.
6. McCourtie J, MacFarlane TW, Samaranayake LP. Effect of saliva and serum on the adherence of *Candida* species to chiorhexidine-treated denture acrylic. *J Med Microbiol* 1986;**21**:209–13.
7. Samaranayake LP, MacFarlane TW. An in vitro study of the adherence of *Candida albicans* to acrylic surfaces. *Arch Oral Biol* 1980;**25**:603–9.
8. San Millán R, Elguezabal N, Regúlez P, Moragues MD, Quindós G, Pontón J. Effect of salivary secretary IgA on the adhesion of *Candida albicans* to polystyrene. *Microbiology* 2000;**146**:2105–12.
9. Samaranayake LP. Nutritional factors and oral candidosis. *J Oral Pathol* 1986;**15**:61–5.
10. Gross KC, Acosta PB. Fruits and vegetables are a source of galactose: implications in planning the diets of patients with galactosaemia. *J Inherit Metabol Dis* 1991;**14**:253–8.
11. Gropper SS, Weese JO, West PA, Gross KC. Free galactose content of fresh fruits and strained fruit and vegetable baby foods: more foods to consider for the galactose-restricted diet. *J Am Diet Assoc* 2000;**100**:573–5.
12. McCourtie J, Douglas LJ. Relationship between cell surface composition, adherence, and virulence of *Candida albicans*. *Infect Immunol* 1984;**45**:6–12.
13. Hawser SP, Douglas LJ. Biofilm formation by *Candida* species on the surface of catheter materials in vitro. *Infect Immunol* 1994;**62**:915–21.
14. Samaranayake LP, MacFarlane TW. The effect of dietary carbohydrates on the in vitro adhesion of *Candida albicans* to epithelial cells. *J Med Microbiol* 1982;**15**:511–7.
15. Hawser S. Comparisons of the susceptibilities of planktonic and adherent *Candida albicans* to antifungal agents: a modified XTT tetrazolium assay using synchronized *C. albicans* cells. *J Med Vet Mycol* 1996;**34**:149–52.
16. Jin Y, Yip HK, Samaranayake YH, Yau JY, Samaranayake LP. Biofilm forming ability of *Candida albicans* is unlikely to contribute to high oral yeast carriage in human immunodeficiency virus-infection. *J Clin Microbiol* 2003;**41**:2961–7.
17. Kuhn DM, George T, Chandra J, Mukherjee PK, Ghannoum MA. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrobiol Agents Chemother* 2002;**46**:1773–80.
18. Tellier R, Krajden M, Grigoriew GA, Campbell I. Innovative endpoint determination system for antifungal susceptibility testing of yeasts. *Antimicrobiol Agents Chemother* 1992;**36**:1619–25.
19. Gracia E, Fernández A, Conchello P, Alabart JL, Pérez M, Amorena B. In vitro development of *Staphylococcus aureus* biofilms using slime-producing variants and ATP-bioluminescence for automated bacterial quantification. *Luminescence* 1999;**14**:23–31.
20. Samaranayake YH, Samaranayake LP, Pow EH, Beena VT, Yeung KW. Antifungal effects of lysozyme and lactoferrin against genetically similar, sequential *Candida albicans* isolates from a human immunodeficiency virus-infected southern Chinese cohort. *J Clin Microbiol* 2001;**39**:3296–302.
21. Veerman EC, van den Keybus PA, Vissink A, Nieuw Amerongen AV. Human glandular salivas: their separate collection and analysis. *Eur J Oral Sci* 1996;**104**:346–52.
22. Zipkes MR, Gilchrist JE, Peeler JT. Comparison of yeast and mold counts by spiral, pour, and streak plate methods. *J Assoc Off Anal Chem* 1981;**64**:1465–9.
23. Kuhn DM, Chandra J, Mukherjee PK, Ghannoum MA. Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infect Immunol* 2002;**70**:878–88.
24. Ramage G, Vande Walle K, Wickes BL, López-Ribot JL. Standardized method for in vitro antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrobiol Agents Chemother* 2001;**45**:2475–9.
25. Wentland EJ, Huang CT, McFeters GA. Spatial variations in growth rate within *Klebsiella pneumoniae* colonies and biofilm. *Biotechnol Prog* 1996;**12**:316–21.

26. Taylor AL, Kudlow BA, Marrs KL, Gruenert DC, Guggino WB, Schwiebert EM. Bioluminescence detection of ATP release mechanisms in epithelia. *Am J Physiol* 1998;**275**:1391—406.
27. Nikawa H, Egusa H, Makihira S, Yamashiro H, Fukushima H, Jin C, et al. Alteration of the coadherence of *Candida albicans* with oral bacteria by dietary sugars. *Oral Microbiol Immunol* 2001;**16**:279—83.
28. Sakakibara T, Murakami S, Hattori N, Nakajima M, Imai K. Enzymatic treatment to eliminate the extracellular ATP for improving the detectability of bacterial intracellular ATP. *Anal Biochem* 1997;**250**:157—61.
29. Douglas LJ. Mannoprotein adhesins of *Candida albicans*. In: Bennett JE, Hay RJ, Peterson PK, editors. *New strategies in fungal disease*. Edinburgh: Churchill Livingstone; 1992. p. 34—50.
30. McCourtie J, Douglas LJ. Extracellular polymer of *Candida albicans*: isolation, analysis and role in adhesion. *J Gen Microbiol* 1985;**131**:495—503.
31. Hawser SP, Baillie GS, Douglas LJ. Production of extracellular matrix by *Candida albicans* biofilms. *J Med Microbiol* 1998;**47**:253—6.
32. Samaranayake LP, McCourtie J, MacFarlane TW. Factors affecting the in vitro adherence of *Candida albicans* to acrylic surfaces. *Arch Oral Biol* 1980;**25**:611—5.
33. Hobden C, Teevan C, Jones L, O'Shea P. Hydrophobic properties of the cell surface of *Candida albicans*: a role in aggregation. *Microbiology* 1995;**141**:1875—81.
34. Skerl KG, Segal E, Sreevalsan T, Scheld WM. In vitro binding of *Candida albicans* yeast cells to human fibronectin. *Can J Microbiol* 1984;**30**:221—7.
35. O'Sullivan JM, Cannon RD, Sullivan PA, Jenkinson HF. Identification of salivary basic proline-rich proteins as receptors for *Candida albicans* adhesion. *Microbiolgy* 1997;**143**:341—8.
36. Gyurko C, Lendenmann U, Troxler RF, Oppenheim FG. *Candida albicans* mutants deficient in respiration are resistant to the small cationic salivary antimicrobial peptide histatin 5. *Antimicrobiol Agents Chemother* 2000;**44**: 348—54.
37. Samaranayake LP, Hughes A, MacFarlane TW. The proteolytic potential of *Candida albicans* in human saliva supplemented with glucose. *J Med Microbiol* 1984;**17**:13—22.