**Candida albicans** biofilm heterogeneity does not influence denture stomatitis but strongly influences denture cleansing capacity

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**Abstract**

Approximately 20 % of the UK population wear some form of denture prosthesis, resulting in denture stomatitis in half of these individuals. *Candida albicans* is primarily attributed as the causative agent, due to its biofilm-forming ability. Recently, there has been increasing evidence of *C. albicans* biofilm heterogeneity and the negative impact it can have clinically; however, this phenomenon has yet to be studied in relation to denture isolates. The aims of this study were to evaluate *C. albicans* biofilm formation of clinical denture isolates in a denture environment and to assess antimicrobial activity of common denture cleansers against these tenacious communities. *C. albicans* isolated from dentures of healthy and diseased individuals was quantified using real-time PCR and biofilm biomass assessed using crystal violet. Biofilm development on the denture substratum poly(methyl methacrylate), Molloplast B and Ufi-gel was determined. Biofilm formation was assessed using metabolic and biomass stains, following treatment with denture hygiene products. Although *C. albicans* was detected in greater quantities in diseased individuals, it was not associated with increased biofilm biomass. Denture substrata were shown to influence biofilm biomass, with poly(methyl methacrylate) providing the most suitable environment for *C. albicans* to reside. Of all denture hygiene products tested, Milton had the most effective antimicrobial activity, reducing biofilm biomass and viability the greatest. Overall, our results highlight the complex nature of denture-related disease, and disease development cannot always be attributed to a sole cause. It is the distinct combination of various factors that ultimately determines the pathogenic outcome.

**INTRODUCTION**

It is estimated that one in five adults in the UK wear some form of dental prosthesis [1]. Given that such a large proportion of the adult population wear a denture, then denture-related oral disease is a substantial healthcare problem. Denture stomatitis (DS) is considered the most common disease to afflict denture wearers and has been shown to affect approximately 50 % of this population [2]. *Candida* spp., primarily *Candida albicans*, are generally acknowledged as the principal microbial agents driving the pathogenesis of DS [3], although other species such as *Candida glabrata* are frequently co-isolated [4]. *C. albicans* is able to adhere to the denture surface and form complex microbial communities known as biofilms [5], surrounded by an extracellular matrix, which confers protection from antimicrobials [6, 7]. These tenacious structures have been shown to adhere strongly to the denture. However, the details of such surface interactions are not yet fully understood [8, 9]. Moreover, this problem is further compounded by the fact that *C. albicans* biofilms are clinically heterogeneous, and this phenotype has been shown to negatively impact patient outcomes [10–12]. Whether this clinical heterogeneity plays a role in the intra-oral denture environment remains to be determined, as the influence of denture surfaces and methods of decolonization needs to be considered.

Poly(methyl methacrylate) (PMMA) is clinically the most commonly used denture material due to low cost and easy manipulation, despite its varied topography, which positively influences microbial colonization and biofilm formation.
development [13–15]. PMMA is a rigid material that, when in close contact with the mucosa, can cause discomfort. Therefore, the softer, less rigid materials are becoming increasingly popular. These include resilient soft liners such as silicone- and acrylic-based liners, which have been shown to improve patient masticatory function and reduce pain and the appearance of sore spots within the oral cavity [16, 17]. However, these liners are not resistant to the colonization of micro-organisms, complicating denture hygiene even further [14, 18]. A number of over-the-counter (OTC) denture cleansers are widely available, although clinical guidelines advising on the most appropriate cleansing regimen are varied and lack a comprehensive evidence base. Denture cleansing alone has demonstrated a significant reduction in microbial viability and biomass, although a reservoir of cells remains upon the surface [19]. Combining mechanical and chemical intervention can further aid the control of biofilm formation on PMMA, although again residual biofilm cells are still capable of persisting [20, 21].

The primary objective of this study was to determine whether quantitative (numbers of biofilm cells) or qualitative (biofilm phenotypic heterogeneity) factors from C. albicans were the primary drivers of DS. A secondary objective was to evaluate whether the biofilm phenotype was influenced by an in vitro denture environment and denture hygiene treatment. Here, we report that C. albicans isolates from dentures are quantitatively associated with disease but not biofilm heterogeneity. In vitro modelling revealed that a denture-related environment impacts biofilm heterogeneity and how this responds to denture hygiene products.

**METHODS**

**Patient sampling**

Samples for this study were obtained from 129 denture wearers attending the Glasgow Dental Hospital and School for routine treatment, as previously described by our group [22]. Written informed consent was obtained from all participants. Ethical approval was granted by the West of Scotland Research Ethics Service (12/WS/0121) for the recruitment of participants to this study, where a clinician was responsible for the collection of clinical samples. The presence or absence of DS was assessed by the clinician, and those patients with DS were grouped according to Newton classification [23]. The following scores were applied: 0 for healthy mucosa, 1 for pin-point hyperaemic lesions (localized erythaema) 2 for diffuse erythaema (generalized simple inflammation) and 3 for pin-point hyperaemic lesions (localized erythaema) 2 for diffuse erythaema (generalized simple inflammation) and 3 for hyperplastic granular surface (inflammatory papillary hyperplasia). Patients were excluded from this study if they had been receiving antimicrobial treatment or using prescription mouthwashes within 6 months prior to sampling. Dentures were removed from the patient’s mouth and placed in sterile bags (Fisher Scientific) filled with 50 ml PBS (Sigma-Aldrich) and placed in a sonic bath (Ultrawave) for 5 min to remove the biofilm from the surface [24]. The denture sonicate was centrifuged for 10 min at 3700 g (6000 r.p.m.), and the plaque pellet was resuspended in 2 ml of RNAlater (Qiagen) and stored at −80°C.

**Molecular quantification of Candida in clinical samples**

The presence of Candida spp. isolated from healthy (n=81) and DS (n=48) patients was assessed by quantitative PCR (qPCR) by amplifying the Candida-specific 18S gene. DNA was extracted using the QIAamp DNA mini extraction kit (Qiagen) according to the manufacturer’s instructions, with a minor modification to include a mechanical disruption step with sterile acid-washed glass beads of 0.5 mm diameter (Thistle Scientific). This was achieved by beating the samples for 3×30 s on a Mini-BeadBeter (Sigma-Aldrich), while intermittently being placed with intermittent cooling. DNA quality and quantity were quantified using the NanoDrop spectrophotometer (Thermo Scientific).

To quantify Candida from each sample, 1 µl of DNA was added to a mastermix containing SYBR GreenER (Life Technologies), UV-treated RNase-free water and 18S forward/reverse primers (10 µM, forward – CTGTAAGTGGACC TTGGGC; reverse – GGCCTGCTTTGAACACTCTA [25]), as per manufacturer’s instructions. The thermal profile used consisted of 50°C for 2 min, 95°C for 2 min followed by 40 cycles of 95°C for 3 s and 60°C for 30 s using a StepOne Plus real-time PCR unit (Applied Biosciences). Cycle threshold values were used to approximate the number of corresponding colony-forming equivalents based on standard curves (Fig. S1, available in the online Supplementary Material).

**Standardized C. albicans biofilm assessment**

To detect and identify the Candida spp. present, 100 µl of the denture sonicate was spread across Colorex Candida agar (E&O Labs) and incubated at 30°C for 72 h. All clinical isolates obtained during this study were stored in Microbank vials (Pro-Lab Diagnostics) at −80°C, until required. Isolates identified as C. albicans from healthy (n=31) and DS (n=37) patients were propagated in yeast peptone dextrose medium (Sigma-Aldrich), incubated at 30°C and vortexed at 150 r.p.m. for 18 h. Following incubation, cultures were washed twice by centrifugation, standardized and grown statically at 37°C for 24 h in 96-well flat-bottomed polystyrene plates (Corning Inc.) and assessed for biofilm formation as previously described [10, 26]. Each isolate was tested in triplicate, and negative controls containing no C. albicans were also included. Following incubation, biofilms were carefully washed twice with PBS to remove any non-adherent cells, before biomass was assessed using the crystal violet (CV) assay, as described previously [27]. Absorbance was read spectrophotometrically at 570 nm, and isolates were grouped based on their level of biomass distribution. Isolates that fell below the first quartile (Q1, OD140=0.382) were classed as low biofilm former (LBF), and strains with a biomass greater than the third quartile (Q3, OD140=1.192) were deemed high biofilm former (HBF), as described previously [10, 11].
Assessment of biofilm formation upon different denture materials

Given the artificial nature of standardized *C. albicans* biofilm testing in RPMI-1640 medium and polystyrene, we next wanted to assess how selected isolates behaved in an environment representative of media and substratum, namely artificial saliva (AS) and denture materials. Two clinical isolates (*n* = 1 for LBF and *n* = 1 for HBF) and one laboratory strain (SC5314) were selected for assessing their biofilm formation upon various denture materials. Isolates were standardized to 10⁶ cells ml⁻¹ in AS (porcine stomach mucins 0.25% (w/v), sodium chloride 0.35% (w/v), potassium chloride 0.02% (w/v), calcium chloride dihydrate 0.02% (w/v), yeast extract 0.2% (w/v), lab lemco powder 0.1% (w/v), proteose peptone 0.5% (w/v) in double distilled water). Urea was then added independently to a final concentration of 0.05% (v/v), as previously described [28], and 1 ml added to each well of a 24-well flat-bottomed polystyrene plate (Corning Inc.) containing the following denture substratum ‘in-house’ manufactured discs (13 × 1 mm): PMMA molloplast B (MOLLO) (Detax dental) and Ufi-gel (UFI) (Voco) soft liners. Biofilms were incubated at 37°C for 4 and 24 h before washing twice with PBS. As the biofilms were grown on the denture material discs, CV could not be used to accurately quantify biomass. Therefore, qPCR was used instead. Biofilms were sonicated at 35 kHz for 10 min in 1 ml PBS to remove the biomass [29]. Samples were centrifuged for 10 min at 10,000 g, the supernatant was discarded and DNA was extracted, as previously described. qPCR was carried out, and *C. albicans* cells were quantified using the 18S primers, as previously described.

Antimicrobial treatment of *C. albicans* biofilms

Five *C. albicans* clinical isolates defined as HBF were selected to test a range of OTC denture cleansers (Steradent, Milton and Poligrip) against pre-formed biofilms. Isolates were grown in a 96-well flat-bottomed plate for 4 and 24 h. Two hundred microlitres of these solutions was added to each biofilm and treated, as defined by the manufacturer (10 min for Steradent; 15 min for Milton; 3 min for Poligrip). Untreated controls were included for comparison. Following treatment, biofilms were washed with PBS, and standardized XTT and CV assays were carried out to assess biofilm metabolic activity and biomass, respectively, as described previously [11, 26]. Scanning electron microscopy (SEM) was performed on the *C. albicans* biofilms grown on PMMA discs to visualize the effects of denture cleansing. Following 4 and 24 h biofilm development, cells were treated with the various denture cleansers before being carefully washed with PBS, fixed, processed and imaged, as previously described [30].

**Statistical analysis**

Graph production, data distribution and statistical analysis were performed using GraphPad Prism (version 5.01). Data were first assessed to see if they configured to a normal distribution and were log transformed where necessary. Student *t*-tests were used to compare candidal loads of healthy and diseased patients. A one-way ANOVA with Bonferroni correction was used to measure statistical differences between biofilms grown on different denture materials and antimicrobial viability testing of the isolates. A Kruskal–Wallis test with Dunn’s post-test was used to measure differences in biofilm biomass following treatment with different denture cleansers.

**RESULTS**

*Candida* burden differs in healthy and DS denture wearers

The candidal burden colonizing the denture surface differed significantly between healthy and DS patients (Fig. 1a; *P* < 0.001). Nonetheless, significant overlap was seen between both groups, emphasizing the variable nature of candidal biofilm formation. Next, the biofilm-forming ability of *C. albicans* denture isolates was assessed to evaluate biofilm heterogeneity (Fig. 1b). Isolates differentially formed
biofilms and were classed as either LBF (Q₁, OD₅₇₀=0.382) or HBF (Q₃, OD₅₇₀=1.192). Isolates in between these groups were classed as intermediate biofilm formers. Optical densities for healthy patients ranged from 0.09 to 2.81 and from 0.1 to 2.06 for DS patients, across the 68 isolates tested. No statistical difference was observed between healthy and DS patients (P>0.05), with biomass regarded as highly variable. This confirms that C. albicans biofilm formation is heterogeneous within both groups.

**Biofilm biomass and viability are impacted by media and denture substratum**

The impact of denture substratum upon C. albicans biofilm formation was assessed next (Fig. 2). At 24 h of biofilm formation, PMMA was the denture material with the most significant C. albicans burden (4.21×10⁶ colony-forming equivalents), this being 2.8 times and 4.1 times greater than those of MOLLO (P<0.05) and UFI (P<0.05), respectively. Unsurprisingly, C. albicans biomass was significantly more abundant in 24 h biofilms than in their 4 h counterparts for each denture substratum: PMMA (P<0.001), MOLLO (P<0.05) and UFI (P<0.001). At the early stages of biofilm development (4 h), no significant candidal burden was observed among the three substrata (P>0.05). When biofilm heterogeneity was assessed upon denture substrata, no significant differences in C. albicans burden were found between LBF and HBF (data not shown).

**Treatment of C. albicans biofilms with antimicrobial denture cleansers**

Finally, three OTC denture cleansers were selected to assess their antifungal activity against C. albicans denture biofilms formed by HBF. Milton was the most effective cleanser against early biofilms (4 h), reducing biomass by 55%, compared to the untreated control (P<0.05), as shown in Fig. 3 (a). Poligrip was the second most active agent, reducing biomass by 28%. However, Steradent led to an increase in the denture biomass by 9%. When mature biofilms were considered (24 h), no significant differences were observed when comparing the denture cleanser treatments to control biofilms. Unsurprisingly, biomass increased between 4 and 24 h by 3.3, 2.8, 5.2 and 5 times for the untreated, Steradent, Milton and Poligrip samples, respectively.

As Milton was the most effective cleanser at disturbing biofilm biomass, it was predicted that it would also be the best at reducing cellular viability. In this study, we have shown that Milton reduces metabolic activity to 17 and 56% in 4 h (P<0.001) and 24 h (P<0.05) biofilms, respectively (Fig. 3b). The cellular viability at 4 h was less than that at 24 h, which is most likely due to early biofilms not being as complex as their 24 h counterparts, meaning antimicrobials are able to penetrate more easily, elicit their activity and reduce viability more readily than they can in mature biofilms. Poligrip had little impact on biofilm development, with 90% viable cells remaining following treatment of 4 h biofilms and 78% at 24 h. Interestingly, Steradent was shown to have the greatest activity against mature biofilms, reducing cell viability to 67% at 24 h, compared to no reduction at 4 h (P<0.001). This being said, no denture cleansing regimens were able to reduce 24 h biofilm viability to less than 50%.

Ultrastructural changes of the treated denture biofilms are illustrated in Fig. 3(c). Steradent-treated early biofilms exhibited minimal changes in appearance compared to the untreated control. However, at 24 h, the biofilms were visually distinct, with a fibrous residue evident along the elongated hyphae. This may be a remnant of the denture cleanser retained within the biofilm as this was also observed in Milton and Poligrip mature biofilms. Interestingly, at 4 h, the hyphal cells appeared to have become embedded with the denture acrylic, which may be due to the cleansers becoming incorporated within the material and changing the surface topography, as suggested elsewhere [31, 32].

**DISCUSSION**

Despite the vast improvements in oral health over the past 50 years, there still remains a large proportion of the adult population that rely on some form of denture prosthesis. Wearing a denture can be an immensely uncomfortable experience [33], with DS being one of the primary complaints affecting half of denture wearers at some point in their lives [3]. C. albicans colonization and subsequent infection of mucosa are the principal causes of DS. Here, we report that, although biofilm heterogeneity impacts overall effective denture cleansing, the quantity of candidal yeasts is the main driver of DS, not the biofilm phenotype nor denture substratum, a finding supported by previous researchers [34].
It is interesting that heterogeneity is apparent among clinical isolates attached to dentures, and this phenomenon may play a yet undefined role in DS. Clinically, the concept that biofilm heterogeneity may play an important role in determining morbidity and mortality levels is worthy of investigation in the context of the oral cavity. We have recently reported an association with this phenotype and mortality in a Scottish candidaemia cohort [10], so therefore we undertook an analogous investigation in a cohort of denture patients. Interestingly, although biofilm heterogeneity was observed using established models, an association with patient outcomes was not shown. Our inability to demonstrate cause and effect may be explained by the fact that this is a relatively mild disease in comparison to candidaemia from a severely immunocompromised group. Nevertheless, a positive correlation between the absolute candidal numbers and disease was observed, suggesting that the physical interaction between large numbers of yeasts and hyphae on the denture surface was more important than the phenotype per se. This is likely influenced by the diverse microbiota in this environment, which we have recently described [22]. Indeed, there is increasing evidence that Candida and bacteria form polymicrobial biofilms that some bacterial species common to the oral cavity can enhance the pathogenicity of C. albicans [35–38] and that the presence of specific oral bacteria is enough to transform a C. albicans LBF into a HBF [39]. We have also reported a significant association between lactobacilli and yeasts in this context, and this may be more important than C. albicans alone, as is the perceived paradigm for this DS. Therefore, the local environment of the denture may play an important role in determining how denture-related stomatitis ensues. Studies in mice infected with HBF strains of C. albicans were shown to survive significantly longer than those infected with LBF strains, suggesting a competitive advantage in the host environment [40].

The microbiology of dentures is dictated by substrata and the surrounding host environment. Changing this environment can vastly influence C. albicans biofilm formation and survival. Initial adhesion of microbes to dentures is key to their pathogenicity, yet denture substrata are highly variable, which may support differential adherence and biofilm formation. PMMA is classically used as a denture substratum, although its chemistry and topography create a preferential surface for biofilm formation [41]. Alternative soft liner materials are increasing in popularity as they are softer against the palate as well as providing improved masticatory function over traditional denture materials [16]. Studies comparing microbial growth on hard and soft denture liners have shown conflicting results, as one study comparing

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**Fig. 3.** Assessing antimicrobial denture cleansers on C. albicans biofilm formation. The effects of three OTC denture cleansers on C. albicans biofilm formation were assessed by biomass (a) and viability (b) at 4 and 24 h. All images are shown at 1000x magnifications (c). Scale bars represent 20 µm. Data represent mean±SD. Significant differences were observed when comparing each cleanser at 4 h to their 24 h counterparts (**P<0.01 and ***P<0.001), as well as comparing cleansers to one another (*P<0.05, **P<0.01 and ***P<0.001).
Candida biofilm growth on various soft denture liners found no significant differences in the quantity of cells recovered between materials [42]. Our study, however, found that, although there was no significant difference in microbial colonization during early biofilm formation, PMMA became the substratum most prone to biofilm formation at later stages of development, regardless of the isolate’s phenotype. Interestingly, analysis of biofilms forming on PMMA and soft liners at 1, 7 and 14 days found no significant differences in adhesion at 24 h, yet colonization on hard acrylic was observed to be lower at all time points [43]. A possible explanation as to why we are seeing higher numbers on PMMA is because soft liners are even more porous in nature than hard acrylics [44], and thus, higher numbers of microbes may become imbedded in these cracks and crevices. Therefore, even a powerful method such as sonication may not be sufficient to completely dislodge the microorganisms hidden within these soft liners. To the authors’ knowledge, this is the first study to use molecular methods such as qPCR to quantify biofilms on denture acrylics, in order to give more accurate quantitative microbial counts. However, the vast majority of studies in this area have relied on culturing and staining techniques for quantification, and thus, the methods used may account for the differences seen across studies [42, 43, 45].

Currently, there is no universal method for cleaning dentures, where it is common to find that the recommended method varies from dentist to dentist. There are numerous OTC denture cleansers available. Therefore, we selected three of the most popular brands – Steradent, Milton and Poligrin – to assess their antifungal activity. Milton was the most effective treatment at reducing biomass and cell viability on early (4 h) biofilms. Yet, it did not completely eradicate the biofilm. Such results are interesting given previous findings showing that this treatment completely inhibited biofilm formation at 4 h [29]. Methodological constraints due to conventional c.f.u. quantification may explain this, since it has been recently demonstrated that counting c.f.u. can give false-negative results. By using live/dead qPCR on the same experiment, ~1×10⁷ cells ml⁻¹ viable cells were detected, where c.f.u. counting detected no live cells [21]. Moreover, as the biofilms matured, Milton lost its effect, most likely because of the thicker biofilm, which would be more difficult to penetrate and disrupt. Yet, previous experiments have shown that denture cleansers and antimicrobial mouthwashes can significantly reduce the viability of mature C. albicans denture isolates [19, 46]. These studies do not confirm the biofilm-forming capacity of the assayed isolates, whereas our study used known HBFs, which could explain why our isolates were more resistant to treatment, a phenomenon reported elsewhere with these phenotypes [11]. Moreover, previous studies have shown that C. albicans is not completely eradicated from the denture, even when using various denture cleansing treatments [29]. Our collection of SEM images supports this as they show that C. albicans cells remain on the denture acrylic irrespective of treatment. We cannot guarantee that all these cells are dead, and therefore, it is likely that some are still alive and could begin to recolonize the denture when placed back into the mouth. Furthermore, full penetration of the biofilms is unlikely due to the protective effects against antimicrobials, such as efflux pumps and extracellular glucans, provided by the extracellular matrix [30, 47].

In conclusion, it was demonstrated that C. albicans isolates reside in higher numbers on dentures on DS-affected individuals. These isolates demonstrated heterogeneity in their biofilm-forming abilities. However, HBFs were not more common to DS sufferers. Therefore, although the particular strain isolated may strongly influence the development of disease, other factors are also involved. The surrounding environment has a strong influence, as we demonstrated that some materials are better at supporting C. albicans biofilm growth. Furthermore, antimicrobial denture cleansing treatments do not fully eradicate HBF C. albicans isolates, leaving behind live cells, which can disperse and recolonize. Therefore, in terms of future studies investigating C. albicans biofilms and denture-related disease, it has to be recognized that various factors influence disease and that it is the culmination of these that determines the pathogenic outcome.

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Conflicts of interest
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