

Dysbiotic subgingival microbial communities in periodontally healthy patients with rheumatoid arthritis

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1 **Title:** Dysbiotic subgingival microbial communities in periodontally healthy patients
2 with rheumatoid arthritis

3
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39 microbiome

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42

1 **Abstract**

2

3 **Objectives**

4 Studies that demonstrate an association between rheumatoid arthritis (RA) and
5 dysbiotic oral microbiomes are often confounded by the presence of extensive
6 periodontitis in these individuals. Therefore, the present investigation sought to
7 investigate the role of RA in modulating the periodontal microbiome by comparing
8 periodontally healthy individuals with and without RA.

9 **Methods**

10 Subgingival plaque was collected from was collected periodontally healthy individuals
11 (22 with and 19 without RA), and 16S gene sequenced on the Illumina MiSeq
12 platform. Bacterial biodiversity and co-occurrence patterns were examined using the
13 QIIME and PhyloToAST pipelines.

14 **Results**

15 The subgingival microbiomes differed significantly based on both community
16 membership and as well as the abundance of lineages, with 41.9% of the community
17 differing in abundance and 19% in membership. In contrast to the sparse and
18 predominantly congeneric co-occurrence networks seen in controls, RA subjects
19 revealed a highly connected grid containing a large inter-generic hub anchored by
20 known periodontal pathogens. Predictive metagenomic analysis (PICRUSt)
21 demonstrated that arachidonic acid and ester lipid metabolism pathways might partly
22 explain the robustness of this clustering. As expected from a periodontally healthy
23 cohort, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* were
24 not significantly different between groups, however, *Cryptobacterium curtum*, another
25 organism capable of producing large amounts of citrulline, emerged as a robust
26 discriminant of the microbiome in individuals with RA.

27 **Conclusions**

28 Our data demonstrates that the oral microbiome in RA is enriched for inflammophilic
29 and citrulline producing organisms, which may play a role in the production of
30 autoantigenic citrullinated peptides in RA.

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1 INTRODUCTION

2 Rheumatoid arthritis (RA) has been associated with periodontal disease (PD),
3 a bacterially initiated chronic inflammation that leads to destruction of tooth-
4 supporting tissues¹. Although PD and RA share similar inflammatory pathways as
5 well as genetic and environmental risk factors, these are insufficient to explain this
6 connection¹.

7 While the cause of RA remains unknown, it has been hypothesized that oral
8 microbiota^{2,3} in particular the periodontal pathogens *Porphyromonas gingivalis* and
9 *Aggregatibacter actinomycetemcomitans*, may play a critical role in its
10 pathogenesis^{4,5}

11 Studies using next generation sequencing methods demonstrate the oral
12 microbiome is altered in RA^{6,7}. However, the majority of these studies included
13 individuals with moderate to severe periodontitis⁷ or individuals whose periodontal
14 health status was not established⁶. Periodontitis, by itself, is a significant modifier of
15 the oral microbiome⁸, making it difficult to dissect the relative contributions of
16 periodontitis and RA to the microbial dysbiosis.

17 Given the potential role of oral bacteria may play in the etiopathogenesis of
18 RA, we set out to characterize the periodontal microbiome in periodontally healthy
19 individuals with and without RA, using next generation sequencing.
20

21 METHODS

22 The study sample included patients with RA and non-RA controls. All participants
23 were periodontally healthy. Subgingival plaque samples were collected and analyzed
24 using 16S rDNA sequencing. Detailed methods are described in supplementary
25 information. The sequences are deposited in the Sequence Read Archive of NCBI
26 (project number: PRJNA391575).
27
28

29 RESULTS

30 We examined 22 patients with RA and 19 non-RA controls. There was a statistically
31 significant but clinically inconsequential difference between groups in periodontal
32 measures, in particular PPD and CAL (Table 1). Principal Coordinate Analysis
33 (PCoA) of both unweighted and weighted UniFrac distances demonstrated significant
34 clustering of the microbiomes based on RA status (Figure 1, $p=0.001$, Adonis test),
35 indicating that these groups differed both in presence or absence of lineages
36 (community membership), as well as in the relative abundances of lineages within
37 communities (community structure).
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41 Table 1: Clinical and demographic characteristics in periodontally healthy subjects
42 with rheumatoid arthritis (RA) and without RA (non-RA). Data represented as mean
43 (25, 75 percentile) for ordinal data and percentage for categorical data. P values are
44 calculated using Mann-Whitney test for ordinal data and Fisher's test for categorical
45 data and significant differences ($p<0.05$) indicated with an asterisk (*).
46 Abbreviations: BMI, body mass index; PPD probing pocket depth; BoP, bleeding on
47 probing; CAL, clinical attachment loss; ESR, erythrocyte sedimentation rate; VAS,
48 visual analogue scale for patients global assessment of disease activity; DAS,
49 disease activity score

	RA n=22	Non-RA n=19
Age in years, mean (IQR)	60 (54.1, 63.4)*	36 (32.9, 41.6)
Gender (% Male)	23	32
Ethnicity (%)		
White	95	89

Asian	5	11
Smoking history (%)		
Never	62	90
Former	29	5
Current	9	5
Alcohol consumption (%)		
Never	11	14
1-4 times/month	73	45
1-4 times/week	16	41
Clinical periodontal characteristics		
PPD in mm, mean (IQR)	2.3 (2.2, 2.4)*	1.6 (1.5, 1.7)
Number of sites with PPD>4mm	1.2 (0, 2)	0.9 (0,3)
Number of sites with BoP, mean (IQR)	6 (0, 19)	4 (1, 16)
Gingival recession in mm, mean (IQR)	0.28 (0.01, 0.26)*	0.13 (0.04, 0.2)
Measures of RA severity		
ESR	8 (8.7, 21.7)	
VAS (global assessment of disease activity)	41 (31.7, 58.5)	
DAS28	3.4 (2.7, 3.9)	

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Since patients with RA differed from controls in both community membership and structure, we identified species level operational taxonomic units (s-OTUs) that contributed to this difference using an increasingly granular top-down approach. Patients with RA presented had greater abundances of obligate anaerobes (both gram-positive and gram-negative) while facultatives (especially gram-negative) were identified in greater abundance in non-RA controls ($p < 0.05$ Wilcoxon signed rank test, Figure 1).

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We then used DESeq2⁹ to identify differentially abundant OTUs; with p-values < 0.05 after adjusting for multiple testing, and Fisher's exact test to examine the frequency of detection. We identified 558 OTUs from 3,963,291 classifiable sequences (mean of 107115 sequences per sample, range 69626-182993). Rarefaction curves demonstrated that all samples approached saturation or had plateaued. 229 OTUs (41.9% of the community) differed significantly in structure and 105 OTUs (19%) differed significantly in membership between groups (Figure 1 and supplementary table 1). Certain species were significantly more abundant in patients with RA, including those belonging to the genera *Actinomyces* (odds ratios (OR) varying from 4-9 for each species within the genus), *Cryptobacterium* (OR=36), *Dialister*, *Desulfovibrio* (ORs of 4 and 26), *Fretibacterium* (OR 9 to 12), *Leptotrichia* (OR 7 to 26) *Prevotella* (OR 0.04 to 6), *Selenomonas* (OR 0 to 7), *Treponema* (OR 0 to 7), and *Veillonellaceae [G1]* (OR 0 to 6).

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In contrast, several species belonging to the genera *Aggregatibacter*, *Gemella*, *Granulicatella*, *Hemophilus*, *Neisseria* and *Streptococci* not only demonstrated lower abundances but also were less frequently detected in RA. These significantly abundant species accounted for a median of 28% (range 12-82%) of each individual's microbiome in patients with RA, indicating that these differences are not attributable to the rare biosphere.

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Since the subgingival microbiome is known to be significantly heterogeneous among individuals¹⁰, we used the core microbiome (suite of species identified in $\geq 80\%$ of subjects) to compare stable associations between groups. 326 OTUs were identified in the core microbiome of all study participants and 364 in patients with RA. 27.7% of the community (101 OTUs) differed significantly in structure and 10.9% (40 OTUs) in membership, with 38 species unique to the RA core microbiome (Figure 1). Importantly, 157 of the 229 species identified above belonged to the core microbiome.

1
2 Sparse, congeneric networks were observed in non-RA controls (Figure 2). On the
3 other hand, the network topology of individuals with RA revealed a highly connected
4 grid with a robust intergeneric hub. 83 of the 157 core species were incorporated in
5 this hub, further reinforcing our observation that in subjects with RA, the environment
6 imposes a selection drive. Importantly, known pathogenic species belonging to
7 *Treponema*, *Selenomonas*, *Filifactor*, *Campylobacter* and *Fretibacterium* were tightly
8 interwoven into this hub, and 12 gram-negative species were identified as network
9 anchors. Interestingly, species traditionally associated with RA, for example,
10 *P.gingivalis* (*Pg*) and *A.actinomycetemcomitans* (*Aa*), were not part of the network
11 cluster.

12
13 Since there is little literature-based information to provide insights into the biological
14 basis for this tight clustering, we combined predictive metagenomic analysis
15 (PICRUST¹¹) with network graph theory and core microbiome analysis to explore if
16 shared functionality could explain co-occurrence (Figure 2). Bacterial arachidonic
17 acid and ether lipid metabolism genes exhibited the greatest betweenness centrality
18 (reflecting the amount of control that these node exerts over the interactions of other
19 nodes in the network¹²), and the highest degree centrality (an indication that they are
20 the central focal point of the structure).

21 22 **DISCUSSION**

23 Gram-negative anaerobes are known to play important roles in initiating periodontitis,
24 and emerging evidence also implicates them in the etiopathogenesis of RA^{6,13}. Our
25 results show that even in periodontally healthy RA patients, gram-negative
26 anaerobes are significantly more abundant in RA, consistent with a dysbiotic state.
27 Such a status might indicate a pre-clinical phase of periodontitis. As expected from a
28 periodontally healthy adult cohort, *Pg* and *Aa* were neither dominant members of the
29 microbiome nor significantly different between groups. Taken together with previous
30 studies¹³, our data implies that gram-negative bacteria other than *Pg* and *Aa* may
31 play a role in initiation of RA, while the evidence from literature suggests that these
32 two species may be critical to disease perpetuation.

33
34 Recent investigations demonstrate that while substantial microbial heterogeneity
35 exists among healthy individuals, a robust core microbiome is identifiable in
36 individuals who smoke or are pregnant¹⁴⁻¹⁶. The findings of the present study parallel
37 these previous observations and support the ecological plaque hypothesis¹⁷,
38 suggesting that RA imposes a habitat filtering on the subgingival environment,
39 preferentially promoting the growth of certain organisms.

40
41 Traditional statistical methods assume bacterial presence and abundance to be
42 independent variables, but in reality bacterial presence in a biofilm is driven by inter-
43 dependent nutritional and metabolic interactions. Therefore, we combined network
44 graph theory with DESeq and core microbiome analysis to examine co-occurrence
45 patterns and identify important community members (network anchors). No network
46 anchors were identifiable in controls (since betweenness centrality was
47 homogeneous between species), indicating that this is an ecological niche in
48 equilibrium. However, the tightly woven hub of anaerobes suggest that a small group
49 of anaerobic bacteria play an important role controlling the flow of resources in the
50 RA-influenced microbiome, implying that even small changes in these anchors could
51 impact upon community assembly in people with RA. These species may be
52 potential targets for microbial disruption.

53
54 Arachidonic acid (AA) is essential for cell membrane integrity. It is metabolized to
55 prostaglandin E2 (PGE₂) and other pro-inflammatory eicosanoids, which are

1 implicated in the development of RA. The ability to metabolize AA into pro-
2 inflammatory eicosanoids is an emergent property of opportunistic pathogens¹⁸. AA
3 is also known to inhibit the growth and epithelial adhesion of beneficial species in the
4 gut¹⁹. Taken together, the data indicate that the subgingival microbiome is both
5 influenced by, and influences, the inflammatory burden of RA.

6
7 One of the most intriguing findings was the identification of *Cryptobacterium curtum*
8 as a predominant member of the RA-influenced periodontal microbiome. This gram-
9 positive, assacharolytic, anaerobic rod (which was previously misclassified as
10 *Eubacterium saburreum*) degrades arginine through the arginine deiminase pathway
11 and produces substantial amounts of citrulline, ornithine and ammonia²⁰. We have
12 previously identified this as a periodontal pathogen²¹, and translocation from oral
13 sources has been implicated in the etiology of distant infections such as pelvic
14 abscesses, gynecologic infections, and wounds²². More importantly, *C. curtum* is
15 enriched in the oral and gut microbiomes of early RA cases^{6,23}. In line with previous
16 studies, we observed that this species was a member of the core microbiome in RA
17 patients. Compared to non-RA controls, this species demonstrated a 100-fold greater
18 abundance in RA with 39-fold greater odds of detection. While this unusually high
19 association does not necessarily suggest an etiopathogenic role for *C.curtum*, this
20 organism is certainly a candidate for further studies. In light of evidence that
21 antibodies against citrullinated protein and peptides (ACPA) precede the clinical
22 onset of RA by several years, have high specificity for RA at over 95%^{24,25} and that
23 we previously observed antibodies characteristic of RA, including citrullinated and
24 uncitrullinated peptides of the RA autoantigens in individuals with periodontitis³, the
25 ability of *C.curtum* to degrade arginine via the arginine deiminase pathway and to
26 produce substantial amounts of citrulline is of particular interest. Presence of
27 *C.curtum* in the plaque may therefore be a contributing factor in the development of
28 RA autoantigens and warrants further investigation.

29
30 In summary, our data suggest that RA plays a major role in shaping the oral
31 microbiome. The microbiome in RA is enriched for pro-inflammatory organisms and
32 those capable of producing substantial amounts of citrulline (pro-antigenic). An ability
33 to metabolize arachidonic acid and ether lipids appears to be a shared function
34 among the species observed in individuals with RA. Our findings lend further
35 credence to a link between the oral microbiome and RA; however, longitudinal
36 studies are needed to understand directionality and causality, and also to
37 characterize potentially "driver species" that could serve as biomarkers for RA.
38

1 TABLES AND FIGURES

2
3 Table 1: Clinical and demographic characteristics in periodontally healthy subjects
4 with rheumatoid arthritis (RA) and without RA (non-RA). Data represented as mean
5 (25, 75 percentile) for ordinal data and percentage for categorical data. P values are
6 calculated using Mann-Whitney test for ordinal data and Fisher's test for categorical
7 data. Abbreviations: BMI, body mass index; PPD probing pocket depth; BoP,
8 bleeding on probing; CAL, clinical attachment loss; ESR, erythrocyte sedimentation
9 rate; VAS, visual analogue scale for patients global assessment of disease activity;
10 DAS, disease activity score.

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13 Figure 1: Differences in alpha and beta diversity metrics between periodontally
14 healthy subjects with and without rheumatoid arthritis (RA). (A): Principal
15 Coordinates Analysis (PCoA) plots of unweighted and weighted Unifrac distances
16 (B): Kernel plots of alpha diversity (Abundance-based Coverage Estimator (ACE)).
17 The peak indicates the median values for each group. The x-axis indicates the data
18 range. (C): Distribution of species by gram staining and oxygen requirement
19 characteristics. Groups that share the same symbol are significantly different from
20 each other ($p < 0.05$, Kruskal Wallis test) (D): Phylogenetic tree representing
21 normalized mean relative abundance (NMRA, stacked bar chart), core species
22 (circles represent species present in $\geq 80\%$ of samples in a group), significant
23 frequency of detection (stars) and phylum-level taxonomic annotation (colored-strips
24 and text) for significantly different and differentially abundant species-level OTUs
25 (tree leaves). Data for figure 1D is presented in supplemental table 1.

26
27 Figure 2: Co-occurrence networks in periodontally healthy subjects with or without
28 rheumatoid arthritis (RA): Each network graph contains nodes (circles) and edges
29 (connections representing Spearman's ρ). Edges are colored green for positive
30 correlation and red for negative correlation. Nodes represent species-level OTUs in
31 2A and 2B and genes encoding for metabolic functions in 2C; and are sized by
32 relative abundance. Edges represent significant and robust Spearman's correlation
33 ($p < 0.05$, $\rho \geq 0.75$). Data for figure 2C is presented in supplemental table 2.

34
35 Supplemental Table 1: Species level OTU matrix highlighting results of abundance
36 analysis (mean \pm standard deviation), differential abundance (DESeq2), differential
37 detection frequency, and presence in core (observed in $\geq 80\%$ of samples in a group)
38 for periodontally healthy subjects with and without rheumatoid arthritis (RA).

39
40 Supplemental Table 2: Correlation matrix of significant ($p < 0.05$) Spearman's
41 correlation among metabolism related KEGG level 3 gene functions of periodontally
42 healthy subjects with rheumatoid arthritis (RA).

43
44 Supplemental File 1: Methods

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