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Denaturing gradient gel electrophoresis profiles of bacteria from the saliva of twenty four different individuals form clusters that showed no relationship to the yeasts present

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ABSTRACT

Objectives: The aim was to investigate the relationship between groups of bacteria identified by cluster analysis of the DGGE fingerprints and the amounts and diversity of yeast present.
Methods: Bacterial and yeast populations in saliva samples from 24 adults were analysed using denaturing gradient gel electrophoresis (DGGE) of the bacteria present and by yeast culture.
Results: Eubacterial DGGE banding patterns showed considerable variation between individuals. Seventy one different amplicon bands were detected, the band number per saliva sample ranged from 21 to 39 (mean ± SD = 29.3 ± 4.9). Cluster and principal component analysis of the bacterial DGGE patterns yielded three major clusters containing 20 of the samples. Seventeen of the 24 (71%) saliva samples were yeast positive with concentrations up to 10^3 cfu/mL. *Candida albicans* was the predominant species in saliva samples although six other yeast species, including *Candida dubliniensis, Candida tropicalis, Candida krusei, Candida guilliermondii, Candida rugosa* and *Saccharomyces cerevisiae*, were identified. The presence, concentration, and species of yeast in samples showed no clear relationship to the bacterial clusters.
Conclusion: Despite indications of *in vitro* bacteria-yeast interactions, there was a lack of association between the presence, identity and diversity of yeasts and the bacterial DGGE fingerprint clusters in saliva. This suggests significant ecological individual-specificity of these associations in highly complex in vivo oral biofilm systems under normal oral conditions.

1. Introduction

The human oral cavity harbours more than 1000 bacterial and yeast species (Wade, 2013), some of which cause the common human oral polymicrobial diseases dental caries and periodontal diseases (Takahashi & Nyvad, 2011; Xu & Gunsolley, 2014) and can be involved in life threatening systemic disease such as endocarditis (Ledic et al., 2013). Oral microbial diseases can result from disturbance of the complex dynamic interactions between the commensal microbiota and the host by environmental factors such as diet and medications (Marsh, 2003). Caries and periodontitis are usually considered primarily bacterial diseases (Takahashi & Nyvad, 2011). However, *Candida albicans* is a highly acidogenic and aciduric yeast (Nikawa et al., 2003), and there is considerable evidence for its involvement in oral biofilms associated with caries (Ghasempour, Sefidgar, Eyzadian, & Gharakhani, 2011) and its presence in periodontopathic plaques (Waltimo, Sen, Meurman, Ørstavik, & Haapasalo, 2003). Yeasts also cause oral mucosal candidosis which is possibly exacerbated by companion bacteria (Diaz, Strausbaugh, & Dongari-Bagtzoglou, 2014). They can undermine immune defenses, invade tissues and the blood stream, and cause disseminated infections with high associated mortality (Dühring et al., 2015).

Knowledge of how the complex oral bacterial and yeast populations relate to each other and their oral cavity environment is important for understanding, and potentially maintaining, a health-promoting microbiota and preventing caries and other diseases. *In vitro*, *C. albicans* co-aggregates, and metabolically interacts, with a range of bacteria and other yeasts (Shirtliff, Peters, & Jabra-Rizk, 2009), in particular during biofilm co-culture (Thein, Seneviratne, Samaranayake, & Samaranayake, 2009; Weerasekera et al., 2016). Studies of *Streptococcus mutans* and *C. albicans* biofilm co-

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culture have provided evidence for enhanced virulence of both *S. mutans* and *C. albicans* (Falsetta et al., 2014; Sztajer et al., 2014). *C. albicans* and other oral yeasts may also be crucial in maintaining the dynamics, diversity and resilience of a normal oral microbiota and hence oral health (Diaz et al., 2014; Krom, Kidwai, & Ten Cate, 2014). In the gut, colonization by *C. albicans* promotes restoration of a diverse gastro-intestinal bacterial flora following antibiotic treatment (Mason et al., 2012). These findings suggest that yeast-bacteria interactions are important in the ecology of the human commensal microbiome.

The diversity of oral yeasts and their relationship to companion bacterial populations in the oral cavity has not been studied extensively. A pyrosequencing and quantitative PCR analysis of saliva from an older population (68–80 year-olds) showed a significant association between *Candida* concentrations and a saccharolytic, acidogenic bacterial microbiota with low species diversity (Kraneveld et al., 2012). However, in oral microcosms that had different arginine exposure and very divergent pH histories, there was no clear association between *C. albicans* concentrations and particular bacterial species (Koopman et al., 2015).

Denaturing gradient gel electrophoresis (DGGE) has been used to profile the eubacteria in saliva, in *in vitro* oral microcosms, and in oral biofilm (Beerens, Ten Cate, & van der Veen, 2017; Ledder et al., 2007; McBain et al., 2003; Rasiah, Wong, Anderson, & Sissons, 2005; Siqueira, Sakamoto, & Rosado, 2017), including analysis before and after clinical interventions to evaluate the shift in bacterial composition (Li et al., 2006). With adequate controls, DGGE is reproducible and allows similarity comparison between complex microbial populations (Piterina & Pembroke, 2013). DGGE pattern analysis has demonstrated profile stability over 7 years in a person's overall oral bacterial flora (Rasiah et al., 2005). DGGE can also be used to characterize, and presumptively identify, much less bio diverse specific microbial populations such as lactobacilli (Walter et al., 2000) and yeast populations in saliva (Weerasekera et al., 2013).

In this study, bacterial populations in the saliva of 24 adult individuals were examined using cluster analysis of DGGE fingerprints and yeast species were identified by *in vitro* culture. The goal was to determine whether there was a relationship between the bacterial population clusters and the presence of yeast and/or with yeast diversity that had been previously established for these samples (Weerasekera et al., 2013).

2. Materials and methods

2.1. Saliva collection

Saliva was collected with informed consent from a convenience sample of 24 adult donors (saliva donors are designated as A–X; 13 female, 11 male) aged 25–65 (median age 46), previously described in a study of yeast diversity (Weerasekera et al., 2013). Ethical approval was obtained from the Wellington Ethics Committee (WGT/04/02/003). Donors abstained from oral hygiene for 24 h prior to saliva collection at 9–11 a.m. Chicle gum was chewed to stimulate salivary flow and increase oral biofilm abrasion. Ten millilitre saliva was collected from each participant. Portions of the saliva samples were used for yeast culture and analysis and nucleic acid extraction as described below. The remainder of the saliva samples were stored at −80 °C.

2.2. Yeast culture and analysis

Fresh saliva samples (50 μL) from each participant were spread on CHROMagar™ *Candida* plates (CHROMagar, Paris, France) in triplicate and incubated at 35 °C for 48 to 72 h. The colony morphologies and colours were recorded. Presumptive yeast species identification was based on the colour of the colony: *C. albicans* or *Candida dubliniensis* (different shades of green), *Candida krusei* (large rough colonies with pale pink colour) *Candida tropicalis* (dark blue-grey hue with a purple halo). The numbers of each type of colony on the agar plate were counted, and the colony forming units (CFUs) per ml saliva sample were calculated. Species identification was confirmed by sequence analysis of yeast DGGE fragments as described previously (Weerasekera et al., 2013).

2.3. Nucleic acid extraction

A 1 mL saliva sample was centrifuged at 12,500 × *g* for 10 min, and stored at −80 °C until analysed. The thawed pellet was washed by re suspension and re centrifugation in sterile water, then TN150 buffer (10 mM Tris-HCl, 150 mM NaCl, pH 8) followed by suspension in 1 mL TN150 buffer. Nucleic acids were extracted from the saliva pellets after bead beating as described previously (Weerasekera et al., 2013). Saliva pellets were resuspended in 1 mL sterile distilled water and 1 mL TN150 buffer [10 mM Tris/HCl (pH 8), 150 mM NaCl] was added to the samples in the bead-beater tubes, which were then vortexed and centrifuged at 11 760*g* for 5 min at 4 °C. The pellets were washed with 1 mL TN150 buffer, resuspended in 1 mL TN150 buffer and subjected to bead beating with 0.3 g of sterile zirconium beads (0.1 mm diameter (BioSpec Products))(model 3110BX; BioSpec Products) at 480 r.p.m. for 3 min. The tubes were placed on ice and then centrifuged at 11,760*g* for 5 min. The supernatant (300 μL) was extracted twice with 200 μL UltraPure buffer-saturated phenol (pH 8; Bio-Rad) and 200 μL chloroform:isoamyl alcohol (24:1), followed by a final extraction with 400 μL chloroform:isoamyl alcohol (24:1). The upper phase was transferred to a sterile microcentrifuge tube, and 1 mL cold ethanol (100%) and 50 μL 3 M sodium acetate was added, and the sample incubated at −20 °C for 18 h. The solution was centrifuged at 11,760*g* for 20 min at −5 °C and the nucleic acid pellet air dried and dissolved in 30 μL TE buffer [10 mM Tris/HCl (pH 8), 1 mM EDTA].

2.4. PCR–DGGE of bacterial DNA

The V2–V3 region of the bacterial 16S rDNA was amplified using universal bacterial primers HDA 1 (forward) (ACT CCT ACG GGA GGC AGC AGT) and HDA 2 (reverse) (GTA TTA CCG CGG CTG CTG GCA C) (Walter et al., 2000). A 40 base pair GC clamp (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G) was attached to the 5′ end of the HDA 1 primer for DGGE analysis. The PCR regime consisted of initial denaturation at 94 °C for 1 min, then 30 amplification cycles of 94 °C for 30 s for denaturation, 56 °C for 30 s for annealing, 72 °C for 30 s for extension, with a final extension at 68 °C for 7 min.

DGGE was performed as previously described (Rasiah et al., 2005): 8% acrylamide (acrylamide to bis-acrylamide, 37.5:1) with a 30–55% gradient of urea and formamide. The gels were prepared and run with 1 x TAE buffer (Tris-acetate-EDTA buffer, pH 8) at a constant voltage of 130 V at 60 °C for 4.5 h. TAE (1 ×) was prepared by diluting 20 mL of 50 x TAE to 1 L with distilled water, where 50 x TAE consisted of 242 g Trizma Base, 57.1 mL glacial acetic acid, 0.5 M EDTA (previously adjusted to pH 8) in 1 L distilled water, and was autoclaved at 121 °C, 1 kg/cm^2 for 20 min.

For bacterial DGGE normalization analysis, a reference panel mixture was constructed from the DNA of the following seven oral bacterial species with differing GC contents separately amplified as above: *Eikenella corrodens* (ATCC 23834) *Streptococcus sanguinis* (ATCC 10556) *Streptococcus vestibularis* (ATCC 49124), *Veillonella parvula* (ATCC 10790), *Propionibacterium propionicum* (ATCC 14517), *Actinomyces odontolyticus* (ATCC 17929), *Actinomyces israelii* (ATCC 12102).

2.5. Statistical analysis

DGGE banding patterns were assessed by cluster analysis with Dice similarity coefficient constructed using the unweighted pair group method with arithmetic average (UPGMA) and their cluster significance

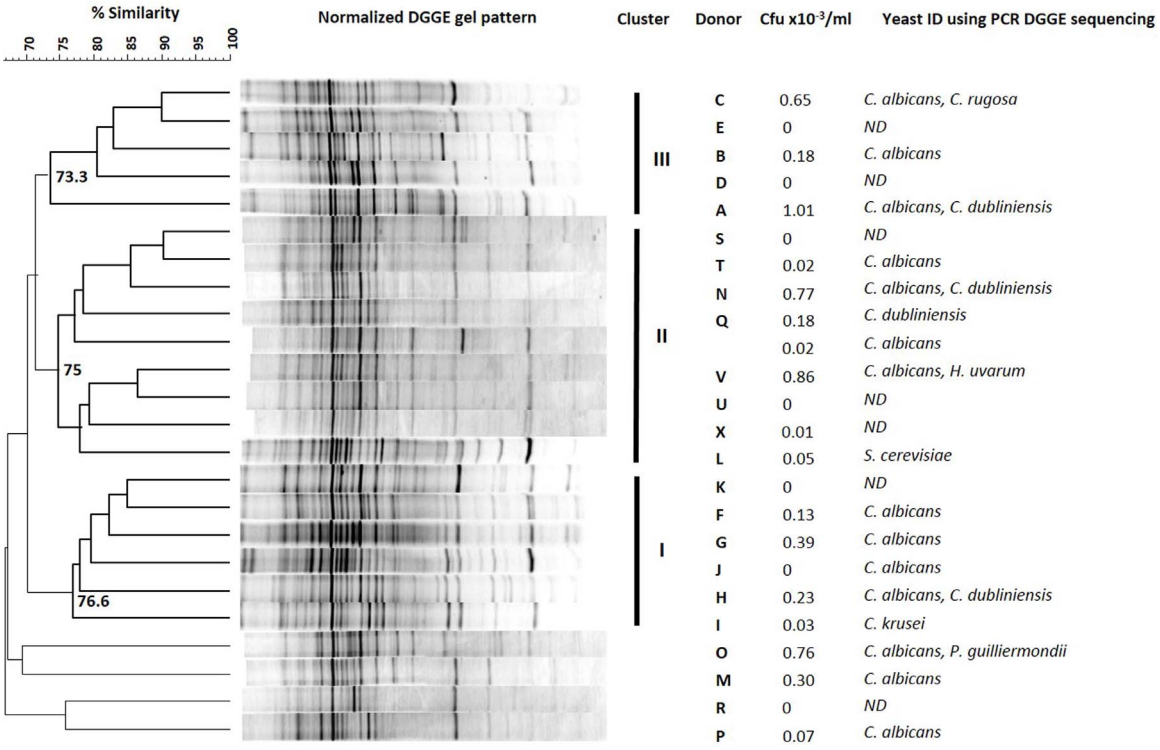

Fig. 1. Cluster analysis of bacterial DGGE profiles for saliva samples from 24 different donors and their total cultivable yeast concentrations. Three clusters identified are designated A, B, and C. The identity of yeasts present by PCR-DGGE-sequencing (Yeast ID) is from Weerasekera et al. (2013). ND – no yeasts detected by PCR-DGGE-sequencing. Sample J was negative for ChromAgar culture but was positive for PCR DGGE. The cultivable yeast in sample X was presumptively *C. albicans* by colour on ChromAgar but was not identified in PCR DGGE.

assessed as $p < 0.05$ by the BioNumerics program, Version 4 (Applied Maths, Kortrijk, Belgium). Differences in cultivable yeast concentrations in saliva associated with donors in each cluster were evaluated by Friedman's non-parametric test.

Binary band matching data (absence or presence) in BioNumerics was further analysed by Principal Component Analysis (PCA) to generate principal components using SPSS software version 13.0 (Statistical Package for Social Science: SPSS Inc, Chicago, IL). PCA was used to identify groups of related bacterial DGGE profiles.

3. Results

3.1. Eubacterial DGGE groups in the saliva from 24 different donors

DGGE banding patterns showed considerable variation between donors (Fig. 1). A total of 71 different amplicon bands were detected, the number per saliva sample ranged from 21 to 39 (mean ± SD = 29.3 ± 4.9). Some dominant DGGE bands with similar intensities were common to all saliva samples. Some bands were present in almost all saliva samples but varied in intensity. Band variation was considerable. The overall similarity between the DGGE banding patterns as determined using BioNumerics cluster analysis was 66.6% ± 4.9%.

Cluster analysis of the banding patterns from individual saliva samples yielded three major clusters and four outliers. The analysis yielded a 'relevance' cut-off of 71% similarity, delimiting three major clusters: cluster I (73.3% ± 2.4% similarity) from six saliva donors Cluster II (75.0% ± 3.6% similarity) from nine saliva donors; Cluster III (76.6% ± 1.7% similarity) from five donors. Saliva samples from donors O, M, R and P were classified as outliers (Fig. 1).

With Principal Components (dissimilarity) Analysis (Fig. 2), Cluster III (donors C, E, B, D, A) from the cluster analysis were well grouped and well separated from the other microbiota. The nine donor Cluster III also fell into a tight group. However the DGGE profiles from the Cluster C saliva samples and the unclustered saliva samples were more widely dispersed, some, overlapping with Cluster B donors (Fig. 2).

3.2. The presence and diversity of yeast showed no detectable relation with the bacterial clusters in the saliva

As previously reported, 17 of the 24 (71%) saliva samples were positive for yeast when analysed by PCR and, in addition to the predominant *C. albicans*, six other yeasts were identified (Weerasekera et al., 2013). Total yeast concentrations ranged up to 10^3 cfu/mL (Fig. 1). The presence, amount and species of yeast present showed no clear relationship to the bacterial clusters (Fig. 1). The three major bacterial clusters had a similar prevalence of yeast (60–67%) as the outliers (75% prevalence). The seven saliva samples in which no yeast was detected by PCR-DGGE sequencing (from donors E, D, S, U, X, K, R) were distributed amongst clusters, apparently at random. Saliva samples with high yeast concentrations (taken to be ≥300 cells/mL; donors C, A, N, V, O, M, G) were likewise associated with all clusters and the outliers. There was an indication of different mean yeast concentrations between clusters (A, 370 cfu/mL; B, 210 cfu/mL; C, 130 cfu/mL, outliers 280 cfu/mL, (including samples with no yeast cultured)) but these differences were not statistically significant.

There was no relationship of the six samples with more than 1 yeast species present, to any particular bacterial cluster. *C. dubliniensis* was the second most common yeast species detected in saliva samples (donors A, H, N, Q). In two saliva samples the non-*C. albicans* yeast could be distinguished by CHROMagar morphology: *Candida rugosa* in donor C and *Hanseniaspora uvarum* in donor V comprised 10% of the total yeast cfu. Neither *C. dubliniensis* nor the other minor yeasts showed a clear association with any of the bacterial clusters.

4. Discussion

The analysis of saliva samples from 24 adult individuals indicated that bacterial communities in saliva differ from person to person but identified three significant sub-groups of individuals having similar

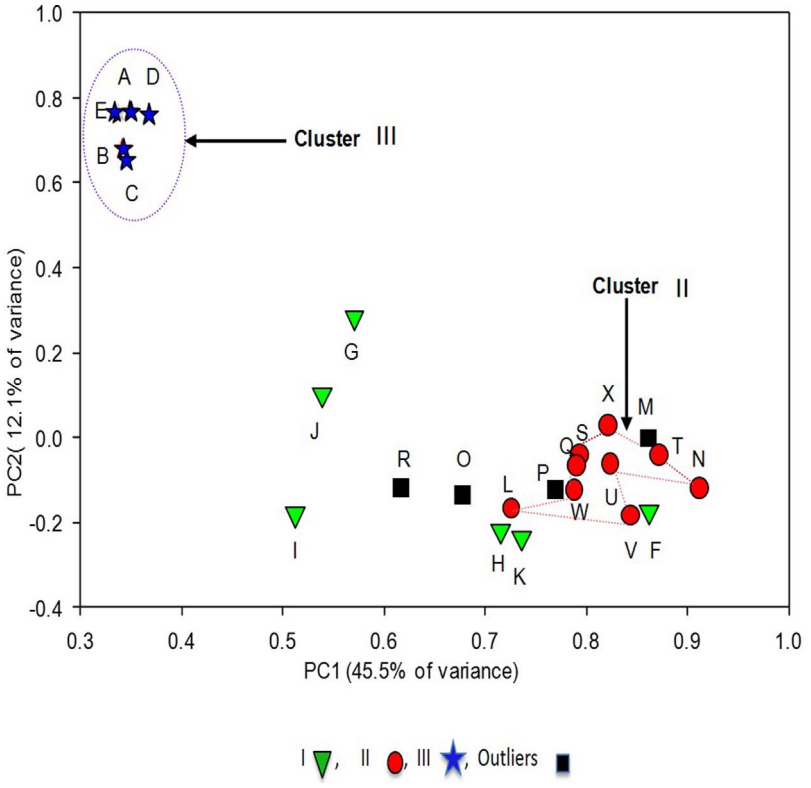

Fig. 2. PCA of the DGGE patterns. Saliva samples identified by letters as in Fig. 1. Symbols identify the Clusters: I, II, III, Outliers.

bacterial populations, plus some outliers. Yeast were detected in 17 individuals and, as expected, *C. albicans* was the dominant yeast (present in 14 samples) with significant variation in the other yeast species present (Weerasekera et al., 2013). The abundance and diversity of these yeasts showed no significant relationship to the eubacterial DGGE pattern clusters at the DGGE level of resolution of the bacterial microbiota. This is in contrast to a previous study of saliva from an older population (68–80 year-olds) which found a significant association between *Candida* concentrations and a saccharolytic, acidogenic bacterial microbiota (Kraneveld et al., 2012). Our study also does not reflect *in vitro* evidence of a range of direct dual species yeast-bacteria binding interactions, inter-species signalling and physiological effects. It is possible that the present negative findings could be the result of technical limitations in the resolution of the DGGE technique, the relatively small number of individuals studied, or fundamentally complex ecological relationships. The samples in the present study had yeast concentrations up to ten-fold lower than those in the samples from the older participants in the Kraneveld et al. study which would have provided potentially greater scope for associations with bacterial populations. The older subjects also may have had conditions such as hyposalivation and edentulism which may have altered ecological interactions. In the comparatively younger population in the present study, interactions between bacteria and yeasts in complex biofilm systems may be weak, subtle, or outweighed by stronger between-individual microbiota differences in less stressed ecological conditions (Koopman et al., 2015).

There are fundamental resolution and technical limitations inherent in the analysis of DGGE fingerprints for bacterial population characterization. DGGE patterns are reproducible, especially within a gel (Rasiah et al., 2005), and also between gels when adequately normalized with reference panels. They are, however, non-quantitative due to biases associated with DNA extraction, differential amplification of the 16S rRNA genes (Machado de Oliveira et al., 2007), template competition, limited gel resolution, and co-migration of bands (Gafan & Spratt, 2005). Some yeast and bacterial species contain multiple rRNA copies yielding DGGE profiles with multiple bands (Machado de Oliveira et al., 2007). Despite these limitations, bacterial DGGE analysis is a powerful technique that can be used to fingerprint the oral bacteria in saliva and oral biofilms, and other complex microbial systems under different environmental and experimental conditions (Lu et al., 2013).

Further technical factors such as individual differences in the contribution of separate oral site-specific biofilms to the salivary microbial pool may affect the population analysis and, although we report a more extensive study than many carried out previously, the number of individuals included is still relatively small. Greater sample numbers may refine the DGGE clusters. Nevertheless there was little sign from the present results that this might detect yeast-bacteria associations, except possibly for differences in yeast abundance.

The lack of concordance of the results of the current study with *in vitro* studies showing close yeast/bacterial relationships in oral microbial communities (Wright et al., 2013) may reflect the limited population diversity in most *in vitro* studies. The yeast-bacterial co-aggregation found *in vitro* may be highly specific to particular yeast and bacterial strains and species chosen for investigation (Thein, Samaranayake, & Samaranayake, 2006). The associations detected between two species may be weak or non-existent in complex oral biofilms, with differing effects of ecological pressures *e.g.* host environment, hyposalivation or diet on bacterial and yeast populations. However, in situations under more extreme ecological pressure such as in a cariogenic acidogenic/aciduric biofilms, or recovery from severe antibiotic pressure, yeasts and major segments of bacterial microbiota may change in concert. Our results suggest that to analyse the complex ecological relationships of fully biodiverse oral biofilms, application of higher-resolution analytical technology than DGGE analysis, such as high-throughput sequencing, is required to adequately dissect yeast-bacteria interactions *in vivo* and their response to normal and stressed oral environments in health and disease. Establishing the degree to which this relationship occurs *in vivo*, and can be disrupted, is important in order to understand yeast involvement in oral and invasive disease.

5. Conclusion

In conclusion, the abundance and diversity of the yeasts in the 24 participants showed no significant relationship to the bacterial DGGE pattern. The lack of association between yeasts and the bacterial fingerprints in the saliva samples in this study suggests significant ecological individual-specificity in highly complex oral biofilm systems under normal oral conditions.

Conflict of interest statement

There are no conflicts of interest.

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