

The Effect of Nutritive and Non-Nutritive Sweeteners on the Growth, Adhesion, and Biofilm Formation of *Candida albicans* and *Candida tropicalis*

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Significance of the Study

- In this study, the non-nutritive sweetener saccharin dampened the in vitro biofilm growth of both *Candida albicans* and *Candida tropicalis*. The finding revealed that saccharin could suppress yeast growth and biofilm formation within the oral milieu and hence indirectly contribute to oral health.

Keywords

Candida species · Biofilms · Adhesion · Growth sucrose · Glucose · Saccharin

Abstract

Objective: To determine the effect of glucose, sucrose, and saccharin on growth, adhesion, and biofilm formation of *Candida albicans* and *Candida tropicalis*. **Materials and Methods:** The growth rates of mono-cultures of planktonic *C. albicans* and *C. tropicalis* and 1:1 mixed co-cultures were determined in yeast nitrogen broth supplemented with 5% (30 mM) and 10% (60 mM) glucose, sucrose, and saccharin, using optical density measurements at 2-h intervals over a 14-h period. Adhesion and biofilm growth were performed and the growth quantified using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The biofilm architecture was visualized using scanning electron microscopy. One- and two-way analysis of variance

(ANOVA) was performed to analyse the differences among multiple means. **Results:** The highest planktonic growth was noted in 5% glucose after 14 h ($p < 0.05$). No significant planktonic growth was observed in either concentration of saccharin. Both the concentrations of glucose and sucrose elicited significantly increased adhesion from MTT activity of 0.017 to >0.019 in mono- as well as co-cultures ($p < 0.05$), whilst the lower concentration of saccharin significantly dampened the adhesion. Maximal biofilm growth was observed in both species with the lower concentration of sucrose (5%), although a similar concentration of saccharin abrogated biofilm development: the highest MTT value (>0.35) was obtained for glucose and the lowest (>0.15) for saccharin. **Conclusion:** In this study, glucose and sucrose accelerated the growth, adhesion, and biofilm formation of *Candida* species. However, the non-nutritive sweetener saccharin appeared to dampen, and in some instances suppress, these virulent attributes of *Candida*.

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Introduction

Candida species are opportunistic fungal pathogens that inhabit 50–60% of the oral cavities of humans [1]. Although *Candida albicans* is the predominant human oral commensal, other species such as *Candida tropicalis* are also commonly isolated from the oral niche [2, 3]. Indeed, it had been reported that 10–15% of oral candidal infections are due to mixed *Candida* species, in particular *C. albicans* and *C. tropicalis* [4]. These fungi cause frequent recalcitrant oral and systemic infections particularly in compromised patient populations such as those with HIV infection and patients on immunosuppressive therapy [5].

Although a yeast of low virulence, *Candida* species possess a range of pathogenic mechanisms, including the ability to form complex biofilms either with other communal bacteria or yeasts on mucosal and tooth surfaces or on acrylic denture surfaces [6, 7]. Both *C. albicans* and *C. tropicalis* are known to produce extensive oral biofilms both in vitro and in vivo, and some are macroscopically visible as white, pseudo-membranes in disease such as oral thrush [8–10]. Furthermore, it is known that fermentable dietary sugars including sucrose and glucose promote *Candida* growth, adhesion, and biofilm development [11] and could aggravate oral diseases, in particular *Candida*-associated denture stomatitis and dental caries [12].

Sucrose is a naturally occurring, universally available, and widely consumed, yet highly cariogenic, dietary sugar [13]. It serves as a substrate for the synthesis of extracellular and intracellular polysaccharides of plaque biofilms [13]. The monosaccharide glucose, a by-product of sucrose fermentation, is also known to contribute equally to the development of oral biofilms [13]. Due to the diverse health concerns associated with rampant and worldwide sucrose consumption, an array of non-nutritive sweeteners such as saccharin, sucralose, aspartame, acesulfame-K, and neotame [14, 15] have been introduced in the food industry and are widely available. Non-nutritive sweeteners are known as chemosensory signalling compounds that influence digestive processes and behaviour [16]. Saccharin is by far the most widely consumed non-nutritive sweetener and is commonly recommended for patients with diabetes as an alternative to the fermentable sugar sucrose [16]. Most of the commercial toothpastes and mouth washes also contain a percentage of non-nutritive sweeteners, especially saccharine [17, 18].

Both nutritive and non-nutritive sweeteners are extensively consumed worldwide [15, 16]. Different environ-

mental conditions such as nutritional sources can influence the activities of micro-organisms. The type of sugar and the concentration are two of the major determinants of biofilm activity of *Candida* species in the oral cavity [11]. The effect of both fermentable natural and non-fermentable synthetic sugars on oral biofilms should be investigated because of the increased consumption of these sugars and artificial sweeteners. Their relative impact on the growth, adhesion, and biofilm development of the opportunistic oral fungal pathogen *Candida* has not been studied. Hence, the objective of this study was to investigate in vitro the effect of 5 and 10% sucrose, glucose, and saccharin on biofilm formation of *C. albicans* and *C. tropicalis*.

Materials and Methods

Candida Strains and Planktonic Growth

C. albicans and *C. tropicalis* (ATCC® 10231 and 13803, respectively) derived from the American Type Culture Collection [19] were used in this study. After confirmation of their identity by using a germ tube test (culture on CHROM agar and cornmeal Tween 80 agar), the yeasts were subcultured on Sabouraud dextrose agar (Sigma-Aldrich, USA) and incubated at 35°C for 48 h. The resultant growth was harvested and subcultured in yeast nitrogen broth (YNB; Sigma-Aldrich) supplemented with either 30 mM (5%) or 60 mM (10%) glucose, 15 mM (5%) or 30 mM (10%) sucrose, or 25 mM (5%) or 50 mM (10%) sodium saccharin (all Sigma Aldrich). Controls were prepared in YNB medium without adding sugars.

Planktonic growth rates of the yeasts were determined using the method initially described by Jin et al., [4, 11] with minor modifications. Briefly, suspensions of *C. albicans* and *C. tropicalis* (10^6 cells/mL) mono-cultures, and a co-culture of the yeasts (1:1 suspension; 10^6 cells/mL) were prepared in YNB and supplemented with glucose, sucrose, or saccharin. A 100- μ L sample of prepared *Candida* cell suspensions was inoculated in triplicate into a 96-well, flat-bottom, microtiter plate. The growth rates were then determined by optical density (OD_{492}) measurements in each well at 492 nm at 2-h intervals for 14 h using a microtiter plate reader (SpectraMax Plus 384; Molecular Devices, Inc., USA) and growth curves were prepared [8, 11].

Candidal Adhesion Studies

Standard cell suspensions (10^7 cells/mL) of *C. albicans* and *C. tropicalis* were prepared in sterile YNB laced with the different concentrations of glucose, sucrose, or saccharin. Both mono-species suspensions of *C. albicans* and *C. tropicalis* and a 1:1 mixture of *C. albicans* and *C. tropicalis* suspensions were prepared.

To examine the initial adhesion phase, 100 μ L of the prepared standard cell suspensions were inoculated in triplicate wells of a microtiter plate and incubated for 90 min at 37°C [6, 8]. After 90 min of incubation, the plates were carefully washed twice with 200 μ L sterile PBS to remove non-adherent cells, and the adherent cells were quantified using a tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich) [20].

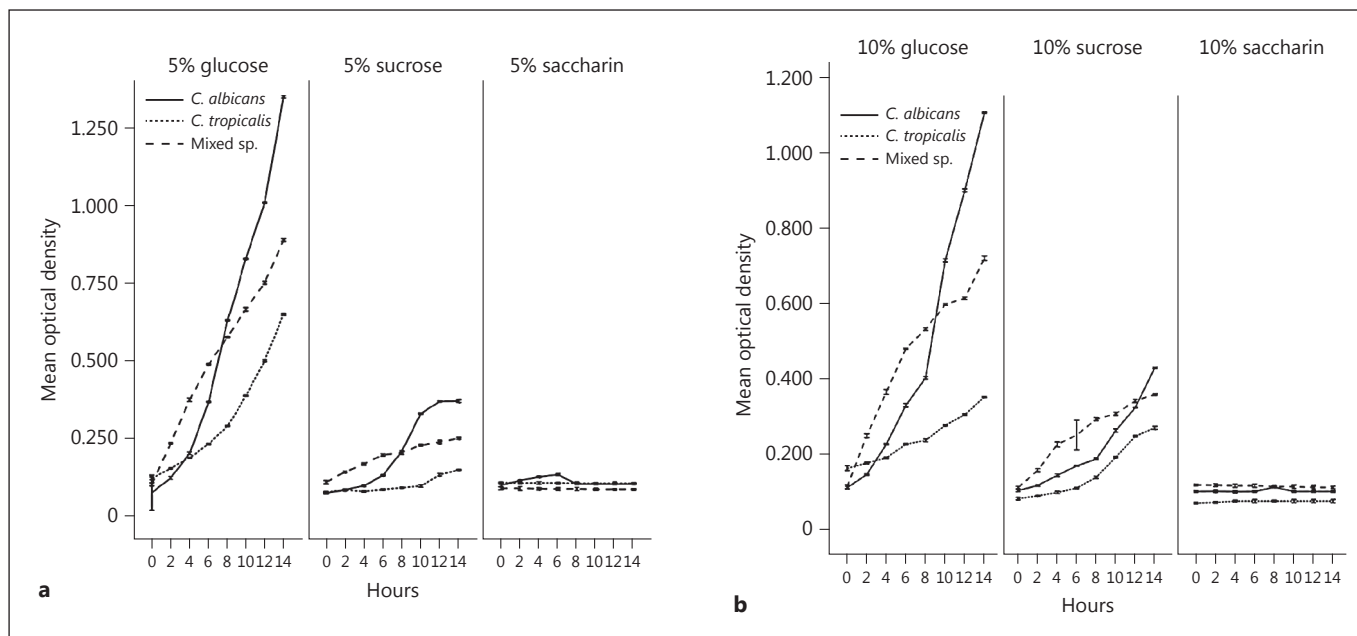


Fig. 1. a Growth curves of planktonic *Candida albicans* and *Candida tropicalis* and 1:1 mixed co-culture grown in glucose, sucrose, and saccharin 5% (mean \pm 2 SD of 3 independent experiments performed in triplicate). **b** Growth curves of planktonic *C. albicans* and *C. tropicalis* and 1:1 mixed co-culture grown in glucose, sucrose, and saccharin 10% (mean \pm 2 SD of 3 independent experiments performed in triplicate).

For the MTT assay, 1 mg/mL aqueous solution of MTT was prepared. To quantify the adherent viable cell mass, 50 μ L of the latter solution was added to each well and incubated at 37°C for 4 h. Afterwards, the MTT solution was carefully removed, 100 μ L of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to solubilize the produced product, and absorbance was measured at wavelengths of 570 and 630 nm.

Candidal Biofilm Growth

The growth rates of the biofilms were determined using the method described previously by Jin et al. [4, 11] with modifications. Standard cell suspensions (10^7 cells/mL) of *C. albicans*, *C. tropicalis*, and mixed species were prepared in sterile PBS, as described above, and 100 μ L was inoculated into wells of a microtiter plate in triplicate. The plates were incubated for 90 min at 37°C to initiate the adhesion phase. Then, the plates were washed twice with 200 μ L of sterile PBS and filled with 100 μ L of sterile YNB supplemented with the appropriate concentrations of the sweeteners. Subsequently, the plates were incubated at 37°C for 96 h and the culture medium was replenished daily. The biofilm biomass was quantified at 24, 48, 72, and 96 h using the MTT assay, and growth curves were constructed. In addition, pH values of the biofilms were measured before and after the addition of sugars.

Scanning Electron Microscopy

In order to examine the ultrastructural features, *Candida* biofilms were developed on 10-mm diameter glass coverslips using the identical procedure described above. The biofilms were fixed with 2.5% glutaraldehyde solution for 2 h and serially dehydrated

with ethanol. After overnight drying in a desiccator, gold-coated biofilms were examined with scanning electron microscopy (SEM) (Hitachi SU 6600, Japan). The matured 1:1 mixed species (*C. albicans* and *C. tropicalis*) biofilm (72 h old) grown with the presence of 5% sucrose and *C. albicans* biofilm grown with the presence of 5% sucrose and 5% saccharin were visualized using SEM [8, 21, 22]. These procedures were done in triplicate.

Statistics

One- and two-way analysis of variance (ANOVA) was performed to analyse the differences among multiple means using the Statistical Package for Social Sciences (SPSS) for windows, version 16.0 (SPSS Inc., Chicago, IL, USA) The *p* value was used to determine the significant differences of adhesion among sugars. *p* values <0.05 were considered statistically significant.

Results

Candidal Growth

The planktonic cultures of *C. albicans* and *C. tropicalis* exhibited copious growth in the two concentrations of both sugars (glucose and sucrose), although much more profuse growth was discernible in glucose. With 5% glucose, *C. albicans* exhibited a maximum growth (1.350 OD₄₉₀) after 14 h followed by 5% sucrose (0.370 OD₄₉₀)

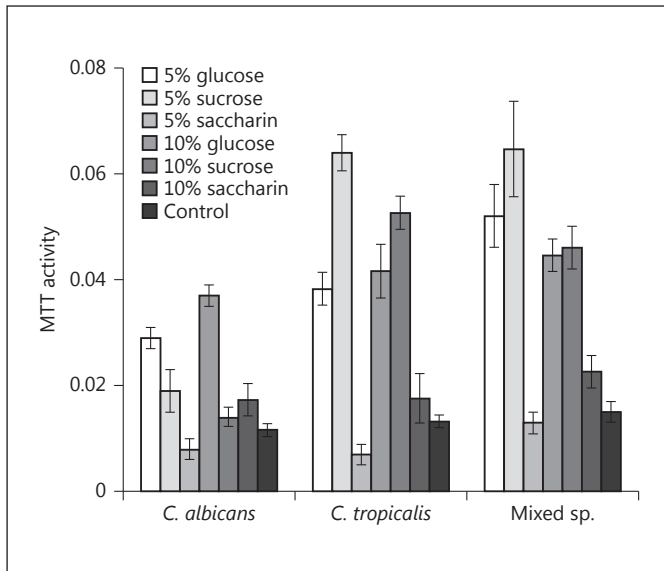


Fig. 2. Results of the MTT assay. Adhesion of *C. albicans*, *C. tropicalis*, and mixed species in yeast nitrogen broth (YNB) supplemented with 5 and 10% glucose, sucrose, and saccharin. In this experiment YNB without sugar was used as negative control. All error bars represent the mean \pm 2 SD.

and 5% saccharine (0.104 OD₄₉₀). With 10% glucose, sucrose, and saccharin after 14 h the growth was recorded as 1.105, 0.425, and 0.100 (OD₄₉₀) respectively. *C. tropicalis* and mixed species also exhibited a similar growth pattern.

Saccharin did not support the planktonic growth of either the mono- or co-cultures of the two species. Both species showed continuous, uninterrupted growth and achieved a maximal cell density at 14 h. In relative terms, the mono-culture growth of *C. albicans* was more intense than that of *C. tropicalis*, whilst the co-cultures demonstrated an intermediate growth profile (Fig. 1).

Candidal Adhesion

Compared to the sugar-free controls, 5% glucose and 5% sucrose (Fig. 2) exposure, either in mono- or co-culture, promoted the adhesion of both *Candida* species ($p < 0.001$). However, 5% saccharin exposure significantly reduced the adhesion of *C. albicans* and *C. tropicalis* compared to the saccharin-free control ($p < 0.05$), although no effect was noted in the co-cultures. An increased concentration of saccharin (10%) promoted the adhesion in both the mono- and co-cultures of *C. albicans* ($p < 0.005$), but not in those of *C. tropicalis* ($p > 0.05$).

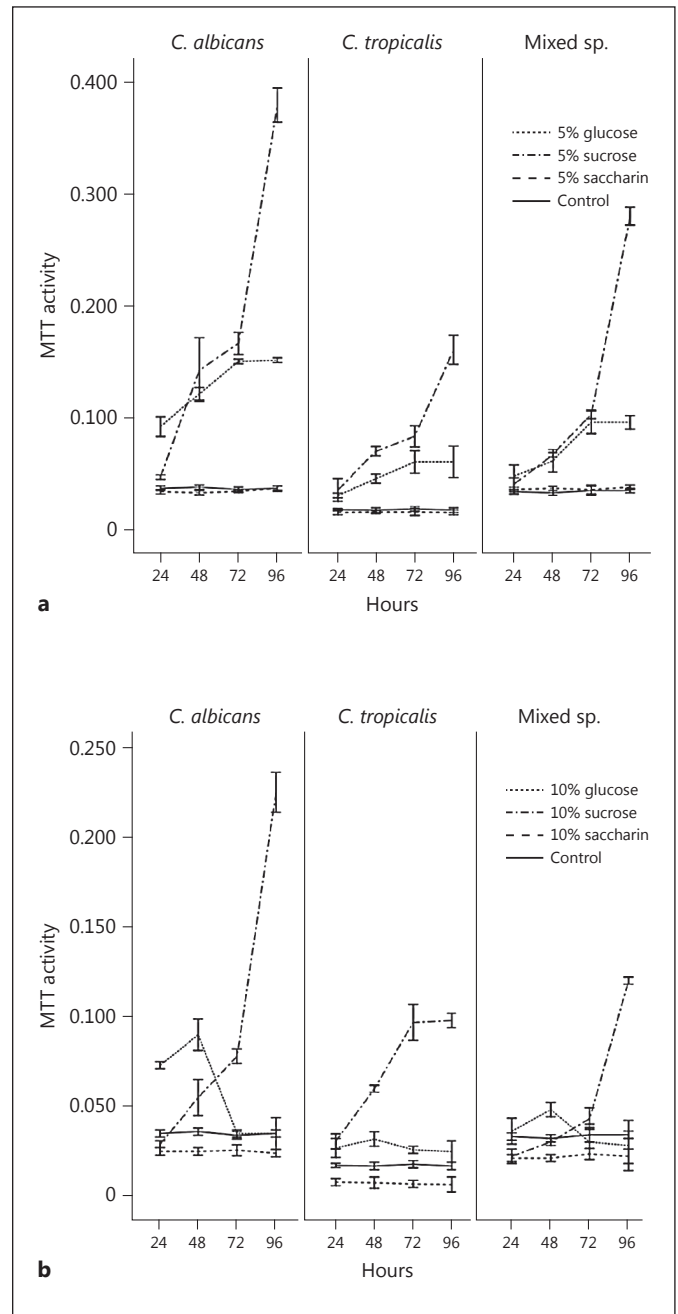


Fig. 3. a Biofilm development of *C. albicans*, *C. tropicalis*, and mixed species in yeast nitrogen broth (YNB) supplemented with 5% glucose, sucrose, and saccharin as determined by MTT assay (mean \pm 2 SD of 3 independent experiments performed in triplicate). **b** Biofilm development of *C. albicans*, *C. tropicalis*, and mixed species in YNB supplemented with 10% glucose, sucrose, and saccharin as determined by MTT assay (mean \pm 2 SD of 3 independent experiments performed in triplicate).

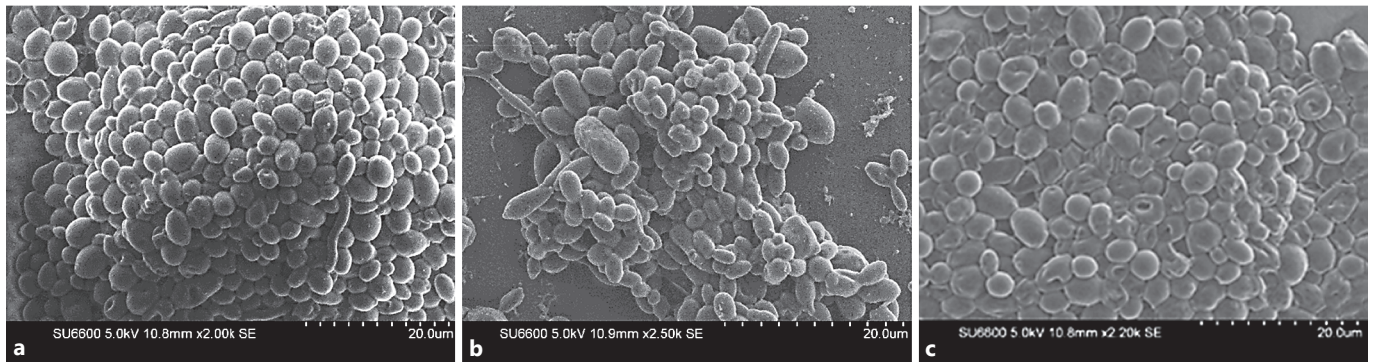


Fig. 4. **a** Electron microscopic appearance of 72-h mature biofilms of *C. albicans* with 5% sucrose. Scale, 20 μ m. **b** Electron microscopic appearance of 72-h mature biofilms of *C. albicans* and *C. tropicalis* mixed species co-culture with 5% sucrose. Scale, 20 μ m. **c** Electron microscopic appearance of 72-h mature biofilms of *C. albicans* with 5% saccharin. Scale, 20 μ m.

Candidal Biofilm Development

In 5% sucrose suspensions, biofilm development in both the mono- and co-cultures of *Candida* was rapid with peak growth at 96 h. The sweetener-free, control suspension was devoid of biofilm growth (Fig. 3a). A similar growth pattern was noted in 10% sucrose, except for *C. tropicalis* biofilm activity which appeared to plateau after 48 h (Fig. 3b). Saccharin did not support candidal biofilm development.

Scanning Electron Microscopy

The 72-h-old biofilms of *C. albicans* and the 1:1 mixed species biofilms of *C. albicans* and *C. tropicalis* grown with the presence of 5% sucrose were subjected to SEM to visualize the ultrastructure, as the highest growth was obtained with the respective concentration of sucrose. Further *Candida* biofilms grown for 72 h with the presence of 5% saccharin were also subjected to SEM as it showed a reduced growth. In a comparison of biofilm formation of *C. albicans* and *C. tropicalis*, *C. albicans* was the most prolific biofilm former based on the SEM observations.

The mature biofilm of *C. albicans* demonstrated a heavy aggregate of almost equal-sized yeast blastospores with very few hyphal elements (Fig. 4a). The co-culture mixed species biofilms had a similar structure, and the species were distinguishable by virtue of the marginally larger-sized *C. tropicalis* blastospores (Fig. 4b). In contrast, the biofilm of *C. albicans* in 5% saccharin was less dense and devoid of either hyphal or budding blastospores. Extracellular polymeric material which is characteristic of biofilms was not visualized in any of the biofilms analysed (Fig. 4c).

Discussion

In this study, *C. tropicalis* adhesion was facilitated in both 5 and 10% sucrose concentration, while *C. albicans* adhesion was promoted with both 5 and 10% glucose concentrations. Although these sugars facilitate the adhesion of the two species, the overall biofilm growth of *C. albicans* and *C. tropicalis* did not get promoted with 10% sucrose, glucose, or saccharine. In high concentrations of sugar, the water concentration is less, and this fact could act as a limiting factor for the biofilm growth of *C. albicans* and *C. tropicalis*. With the increase of sugar concentration in the medium, biofilm/micro-organism respiration and bio-synthesis rates will increase. However, in this situation water became a limiting factor due to the high sugar concentration in the surrounding media. These data are similar to previous findings of a number of researchers [23–25]. Both sugars are known to promote the production of a floccular, fibrillar layer, especially in *C. albicans*, which is thought to mediate adhesion and subsequent biofilm formation [26]. The adhesion of suspended, planktonic cells onto either a biotic surface such as the oral mucosa or an abiotic surface such as an acrylic denture is the initial step in biofilm formation.

Saccharin 5% reduced adhesion of both *C. albicans* and *C. tropicalis*. However, 10% saccharin facilitated the adhesion of both *Candida* species. Saccharin had a paradoxical effect on yeast adhesions, as lower concentrations (5%) of this non-nutritive sweetener reduced the adhesion of both yeast species whereas higher concentrations (10%) facilitated the adhesion of both *Candida* species. The attachment of *Candida* species to the surface is medi-

ated by electrostatic forces and certain other non-specific interactions. Further, the contribution of a high concentration of sodium in 10% saccharin could influence the electrostatic interactions between the cell surface and the polystyrene plate surface compared to other non-polar sugar molecules which promote candidal adhesion.

Fermentable sugars such as sucrose and glucose play a critical role in the initiation and development of major oral diseases such as caries and *Candida*-associated denture stomatitis. In an early study it was demonstrated that dietary carbohydrates, also termed nutritive sweeteners, including glucose and sucrose promoted adhesion and colonization of *Candida* species on both biotic and abiotic surfaces of the oral cavity [27]. Available data revealed that there is a strong relationship between quorum sensing, quorum sensing inhibition/quorum quenchers, and environmental and nutritional conditions including carbon source (type of sugar) [28]. Quorum sensing is a key contributory factor of microbial social behaviours and biofilm development. As regards planktonic growth of the yeasts, our data confirm the previous findings that both glucose and sucrose promote yeast growth, and glucose is better at fostering such growth. Noteworthy, however, was the fact that saccharin did not support the planktonic growth of either the mono- or co-cultures of the two species, implying that the yeasts are incapable of metabolizing this substrate.

The finding that both glucose and sucrose facilitated candidal biofilm development confirmed the findings of a previous study [12]. Equally, the finding that *C. albicans* was a superior biofilm former to *C. tropicalis* corroborated previous findings [10] because *Candida* is a yeast which prefers to grow under low pH conditions, but pH 5 created by 10% sucrose did not foster its growth. Akin

to planktonic cultures, saccharin did not support biofilm growth of either *Candida* species.

The co-culture studies of the two yeast species confirmed that they grew well together and had the ability to form multi-species biofilms. Clinical findings have also indicated that both *C. albicans* and *C. tropicalis* could cause poly-fungal infections in the oral cavity and elsewhere [29].

The ultrastructural findings of the biofilms confirmed the classic architecture of candidal biofilms. Extracellular polysaccharide material covering the biofilms (an integral biofilm component) was not visualized probably due to a processing artefact such as high vacuum and to the electron beam of SEM. Most importantly, the candidal cells were crenate, some demonstrating pockmarks with the presence of 5% saccharine, possibly indicating cell death due to loss of contents.

Clinically, these findings could imply that saccharin might also suppress yeast growth and biofilm formation within the oral milieu, and indirectly contribute to oral health.

Conclusion

Both glucose and sucrose accelerated the growth, adhesion, and biofilm formation of *Candida* species, either in mono- or co-cultures, to varying degrees. However, the widely used artificial, non-nutritive sweetener saccharin appeared to dampen, and in some instances suppress, these virulent attributes of *Candida*. Further studies are recommended to clarify the mechanism underlying this phenomenon, which has not been previously reported.

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