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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