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## **Apical periodontitis microbiome association with salivary and serum inflammatory burden**

### **Sampling Protocol**

#### ***Saliva sample Collection***

Unstimulated pre-operative saliva samples were collected from all three groups in a polystyrene 30 ml universal tube (Fisher Scientific, Waltham, MA, USA) for 10 min. The collected saliva was transported on ice and stored in a -80 °C freezer (Sanyo Electric Co, Japan).

#### ***Blood sample collection***

Blood samples from the control group were collected using BD Vacutainer SST II Advance 8.5 ml tube (Becton Dickinson, NJ, USA). In contrast, for both treatment groups, pre- and post-operative blood samples were collected using a 4 ml Vacuette K3 EDTA blood tube (Greiner Bio-One, Austria) and BD Vacutainer SST II Advance 8.5 ml tube (Becton Dickinson). For bacterial culture, blood was also withdrawn into BD Bactec ANO2 10 ml bottle (Becton Dickinson).

Whole blood collected in the 4 ml Vacuette K3 EDTA tube (Greiner Bio-One) was then separated into aliquots of 0.5 ml in a 1.5 ml microcentrifuge tube (Fisher Scientific) then stored in a -80 °C freezer. Blood samples collected in the BD Vacutainer SST II Advance 8.5 ml tube (Becton Dickinson) from all three groups were centrifuged for 10 min at a speed of 2000 x g and a temperature of 4 °C using Eppendorf centrifuge 5810R (Eppendorf, Hamburg, Germany) to separate the serum from the blood. The separated serum was then stored in a -80 °C freezer in an aliquot of 0.5 ml in a 1.5 ml microcentrifuge tube (Fisher Scientific) for further processing.

#### ***Root canal sample collection***

A single, trained operator collected all the samples under aseptic conditions. Local anaesthesia was administered, and each target tooth was cleaned with pumice. This was followed by rubber dam isolation (UnoDent, UK) with an appropriate clamp and sealed with a light-cured gingival barrier, OpalDam Green™ (Ultradent Products, Inc., South Jordan, UT, USA). The tooth surface was disinfected using a small cotton pellet immersed in 2.5% sodium hypochlorite (NaOCl) (Milton Laboratories, Rivadis, Louzy, France), followed by 5% sodium thiosulfate swabbed for at least 30 s. A contamination control sample was taken using sterile swab rubbed on the tooth surface for 30 s (These were checked later by culture and DNA extraction. All these control samples showed negative results).

A sterile bur cooled with saline delivered with a separate syringe was used to remove all restorations and caries. The rubber dam and tooth were again disinfected with 2.5% NaOCl (Milton Laboratories). The tooth was then built up using composite restoration (SDR flow<sup>+</sup>; Dentsply Sirona, Baillagues,

Switzerland) to avoid leakage. Access was gained with a new sterile bur and saline (Sterets Normasol, Oldham, UK) exposing the pulp chamber. Single-use sterile Gates Glidden burs, Hedstrom files (H-files), Flexofiles (Dentsply Sirona), and rotary files were used to remove existing root canal filling with the use of sterile saline (Sterets Normasol). Instruments were separated from their heads using a sterile bur and were placed into a 7 ml bijoux tube (Greiner Bio-One Ltd) along with the root canal filling material; the bijoux tube also contained 1 ml of fastidious anaerobic broth (Lab M). Canals were filled with saline (Sterets Normasol), and paper points (Dentsply Sirona) samples were collected. Samples were transported in ice and stored in a -80 °C freezer for further processing.

### **Phyla identified in different sample sources**

Using the HOMD database, 201 different genera belonging to 12 distinct phyla including *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Fusobacteria*, *Saccharibacteria (TM7)*, *Spirochaetes*, *Synergistetes*, *Abconditabacteria (SR1)*, *Gracilibacteria (GN02)*, *Chloroflexi* and *Cyanobacteria* were identified from all three sample sources. *Actinobacteria* were significantly higher in abundance than in blood samples than in saliva samples and intracanal samples ( $p < 0.0001$ ,  $p < 0.0001$ , respectively), while a significant difference was also present between saliva samples and intracanal samples ( $p < 0.0001$ ). In contrast, *Bacteroidetes* were significantly higher in saliva samples and intracanal samples than in blood samples ( $p < 0.0001$ ). A significant difference was also found in *Firmicutes* between saliva samples and blood and intracanal samples ( $p < 0.0001$ ,  $p = 0.03$ , respectively). *Firmicutes* were also significantly higher in intracanal samples than in blood samples ( $p = 0.01$ ). Abundance of the *Fusobacteria* phylum was found at significantly higher levels in saliva samples than in blood and intracanal samples ( $p = 0.007$ ,  $p < 0.0001$ , respectively), whilst *Proteobacteria* were significantly higher in blood than in intracanal and saliva samples ( $p = 0.002$ ,  $p < 0.0001$ ) and higher in intracanal than in saliva samples ( $p = 0.0006$ ). *Saccharibacteria (TM7)* was significantly higher in blood than in intracanal samples and significantly higher in saliva samples than in intracanal samples ( $p = 0.0004$  and  $p < 0.0001$ , respectively). Moreover, *Spirochaetes* was significantly lower in saliva samples than in blood and intracanal samples ( $p = 0.006$  and  $p = 0.0004$ ), while *Synergistetes* was significantly higher in intracanal samples than in saliva samples ( $p < 0.0001$ ).

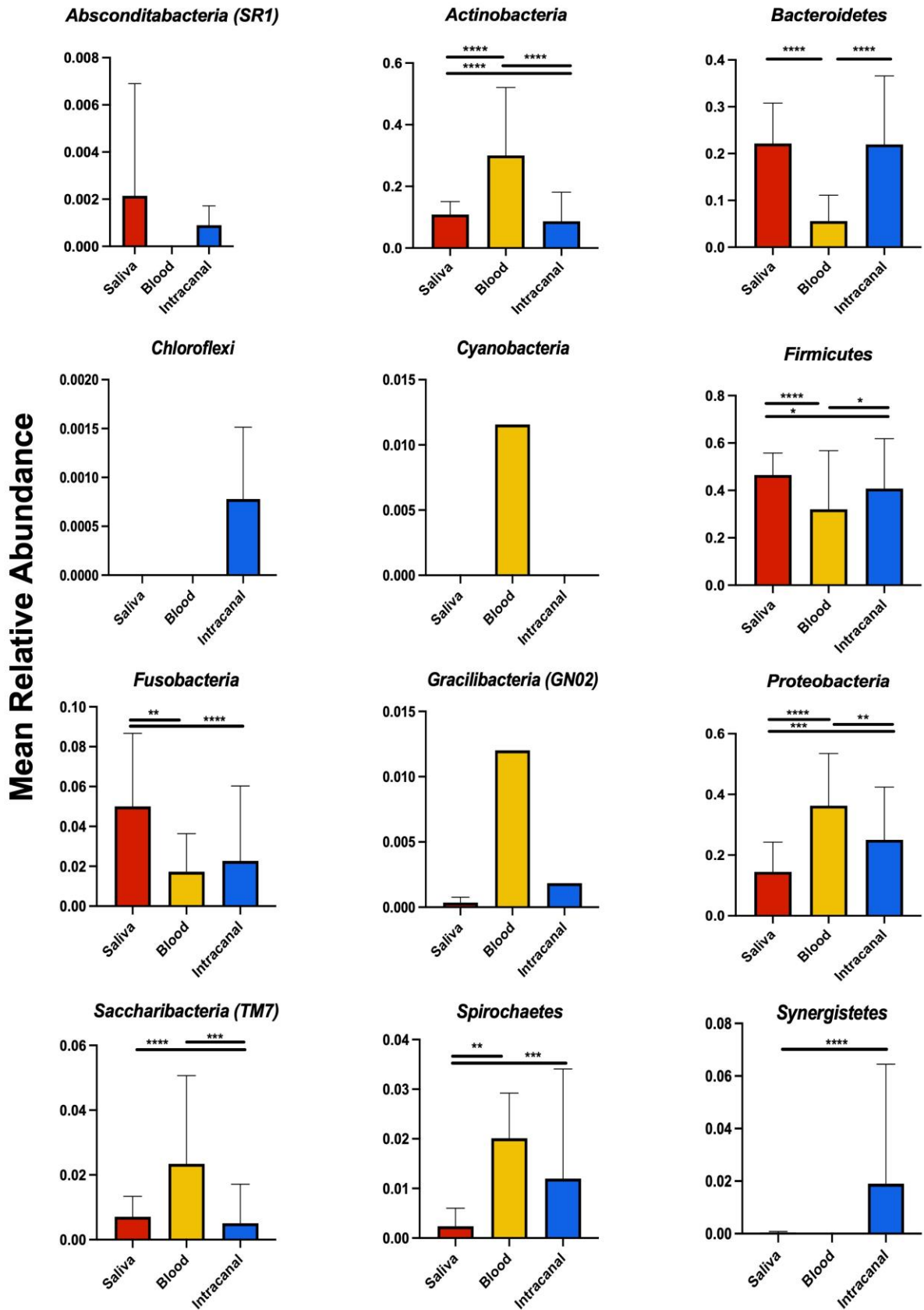
## **Microbiome analysis**

All reads passing the standard Illumina filter procedure (chastity filter) were demultiplexed and corresponded with the sequence of their sample. The region-specific forward and reverse primer sequence were identified and clipped from the start of raw forward and reverse reads. Unmatched read pairs were removed to maintain high-quality ones. The forward and reverse clipped FASTQ reads were provided by Eurofins for each sample. The FASTQ files were processed using *DADA2* (version 1.2) in R (version 4.1). After importing the FASTQ files, the quality profile of each forward and reverse was obtained by plotting the average quality score. The low-quality sequences were filtered, trimmed, and truncated. Truncation was set to 200bp for the forward files and 175bp for the reverse files. Error frequency was checked. Identical sequence reads were dereplicated into a single unique sequence with a corresponding abundance. Forward and reverse reads were merged to generate amplicon sequence variants (ASVs). Chimeric reads were identified and removed. The counts were normalised using the median of ratios method in *DeSeq2* (version 1.32.0) package in R. The information on the FASTQ Read statistics is provided in supplementary Table 1.

## **Microbiome profiling**

The merged and clipped pair reads were used as input for microbiome profiling. Each sequence variant was classified by comparison with a specific database using the Naïve Bayesian Classifier Method. The Human Oral Microbiome Database (HOMD) (version 15.1) was used for assigning taxonomy after generating the *phyloseq* object using the *phyloseq* package (version 1.36.0) and plots were generated using *ggplot2* (version 3.35) for the generated ASVs.

## Comparison between different phyla in sample sources



Supplementary figure 1: Comparison between different phyla in sample sources

**Supplementary Table 1:** FASTQ processing results.

	<b>Read Pairs</b>	<b>Filtered</b>	<b>Denoised Forward</b>	<b>Denoised Reverse</b>	<b>Merged</b>	<b>Nonchimera</b>	<b>Percentage kept</b>
1	39054	33182	33015	32991	32502	32195	82.43713832
2	39992	34371	34135	34218	33519	33467	83.68423685
3	29778	25030	24773	24709	23954	23909	80.29081873
4	18347	15661	15418	15491	14984	14982	81.65912683
5	44231	37499	37330	37319	36483	36477	82.46930886
6	37737	31488	31297	31270	30557	30451	80.69268887
7	33918	28753	28497	28528	27223	27189	80.16097647
8	32963	28022	27839	27863	27380	27213	82.55619938
9	33970	28201	28122	28133	27925	27842	81.96055343
10	24099	20585	20347	20384	19599	19567	81.19424042
11	41614	35574	35236	35225	34191	34057	81.84024607
12	21901	18441	18203	18194	17852	17791	81.23373362
13	44242	37418	37238	37223	36462	36171	81.75715384
14	29759	25285	25066	25027	24346	24341	81.79374307
15	37289	31898	31664	31761	30996	30886	82.82871624
16	37398	32090	31607	31735	30068	30036	80.31445532
17	38662	32856	32624	32689	31854	31842	82.35993999
18	34877	28327	28215	27632	27462	27385	78.51879462
19	40692	34796	34471	34700	34023	33995	83.5422196
20	32495	27291	27092	27102	26361	26345	81.07401139
21	33588	28541	28380	28446	28111	28024	83.43455996
22	33067	27915	27817	27770	27358	27253	82.41751595
23	32744	27897	27760	27820	27552	27412	83.71610066
24	39030	33164	32982	32941	32295	32043	82.09838586
25	34969	29923	29687	29737	29099	28947	82.77903286
26	35310	29960	29751	29757	28861	28851	81.70773152
27	25537	21511	21328	21388	20741	20607	80.69467831
28	40509	33894	33762	33754	33285	33266	82.12002271
29	28872	24743	24527	24564	23878	23873	82.68564699
30	43784	37253	37112	37172	36424	36403	83.14224374
31	41618	36114	35758	35880	35054	35033	84.17751934
32	28964	24284	24046	24052	23393	23387	80.74506284
33	38743	33595	33325	33474	32922	32876	84.85661926
34	41421	35156	34927	35013	34008	33724	81.4176384
35	31138	26483	26257	26291	25717	25641	82.34632924
36	39096	33021	32819	32816	32342	31909	81.61704522
37	39665	33145	32939	32962	32274	32251	81.30845834
38	31389	26661	26526	26500	26109	26099	83.14696231
39	40790	34666	34415	34467	33465	33437	81.97352292

40	39228	32990	32629	32737	31385	31229	79.60895279
41	12118	10348	10248	10243	9883	9839	81.19326622
42	20885	17795	17656	17632	17201	17184	82.27914771
43	40240	34165	33850	33903	33145	33064	82.16699801
44	42461	36003	35759	35821	34956	34613	81.51715692
45	48919	41794	41561	41558	40704	40532	82.85533228
46	34908	29277	28903	29025	28399	28389	81.32519766
47	41832	35702	35275	35440	34037	33852	80.92369478
48	32797	27832	27617	27671	27167	27113	82.66914657
49	33215	28411	28125	28202	27608	27558	82.96853831
50	26669	22614	22395	22397	22164	22157	83.08148037
51	26166	22336	22139	22201	21619	21611	82.59191317
52	43725	36948	36647	36727	36093	36053	82.4539737
53	36752	30929	30779	30701	30284	30281	82.39279495
54	32465	26994	26714	26726	26084	26054	80.2525797
55	40444	34359	34209	34153	33419	33408	82.60310553
56	36414	30585	30343	30427	29779	29637	81.3890262
57	33034	28083	27921	27902	27278	27256	82.50893019
58	35140	30169	29968	29955	29468	28865	82.14285714
59	44022	37057	36854	36874	36235	36172	82.16800691
60	37032	30662	30345	30338	29046	29007	78.32955282
61	42844	36051	35834	35886	35232	35129	81.99281113
62	36283	30802	30649	30624	29589	29378	80.96904887
63	41616	34987	34782	34775	34163	33936	81.5455594
64	37754	32150	31954	31920	31034	30993	82.09196377
65	42482	36057	35804	35914	35188	35118	82.66559955
66	42122	35598	35519	35573	34811	34684	82.34176915
67	45777	38872	38741	38762	38028	37332	81.55187103
68	53396	45038	44873	44889	44337	44170	82.72155218
69	47456	40449	40258	40374	39610	39284	82.77983817
70	46322	39051	38932	38805	37906	37791	81.58326497
71	50374	42968	42907	42918	42495	41901	83.17981498
72	41870	35636	35550	35566	35235	34935	83.43682828
73	42610	35940	35795	35798	34605	33775	79.26543065
74	39117	33401	33177	33317	30735	30696	78.47227548
75	44374	38220	38132	38107	37749	37663	84.8762789
76	42894	34303	34244	34272	34215	34185	79.69646104
77	51738	44421	44200	44311	43730	43140	83.38165372
78	51485	43338	43263	43192	42785	42665	82.86879674
79	51980	43342	43236	43149	42119	41682	80.18853405
80	47796	40389	40226	40336	39887	38114	79.74307473
81	47302	39766	39595	39682	39240	38889	82.21428269

82	57160	47844	47662	47660	46647	46525	81.3943317
83	43521	36960	36876	36764	35972	35908	82.50729533
84	52701	44508	44358	44339	43786	43249	82.06485645
85	46419	37287	37213	37228	36152	36104	77.77849587
86	47755	39811	39624	39609	39209	38982	81.62914878
87	44435	37609	37476	37481	36939	36377	81.86564645
88	53609	46029	45851	45967	45406	40348	75.26348188
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90	42234	35469	35448	35445	34997	32164	76.15665104
91	47314	40621	40604	40571	40401	40027	84.59863888
92	34809	29937	29917	29920	29777	28278	81.23761096
93	53005	44933	44695	44740	43989	42310	79.82265824
94	49998	43293	43279	43272	43067	43067	86.1374455
95	48460	40998	40837	40965	40714	40543	83.66281469
96	42356	35641	35530	35538	35016	34899	82.39446596
97	41337	35393	35313	35291	34853	34797	84.17882285
98	45389	38043	38002	37964	37743	37737	83.14128974
99	45778	38557	38371	38265	37801	37414	81.72921491
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110	45861	39073	38664	38760	36940	36594	79.79328841
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112	30839	26396	26155	26240	25488	24490	79.41243231
113	35780	30437	30197	30106	29001	28702	80.21799888
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121	44661	38572	38239	38327	37387	37209	83.31430107
122	52056	43961	43611	43713	42016	41308	79.35300446
123	60761	52135	51588	51810	49641	49268	81.08490644



124	37465	32172	31780	31828	30619	29160	77.8326438
125	41722	35805	35544	35580	34136	32383	77.61612578
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151	42970	36533	36175	36258	34885	34495	80.2769374
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