Polymicrobial denture-associated biofilms: *in vitro* interactions and infection modelling

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Introduction

In addition to the hundreds of bacterial species known to be present in the oral cavity[1,2], the mouths of up to 50% of individuals are colonised by the fungus *Candida albicans*[3,4]. Although typically a commensal microorganism, with no negative implications toward the host, changes in local host environments can lead to development of *Candida*-associated infections, known as candidoses.

Several forms of oral candidoses are recognised; with the most prevalent form, chronic erythematous candidosis (*Candida*-associated denture stomatitis, DS; Fig 1), affecting up to 60% of denture wearers[5]. The condition is associated with polymicrobial biofilms that exist on the fitting-surface of the denture, often arising from poor oral hygiene and denture care. Clinical symptoms of DS primarily include areas of inflammation on the palatal mucosa overlying the denture acrylic, but also general discomfort and a burning or itching sensation. Many predisposing factors are known to contribute to the development of the condition and include use of tobacco, antibiotics and steroids, and extended and nocturnal denture wearing.

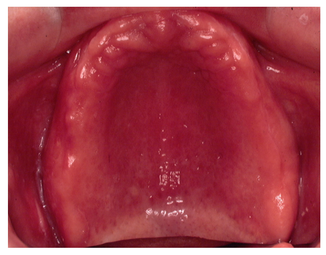

Fig 1. Clinical presentation of *Candida*-associated denture stomatitis
Note the redness of the palate as a result of inflammation from contact with the denture fitting surface.

Whilst *Candida* are considered the main causative agents of DS, the role of the residing oral bacteria and their effect on *Candida* in the infection remains unclear. Studying interactions between *Candida* and bacteria in denture biofilms *in vitro* is important, and could have significance to both prognosis and management of DS; particularly as current treatments are largely targeted toward the fungal component of the infection. **This study aimed to evaluate the effects of the presence of oral bacteria toward *C. albicans* virulence factors in *in vitro* biofilms, the subsequent effects and tissue damage in *in vitro* infections with tissue models, and characterise the bacterial microbiota of denture-associated biofilms in patients with and without DS.**

Materials & Methods

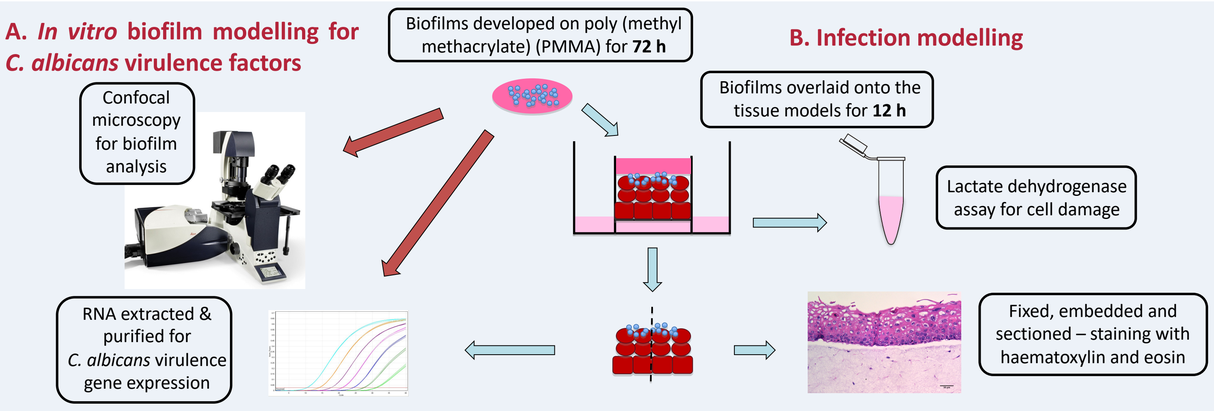

Fig 2. *In vitro* biofilm culture, tissue model infection, and analyses
Biofilms cultured on PMMA for 72h and analysed for **A)** expression of *C. albicans* virulence factors, or **B)** overlaid on to 3D oral mucosal tissue to model infections. Infected tissues were bisected for gene expression and histological analyses, and the supernatant collected for quantification of cell damage.

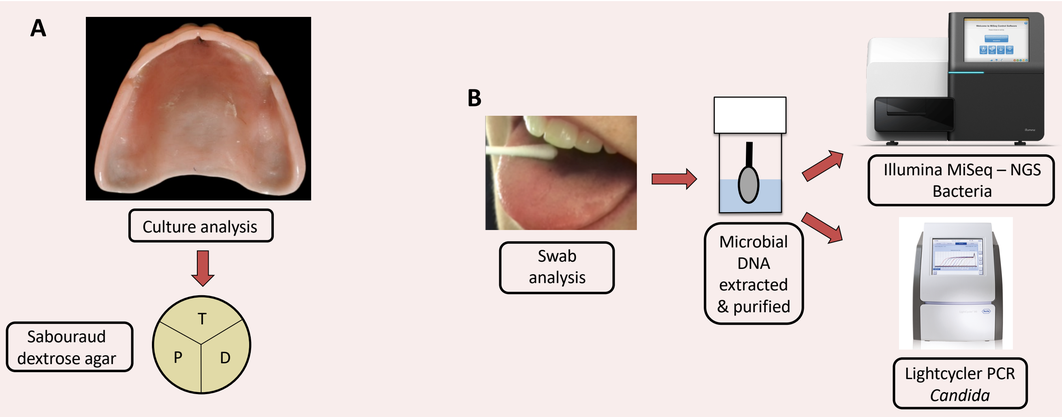

Fig 3. Laboratory processing and analyses of clinical samples
For the clinical study, eligible patients attending the School of Dentistry, Cardiff University for normal, routine treatment were recruited following informed consent (ethical approval *REC Reference 14/WA/0023, Protocol Number SPON 1265-13, IRAS Project ID 137108*). Samples were obtained for **A)** culture analysis for *Candida* species, and **B)** molecular analyses of microbial DNA for bacterial microbiota characterisation.
Analysis of the next generation sequencing (NGS) data included estimation of the biodiversity within a community (Chao index), groupings of communities (non-metric multi dimensional scaling, NMDS plot), and changes in top ten bacterial species (denture surfaces). Data were statistically analysed at 95% confidence.

Results

Modulation of expression of *C. albicans* virulence factors by oral bacteria

Biofilms cultured *in vitro* on denture acrylic, analysed for transition from yeast cells to hyphae, and expression of virulence genes

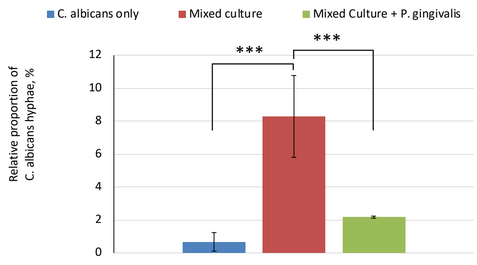

Fig 4. Morphological transition of *C. albicans* ATCC 90028 from yeast to hyphae is modulated by oral bacteria when cultured in biofilms. Increased number of hyphae in mixed-species biofilms relative to *C. albicans*-only, but no significant increase when also including *P. gingivalis* in biofilm culture.

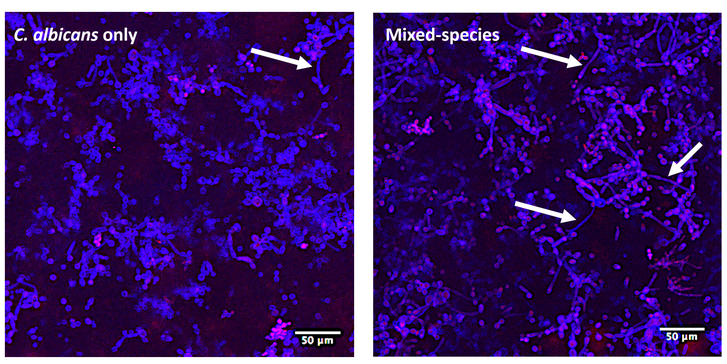

Fig 5. Confocal microscopy image of *C. albicans*-only and mixed-species (*C. albicans* plus four oral bacterial strains) biofilms. White arrows indicate *C. albicans* hyphae. *C. albicans* stained with calcofluor white (blue) and all microorganisms counter-stained with propidium iodide (red).

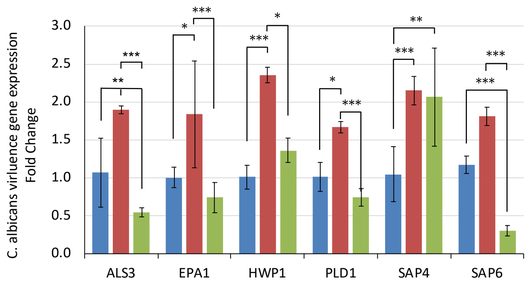

Fig 6. Relative expression of *C. albicans* virulence genes differs when cultured with different oral bacteria in biofilms. Significant increases in gene expression for all virulence genes tested in mixed-species biofilms relative to *C. albicans*-only, but no change, or a decrease when also including *P. gingivalis* in biofilm culture.

ALS: agglutinin like sequences, EPA: epithelial adhesin, HWP: hyphal wall protein, PLD: phospholipase, SAP: secreted aspartyl proteinase

	Ca+P1	Ca+P2	Ca+P3	Ca+P4	Ca+P5	Mix+P1	Mix+P2	Mix+P3	Mix+P4	Mix+P5
ALS3	0.39	0.90	2.03	0.51	2.40	0.92	1.34	0.71	0.90	2.09
HWP1	0.67	2.59	4.94	0.82	4.79	0.46	1.27	1.32	0.57	4.96
SAP4	2.03	0.57	1.23	0.32	1.23	0.67	0.34	0.55	0.56	4.42
SAP6	0.60	1.95	2.61	0.78	2.27	0.45	0.97	1.03	0.46	2.45
PLD1	0.51	0.70	1.11	0.42	1.99	0.42	0.70	0.56	0.53	3.04
ECE1	0.67	1.42	2.79	1.01	1.02	0.34	0.48	1.13	0.43	1.06

Increased
No change
Decreased

Table 1. Fold change values for putative *C. albicans* virulence genes determined using clinical isolates from patients with (DS) or without (NoDS) denture-associated stomatitis in the mixed-species biofilm model. Values calculated relative to a *C. albicans*-only biofilm, normalised to *ACT1* housekeeping gene control. Green indicates decrease in gene expression, yellow indicates no change, orange indicates slight increase, red indicates greater increase. *ECE1: extent of cell elongation (*encodes for candidalysin toxin)
P1, *Lactobacillus fermentum*; P2, *L. delbrueckii*; P3, *L. plantarum*; P4, *L. acidophilus*; P5, *L. plantarum* (food isolate)

Modulation of *C. albicans* virulence factors by oral bacteria

Biofilms cultured *in vitro* on denture acrylic, analysed for transition from yeast cells to hyphae, and expression of virulence genes

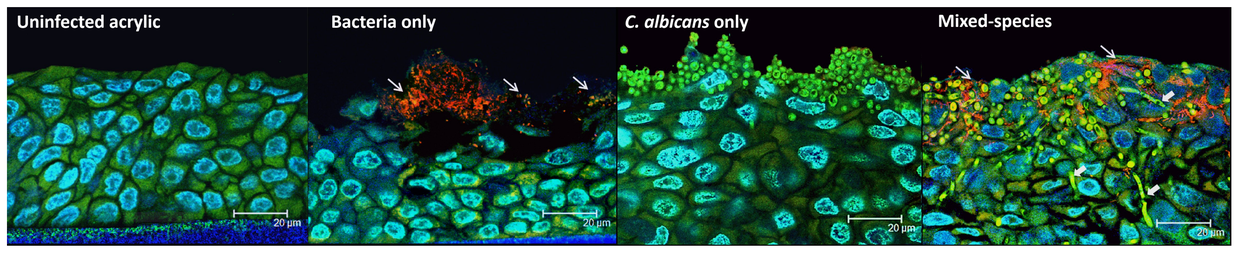

Fig 8. Confocal laser scanning microscopy of epithelial tissue sections, post-biofilm infection. **Uninfected acrylic**, PMMA (poly(methyl methacrylate)) only; **Bacteria only** (*Streptococcus sanguinis, S. gordonii, Actinomyces viscosus, A. odontolyticus*); ***C. albicans*-only**; **Mixed-species,** bacterial species + *C. albicans*. Tissue model cells stained with pan-cytokeratin antibody (green) and hoescht (nuclei, blue), bacteria stained with PNA probe (red), *C. albicans* stained with Yeast Traffic Light PNA (bright green). Damage to tissues evident in bacteria-only and *C. albicans*-only infections, but biofilms remain on surface, whereas mixed-species biofilms resulted in substantially more damage relative to all other conditions, and invasion of all microorganisms within the tissue.

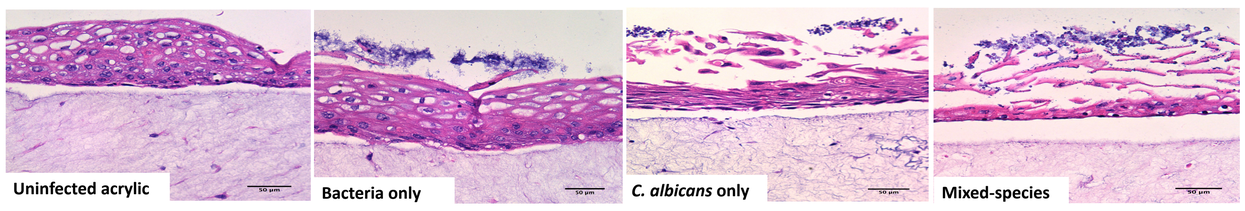

Fig 9. Light microscopy of full-thickness tissue sections, post-infection with acrylic biofilms. Tissues fixed and paraffin wax embedded, sectioned and stained with haematoxylin and eosin. Similar pattern of damage and tissue invasion as observed in Fig 8.

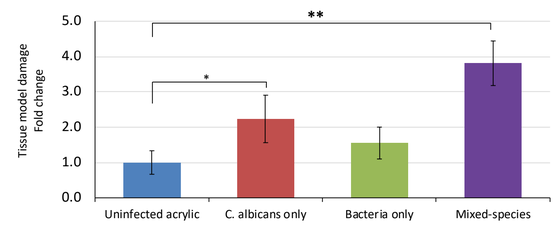

Fig.10. Tissue damage profiles showed tissue damage caused by *C. albicans*-only and oral bacteria-only biofilms, and a substantial increase in damage caused by mixed-species biofilm infections.

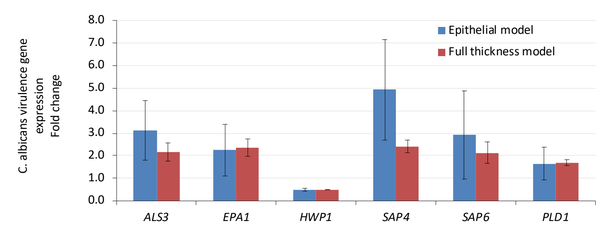

Fig 11. Expression of *C. albicans* virulence genes did not differ between infections of different tissue model types, indicating consistency between the tissue models for infections, showing the same enhancement as *in vitro* biofilm analyses.

Clinical study – characterisation of the bacterial microbiota of DS vs Non-DS patients

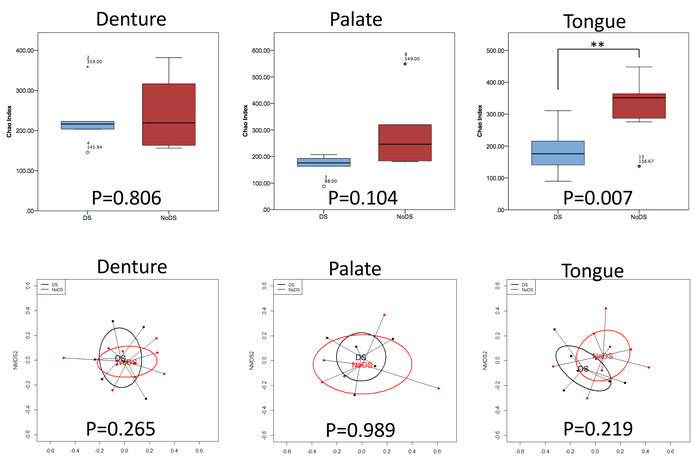

Fig 12. Number of unique bacterial species present in patients with (blue) and without (red) clinical symptoms of DS.
Reduced biodiversity was observed in patients with DS for both the palate and significantly reduced in samples from the tongue.

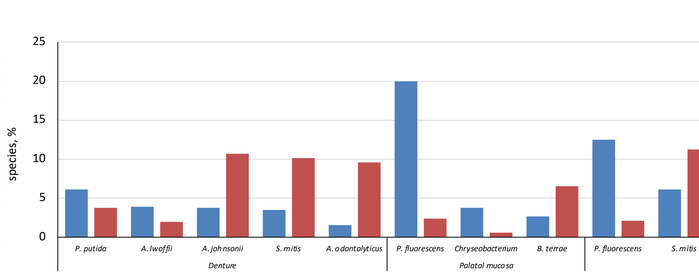

Fig 13. Principle component analysis to identify distinct microbiota of patients with (black) and without (red) clinical symptoms of DS at each sample site.
Overlapping plots indicate non-distinct whole microbiota at any sample site.

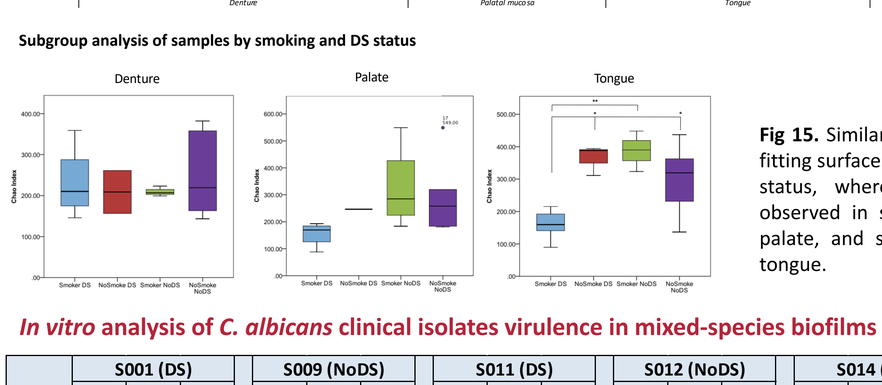

Fig 14. Relative abundance of individual bacterial species common to patients with and without DS. Substantial differences evident for several species within the top 25 bacterial species overall by mean relative abundance at each sample site. There is potential for these species to differentially modulate *Candida* virulence within *in vivo* biofilms, and thus influence the risk or severity of DS.

Fig 15. Similar biodiversity in samples of the denture-fitting surface in patients irrespective of smoking or DS status, whereas a reduction in biodiversity was observed in smokers with DS of samples from the palate, and significant reduction in samples of the tongue.

In vitro analysis of *C. albicans* clinical isolates virulence in mixed-species biofilms

	S001 (DS)			S009 (NoDS)			S011 (DS)			S012 (NoDS)			S014 (DS)			S019 (DS)		
	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
ALS3	0.93	1.16	6.13	0.28	1.91	1.62	0.74	1.04	1.52	0.25	3.19	1.18	0.24	1.79	0.97	0.43	1.32	0.83
HWP1	0.17	1.48	2.21	0.15	1.91	0.98	0.38	1.04	2.88	0.12	3.19	0.55	0.13	1.79	0.95	0.34	1.32	1.31
SAP4	0.65	21.48	2.68	0.36	0.71	0.51	0.49	0.82	3.13	1.74	1.09	1.44	0.33	0.68	0.37	0.10	1.42	3.28
SAP6	0.26	0.70	1.32	1.05	3.54	0.91	0.49	2.63	2.20	9.35	2.01	1.38	0.34	0.82	0.65	0.28	2.12	1.26
PLD1	2.33	1.56	8.08	2.63	1.87	1.83	1.32	1.21	2.41	5.08	2.01	51.09	1.31	0.82	0.46	0.60	2.12	0.81
ECE1	0.17	0.96	1.55	0.28	0.43	1.15	0.42	0.88	1.47	0.16	0.54	1.17	0.12	0.92	0.64	0.09	0.59	1.74

Increased
No change
Decreased

Table 2. Fold change values for putative *C. albicans* virulence genes determined using clinical isolates from patients with (DS) or without (NoDS) denture-associated stomatitis in the mixed-species biofilm model. Values calculated relative to a *C. albicans*-only biofilm, normalised to *ACT1* housekeeping gene control. Green indicates decrease in gene expression, yellow indicates no change, orange indicates slight increase, red indicates greater increase.

Conclusions

- Oral bacteria **modulate** the local environment and **influence** *C. albicans* virulence in *in vitro* biofilms
- Enhanced *C. albicans* virulence leads to **increased tissue damage** in tissue infections, with **substantial invasion** of microorganisms
- Oral bacterial microbiota **is different** between patients with and without denture-stomatitis, indicating an **important role** for the presence and abundance of specific bacterial species to modulate the local environment
- **No clear association** between *C. albicans in vitro* virulence profile and DS incidence in this biofilm model
- Probiotic bacteria **can be used to inhibit enhancement** of specific *C. albicans* virulence in biofilms

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