1566

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INTRODUCTION

Imbalances of the oral microbiota, commonly referred to as microbial dysbiosis, have been associated with a wide range of oral diseases (Kilian et al., 2016). Most oral microbiota studies have focussed on plaque-induced diseases and have demonstrated a key role for dysbiosis in the pathophysiology of periodontitis and caries. Clinically important changes of the structure of the microbiota are induced by a variety of environmental and host factors (Marsh, Head, & Devine, 2015) leading to the enrichment of species which for example are tolerant to an acidic environment in caries (Liu, Nascimento, & Burne, 2012), or subvert immune responses leading...
to destructive inflammation of the periodontium (Darveau, 2010). In addition, low-abundance pathogens themselves can cause microbiota imbalances which in turn induce inflammatory responses in periodontitis (Hajishengallis et al., 2011).

The role of microbial dysbiosis in oral disease may be not confined to plaque-induced oral diseases where the role of microbial biofilms has been long established. A number of studies have reported enrichment or depletion of specific bacterial genera and species in oral samples from patients suffering from immune-mediated oral mucosal diseases, such as recurrent aphthous stomatitis (RAS) (Bankvall et al., 2014; Hijazi et al., 2015; Kim et al., 2016; Marchini, Campos, Silva, Paulino, & Nobrega, 2007; Seoudi, Bergmeier, Drobniewski, Paster, & Fortune, 2015) and oral lichen planus (LP) (Choi et al., 2016; Kazanowska-Dygdała et al., 2016; Pankhurst, Auger, & Hardie, 1988; Wang et al., 2016), for which historically the role of bacteria has been debated but never confirmed. Oral mucosal diseases, such as RAS and LP, have a proven negative impact on patient quality of life (Hegarty, McGrath, Hodgson, & Porter, 2002; Liu, Xiao, He, & Jiang, 2012), but with little knowledge of the events at the mucosal interface that initiate and sustain inflammation, the treatment of these conditions remains untargeted and often ineffective (Belenguer-Guallar, Jimenez-Soriano, & Claramunt-Lozano, 2014).

A range of bacterial species including Streptococcus species and Helicobacter pylori were historically implicated in RAS (Hasan et al., 1995; Riggio, Lennon, & Wray, 2000), but their role has remained controversial. More recently, case–control studies of the oral microbiota have reported significant enrichment of other species (Rothia dentocariosa and Acinetobacter johnsonii) as well as decreased abundance of Streptococcus salivarius, Neisseria and Veillonella species in patients suffering from RAS compared to controls (Kim et al., 2016; Seoudi et al., 2015). Equally, comparative analyses of mucosal and salivary samples from patients suffering from oral LP and healthy controls have shown a positive association with periodontal disease-related species as well as decreased abundance of Streptococcus, Haemophilus, Corynebacterium, Cellulosimicrobium and Campylobacter (Choi et al., 2016; Wang et al., 2016). While the cause–effect relationship between these species and the disease is yet to be elucidated, it has been long known that patients suffering from immune-mediated oral mucosal disease can respond well to a range of broad-spectrum anti-microbials (Ranganath & Pai, 2016). For example, chlorhexidine and tetracyclines are formally recommended for routine management of mild forms of RAS (Pemberton, 2014) and snap-frozen for quantitation of cytokines. Another whole saliva sample (5 ml) was collected for Candida colony quantitation which was carried out by sample concentration in 500 µl of fresh saline before serial ten-fold dilution plating onto Brilliance Candida Agar (Oxoid, Thermo Fisher Scientific). Plates were incubated at 37°C for 48 hr.

2.3 | Sample collection

Swabs of the lower left buccal sulcus were taken from participants in the morning before breakfast. Ulcerated areas were avoided to maintain consistency of sampled tissue type amongst the three groups. As such, the patients who presented with ulceration along the lower left buccal sulcus were sampled at the right lower buccal sulcus. Swabs were immersed in 500 µl of sodium phosphate (SP) buffer and the suspension was stored at 4°C for a maximum of 2 hr postcollection until DNA extraction was performed.

Whole unstimulated saliva samples (1.5 ml) were obtained over 10 min under strictly controlled conditions as previously described (Navazesh, Kumar, & University of Southern California School of Dentistry, 2008) and snap-frozen for quantitation of cytokines. Genomic DNA was extracted from swabs using FastDNA SPIN Kit for soil (MP Biomedicals) following the manufacturer’s instructions. Full detail of the DNA extraction protocol is reported in the Supporting information. To assess the bacterial diversity by 16S rRNA high-throughput sequencing, fusion primers consisting of Th1, Th2 and Th17 inflammatory cytokines implicated in LP and RAS.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Ethical approval for involvement of patients in this study was granted by North of Scotland Research Ethics Service (reference 12/NS/0006). Written informed consent was obtained from all participants according to the World Health Organization guidelines for Good Research Practice.

2.2 | Patient recruitment

Cases and controls were recruited at the Maxillofacial Unit of Aberdeen Royal Infirmary and the Institute of Dentistry of the University of Aberdeen, respectively, using stringent criteria as previously described (Hijazi et al., 2015) with some modifications. Full detail of the recruitment methodology is reported in the Supporting information.

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2.4 | DNA extraction and amplicon generation for sequencing

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illuminating the overhang nucleotide sequences added to universal primers 27F and 338R were used to amplify the V1-V2 hypervariable regions of the 16S rRNA gene. Full detail of the PCR protocol is reported in the Supporting information.

### 2.5 Library preparation and sequencing

The purified amplicons containing the Illumina overhang were indexed with Illumina Nextera XT v2 indices (Illumina). Full detail of library preparation is reported in the Supporting information. The libraries were equimolar pooled and sequenced on an Illumina MiSeq Sequencing System using MiSeq v3 chemistry with 300 bp paired-end reads. Base calling and FASTQ output files were generated by the MiSeq instrument. Sequences have been submitted to the BioSample database through Sequence Read Archive (Bioproject ID PRJNA609244).

Bioinformatic analyses are described in the Supporting information.

### 2.6 Cytokine measurements

Frozen saliva samples were thawed on ice and centrifuged at 6,000 g for 20 min. The supernatants were used for cytokine quantitation. Total protein in the saliva samples was quantified with the BCA protein assay kit (Pierce, Thermo Fisher Scientific) according to manufacturer's instructions. Concentrations of tumour necrosis factor-α (TNF-α), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), interferon-γ (IFN-γ), interleukin-17A (IL-17A) and interleukin-1β (IL-1β) were measured using the BD CBA Human Enhanced Sensitivity Flex Sets (Catalog no. 561523, BD Biosciences) strictly according to manufacturer's instructions with no modifications. Clarified saliva supernatants (50 μl) and standards for each of the above cytokines (274–200,000 fg/ml) were added to prewashed antibody-coated plates. Capture Beads, Human Detection Reagent and the Enhanced Sensitivity Detection Reagent were sequentially incubated with samples and standards before acquisition. Sample and standard acquisition was carried out with the FACSCalibur flow cytometer (BD Biosciences), and output data analysed using the FCAP Array software, version 3 (BD Biosciences).

### 2.7 Statistical methods

Alpha diversity indexes (Shannon, Simpson’s Index of Diversity, Chao1, phylogenetic diversity and number of observed OTUs) of the RAS and LP groups were compared to controls using Kruskal-Wallis test with Dunn’s multiple comparison test. Correlation matrices reporting (a) alpha diversity indexes, (b) cytokine concentrations and (c) clinical severity scores of RAS and LP for each sample were designed. Correlation scores were calculated using Spearman’s rank correlation, since values were mostly not normally distributed. Relative abundances of genera and species amongst different groups of samples were compared using the Kruskal-Wallis test with Benjamini-Hochberg correction for multiple testing; GraphPad Prism (v 8.3.0) and the pairs.panels function of the R package psych were used for statistics and figures. Cytokine concentrations amongst the three patient groups were compared by ANOVA with Dunn’s post hoc test to allow multiple comparisons; graphs were created using SPSS. Two-sided statistical significance was set at p < .05.

### 3 RESULTS

#### 3.1 Patient characteristics

Fifteen RAS patients (age (46.13 ± 11.84) years), 21 LP patients (age (50.17 ± 8.64) years) and 13 controls (age (48.62 ± 9.47) years) were recruited with no significant difference in age distribution amongst the groups (p = .372). Three LP patients were subsequently excluded from the study as the respective samples did not meet sequencing quality control standards; thus, 18 LP patients were included in the study. Sex distribution was similar amongst the three groups of patients included in the study (11 female, seven male in the LP group; 10 female, five male in the RAS group; nine female, four male in the control group). Body mass index was between 20.56 and 29.98 with no significant difference in BMI distribution amongst the groups (controls: 24.31 ± 2.35, LP: 24.36 ± 2.17, RAS: 24.20 ± 2.21, p = .98). RAS disease scores were between 18 and 37. LP disease scores were between 9 and 29. Whole unstimulated saliva flow values were between 0.32 and 0.78 ml/min with no significant differences amongst the groups (controls: 0.53 ± 0.11 ml/min, LP: 0.54 ± 0.13 ml/min, RAS: 0.50 ± 0.11 ml/min, p = .62). Colony counts of Candida species cultured from whole saliva samples were between 14 and 624 CFU/ml. Key general and oral health characteristics of recruits included in the study are reported in Table S1.

#### 3.2 Composition and diversity of the oral mucosal bacterial community in oral mucosal disease patients and healthy controls

A total of 5,369,503 sequences (range 40,280–210,818, median 128,058) from 43 samples were obtained after chimera filtering and removal of low confidence OTUs (i.e. OTUs containing less than 122 sequences). These sequences were assigned to 537 different OTUs, 14 (2.6%) of which were present in all samples. The genera present in all samples were as follows: Actinomyces, Alloprevotella, Atopobium, Capnocytophaga, Fusobacterium, Gemella, Granulicatella, Haemophilus, Leptotrichia, Porphyromonas, Prevotella, Rothia, Selenomonas, Streptococcus. The most abundant were Streptococcus, Haemophilus, Gemella, Veillonella and Prevotella. There were no statistical differences in the abundance of these genera amongst the three sample groups (Figure S1).
Bacterial diversity was determined calculating the Shannon index and Simpson’s Index of Diversity. Chao1 index, phylogenetic distance and number of observed OTUs, which are based on the presence/absence of a given species, were also calculated. Comparative analyses of the LP and RAS groups against the control group (Kruskal–Wallis non-parametric test) showed that both had a significantly lower Chao1 index ($p = .021$ and $p = .044$ for LP and RAS, respectively). All the other indices were comparable amongst the study groups (Figure 1).

Species- and genus-level abundance comparisons were made between each of the three pairings of sample groups (LP versus RAS; LP versus controls; RAS versus controls) against all species-level matches using the Kruskal–Wallis test followed by Benjamini–Hochberg correction for multiple testing. There was no significant difference in the abundance of any genus or species between cases and controls or amongst the three groups. Table S2 shows the abundance of individual genera and their prevalence in case and control samples. Principal component analyses undertaken on raw OTU abundance data showed no distinct group clustering within any samples group (Figure S2).

### 3.3 Correlation of bacterial diversity and clinical severity of disease

The clinical severity score of individual RAS and LP patients was correlated to the alpha diversity indices within each group using Spearman’s rank correlation. An inverse correlation was found between the clinical severity and diversity indices. The correlation of RAS severity was significant with the Chao1 index ($p = −0.714$, $p = .008$) and with the number of observed OTUs ($p = −0.601$, $p = .033$). LP severity was significantly correlated with all five alpha diversity indices (Shannon’s $p = −0.587$, $p = .015$; Simpson’s Index of Diversity $p = −0.551$, $p = .024$; phylogenetic distance $p = −0.557$, $p = .022$; Chao1 $p = −0.624$, $p = .009$; observed OTUs $p = −0.636$, $p = .007$) (Figure 2). This suggests that mucosal microbial population diversity is reduced in active disease.

### 3.4 Correlation of bacterial diversity and salivary cytokines

The concentration of TNF-α, IL-4, IL-6, IFN-γ, IL-10, IL-17A and IL-1β in whole saliva was measured using cytometric bead assays. Findings are presented as cytokine concentration absolute values in view of equal total protein concentrations in the three groups (controls: 1675.16 ± 281.54 µg/ml, LP: 1646.05 ± 584.28 µg/ml, RAS: 1,490.54 ± 1,028.34 µg/ml, $p = .417$). Concentrations of TNF-α, IL-4, IL-6, IL-17A and IL-1β in LP and RAS patients were significantly raised compared to the control group (Figure 3). IFN-γ was significantly raised in LP ($p < .001$) compared to RAS and controls while no statistically significant differences in IFN-γ levels were observed between the latter two groups (Figure 3). There were no significant differences in IL-10 concentration amongst the three groups (data not shown).

Correlation studies of salivary cytokine concentration and bacterial diversity values amongst all groups were carried out. As shown in

![Figure 1](https://via.placeholder.com/150)  
**Figure 1** Alpha diversity amongst oral mucosal disease groups and controls. Boxes show the 25–75 percentile interval of data, and whiskers show the minimum and maximum ranges. Data from single patients are shown as black dots (RAS group), squares (LP group) or triangles (control group). Mean values for each index in each group are indicated by the symbol “+”. Boxes are colour-coded according to the group (dark grey: RAS; light grey: LP; white: control). Statistical significance was calculated for comparisons of the two disease groups versus controls using the Kruskal–Wallis test and is indicated by asterisks (*$p < .05$)
Figure 4 there was a moderate to strong negative correlation of IFN-γ ($\rho = -0.449, 95\% CI: -0.680$ to $-0.186, p = .003$), IL-17A ($\rho = -0.401$, $95\% CI: -0.616$ to $-0.134, p = .009$) and IL-1β ($\rho = -0.418, 95\% CI: -0.666$ to $-0.103, p = .007$) concentrations with Chao1 values. A significant and direct correlation of IL-17A levels and clinical disease scores in both RAS and LP patients was observed (RAS: $\rho = 0.58, 95\% CI: 0.5$–$0.85, p = .032$; LP: $\rho = 0.61, 95\% CI: 0.17$–$0.85, p = .011$) (Figure S3).

Altogether, these findings (Figures 2 and 4) show that mucosal disease severity and inflammation, measured as concentration of salivary pro-inflammatory cytokines, correlate with reduced bacterial diversity.

4 | DISCUSSION

The study of oral microbiota-immunity interactions in the context of inflammatory oral mucosal diseases is critical to inform novel
therapeutic strategies beyond the current palliative options. In addition to the discovery of new treatment targets, our increased understanding of the relationship of the oral microbiota and immune responses driving oral mucosal diseases is important to identify markers of disease activity and thus monitor response to treatment.

There has been lack of consistency in the findings of microbiome studies of immune-mediated oral mucosal disease published to date (He et al., 2017; Hijazi et al., 2015; Kim et al., 2016; Seoudi et al., 2015; Wang et al., 2016), and a microbial signature for LP and RAS patients has not been confirmed. Notwithstanding that a plethora of factors relating to the high-throughput sequencing approach may be explanatory of the inconsistent results, it is critical to reflect on the role of aspects relating to study design itself including recruitment strategy and sample collection. Although all studies have acknowledged the limitation of small sample sizes, we suggest that small oral microbiota studies must adhere to stringent recruitment criteria to minimise local and systemic factors known to influence the composition of the oral microbiota independently of immune-mediated mucosal disease, while larger studies may use regression models to adjust for these factors. In this study, patients with the range of host phenotypic characteristics known to influence the microbiome structure as reported by the Human Microbiome Project Consortium (Human Microbiome Project Consortium, 2012) were excluded, while ensuring equal distribution of age, sex, ethnicity and body mass index amongst the study groups. Patients were also screened for subclinical conditions, such as haematmic deficiencies, long known to affect the oral epithelial turnover (Challacombe, Barkhan, & Lehner, 1977) and likely to affect the structure of the oral mucosal microbiota. Further, control of local environmental factors was extended to regulation of sugar consumption levels and oral hygiene practices prior to sampling.

Host immunogenetics likely play a critical role in the pathogenesis of oral mucosal inflammatory disease as recently confirmed by a genome-wide association study which identified 97 genetic variants independently associated with recurrent aphthous stomatitis (Dudding et al., 2019). Future studies should investigate how these genetic variants relate to the structure of the oral microbiota.

The choice of sample site in oral microbiota studies also merits careful consideration. The composition of the microbiota in healthy individuals shows great diversity between different oral micro-environments with the subgingival environment displaying the greatest level of diversity (Dewhirst et al., 2010), hence our consistent sampling approach and exclusion of patients presenting with gingival inflammation, being it plaque-induced or not. Significant differences in the composition of the oral microbiome between local mucosal samples and whole saliva have been confirmed in a recent study (Kragelund & Keller, 2019). It is reasonable to speculate that microbiome sampling from oral mucosa is more likely representative of the microbial community directly involved in mucosal inflammation, while whole saliva samples capture the microbiota from the range of oral micro-environments, including those that play no role in immune-mediated mucosal disease, namely subgingival and hard tissue-associated microbiota. To maintain homogeneity of sampling substrate, analyses were limited to non-ulcerated mucosa. Microbiota analysis of ulcerated oral mucosa by us and another group (Hijazi et al., 2015; Kim et al., 2016) has shown substantial
Phylum-level changes with decreased *Firmicutes* and increased *Proteobacteria* consistently with the well-established microbiota changes associated with bowel ulceration (Frank et al., 2007). This major shift in the microbiota populating the ulcerated mucosa is likely heavily influenced by the different tissue substrate (fibrin layer) replacing the breached epithelium at ulcerated mucosa and hence more likely to be an effect of the change of tissue substrate rather than a player in disease activity.

In the present study, composition differences of the bacterial community between controls and patients suffering from RAS or LP ascribable to individual species, as reported in previous studies, were not confirmed. The variance across individuals within each group, likely
reflective of the breath of disease activity, was larger than the inter-group differences suggesting that considerably larger sample sizes may be required to identify species-level differences due to disease. Nonetheless, a significant overall reduction of bacterial taxonomic diversity in patients suffering from LP patients compared to controls was observed. This finding is in keeping with a previous report of reduced bacterial richness and diversity in erosive lichen planus (Wang et al., 2015). Reduced bacterial diversity compared to healthy controls has been reported in multi-system conditions characterised by recurrent oral ulceration, for example Behcet's disease (Coit et al., 2016).

For the first time, this study demonstrates a significant correlation between the reduced bacterial diversity in LP and RAS (measured by a range of diversity indices) and disease activity quantified using validated disease scores for LP and RAS. Both scores have previously shown sufficient sensitivity to detect response to treatment (Escudier et al., 2007; Tappuni, Kovacevic, Shirlaw, & Challacombe, 2013). The data here presented demonstrate an inverse correlation of the two scores with bacterial diversity panning the way for further studies of oral microbiome-based markers of disease activity. Both clinical scores were directly correlated with salivary IL-17A. The correlation with the other inflammatory cytokines did not reach statistical significance. The important role of IL-17 in oral mucosal inflammation is supported by evidence of favourable response to the therapeutic targeting of IL-17+T cells in recalcitrant erosive lichen planus (Solimani et al., 2019).

Correlation studies disclosed an inverse relationship between bacterial diversity and salivary concentrations of key Th1, Th2 and Th17 inflammatory cytokines implicated separately in microbial dysbiosis (mostly in the gut) and oral mucosal inflammatory disease. A previous report showed a negative correlation of salivary IL-17 and bacterial diversity in patients suffering from erosive lichen planus (Wang et al., 2015). This study shows a correlation of reduced bacterial diversity with pro-inflammatory cytokines IFN-γ, IL-17A and IL-1β. There was no statistically significant correlation with TNF-α, the role of which has been long established in RAS (Sand & Thomsen, 2013) or IL-6 shown to be elevated in saliva of LP patients in a recent meta-analysis (Mozaffari, Sharifi, & Sadeghi, 2018). Future studies should extend analyses to other Th17-activating cytokines and inflammatory cytokines implicated in immune conditions associated with oral mucosal disease such as IL-21 (Geri et al., 2011) as well as effector cytokines known to modify the composition of mucosal microbiota such as IL-22 (Korn et al., 2014).

Our findings of inflammatory cytokine-bacterial diversity correlation studies support the suggestion that oral mucosal inflammation provides a novel source of nutrients which result in major compositional changes of the microbiota (Marsh et al., 2015). On the other hand, it is plausible that imbalances of oral mucosal microbiota may themselves regulate mucosal inflammatory responses in line with current theories of host-microbiota interactions in the gut (Grigg & Sonnenberg, 2017). Indeed, repopulation of the oral cavity with a healthy microbiota has been proposed for the tackling of oral diseases (Zhang et al., 2018).

Future studies should also investigate the role of the oral mycobiome. In this study, the detection rate of Candida species and colony counts using standard culture methods were similar in the three groups and below clinically significant counts (Tooyama et al., 2015). Nonetheless, mycobiome analyses would be important to complement the data of this study in order to determine the role of fungal diversity in the modulation of oral mucosal inflammation. A recent study has found reduced fungal diversity in LP patients compared to controls and correlations of a range of fungal genera with IL-17 (Li et al., 2019). Future studies should further investigate the clinical relevance of this finding and the relationship with oral mucosal immune responses in the context of oral mucosal disease.

The correlation of reduced bacterial diversity with increased clinical scores and pro-inflammatory cytokines in saliva points to the potential value of oral microbiome diversity as marker of disease activity for control of immune-mediated oral mucosal diseases. We speculate that taxa-level changes underpinning reduced diversity in inflammation vary depending on disease activity. Future studies comprising larger groups of patients representing different stages of disease may disclose specific taxa-level changes that will aid the development of microbiota-targeted treatments.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

AUTHOR CONTRIBUTIONS

Karolin Hijazi: Conceptualization; data curation; funding acquisition; investigation; methodology; project administration; resources; supervision; writing – original draft; writing – review and editing. Roderick W. Morrison: Investigation; methodology; supervision; writing – original draft; writing – review and editing. Indrani Mukhopadhyya: Formal analysis; methodology; validation; visualization; writing – original draft; writing – review and editing. Brennan Martin: Methodology; resources; validation; visualization; writing – original draft; writing – review and editing. Matthew Gemmell: Formal analysis; methodology; software; visualization; writing – original draft; writing – review and editing. Sophie Shaw: Data curation; formal analysis; methodology; software; visualization; writing – original draft; writing – review and editing. Francesco Santoro: Data curation; formal analysis; investigation; methodology; software; validation; visualization; writing – original draft; writing – review and editing.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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