

A randomised, double-blind clinical study into the effect of zinc citrate trihydrate toothpaste on oral plaque microbiome ecology and function

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Supplementary Material

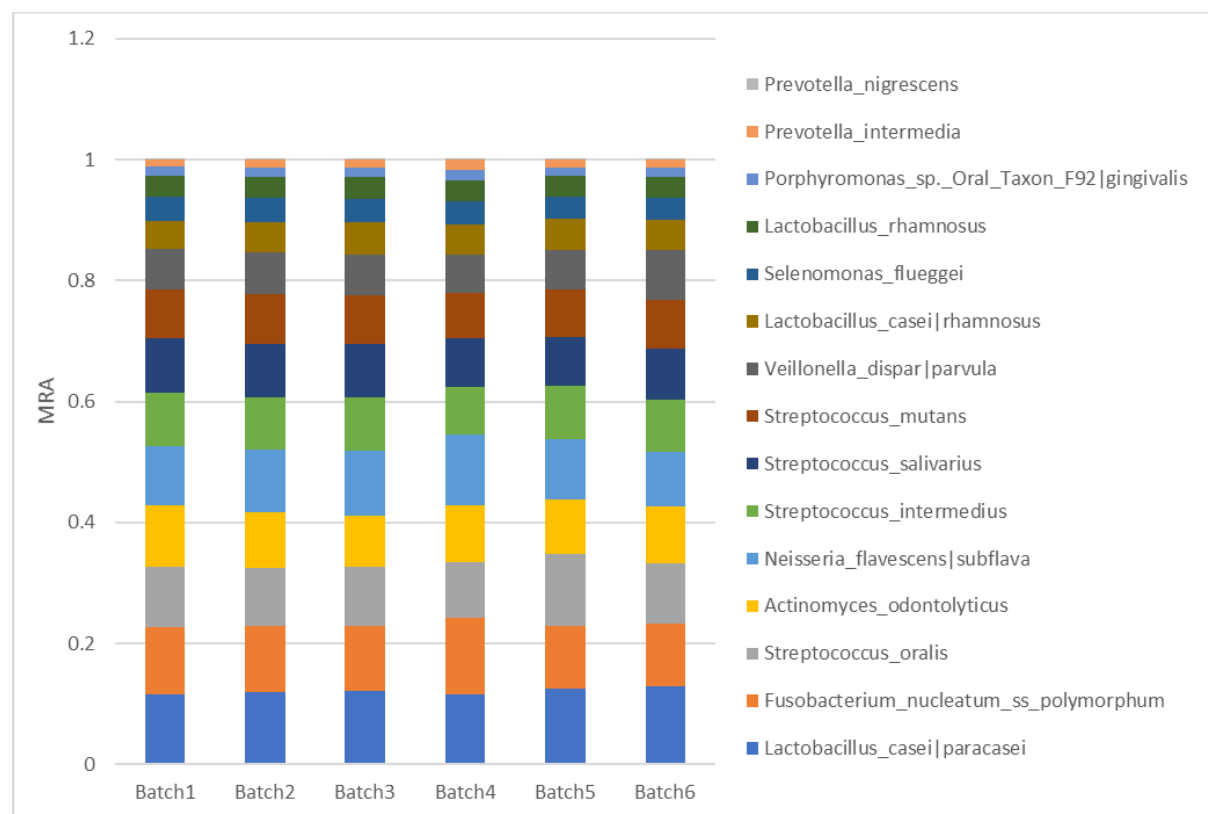


Figure S1 - Batch to batch comparison of the positive control mock communities, based on the mean relative abundance (MRA) for the 15 species present. The mock community was present in all the 6 sequencing runs, with excellent consistency between batches.

Table S1 - Changes in mean relative abundance observed for the significantly different taxa following use of the zinc toothpaste at 2 and 6 weeks compared to baseline (p<0.05 and q<0.05).

Taxa	Change in mean relative abundance baseline - 2 weeks (%)	Mean relative abundance at baseline (%)	Mean relative abundance after 2 weeks (%)	p values	q values	Change in mean relative abundance baseline - 6 weeks (%)	Mean relative abundance after 6 weeks (%)	p values	q values
<i>Veillonella dispar</i> <i>parvula</i> sp._Oral_Taxon_E53 sp._str._3144 sp._str._6127_OTU_73	3.830	5.057	8.887	0.000294	0.00692	4.104	9.160	0.000246	0.003459
<i>Actinomyces</i> _sp._oral_taxon_448_OTU_29	1.185	0.775	1.959	0.018086	0.137686	1.789	2.564	8.35E-05	0.001485
<i>Veillonella parvula</i> _OTU_94	1.424	1.100	2.524	3.43E-05	0.001159	1.190	2.290	0.000307	0.004152
<i>Campylobacter gracilis</i> sp._Oral_Taxon_E67_OTU_39	0.665	0.879	1.545	0.000511	0.009527	0.764	1.643	0.001327	0.015057
<i>Streptococcus tigurinus</i> _OTU_315	0.385	0.264	0.649	0.025578	0.17725	0.632	0.896	0.000311	0.004152
<i>Prevotella histicola</i> _OTU_314	0.119	0.070	0.188	0.209696	0.563032	0.591	0.661	0.00163	0.017383
<i>Campylobacter gracilis</i> _OTU_132	0.257	0.099	0.356	0.00265	0.03581	0.407	0.506	0.000157	0.002322
<i>Streptococcus anginosus</i> _OTU_229	0.487	0.064	0.550	5.57E-07	3.01E-05	0.382	0.445	2.91E-08	1.03E-06
<i>Prevotella</i> _sp._oral_taxon_313_OTU_309	0.201	0.010	0.211	0.000738	0.012473	-0.001	0.009	0.955403	0.923424
<i>Streptococcus salivarius</i> <i>vestibularis</i> _OTU_111	0.197	0.016	0.214	0.000398	0.007676	0.085	0.101	0.034368	0.172422
<i>Megasphaera micronuciformis</i> _OTU_100	0.103	0.036	0.139	0.001217	0.018796	0.158	0.194	0.00015	0.002289
<i>Atopobium parvulum</i> _OTU_353	0.049	0.015	0.064	8.71E-05	0.00277	0.100	0.115	1.87E-07	6.24E-06
<i>Selenomonas</i> _sp._oral_taxon_134_OTU_78	0.045	0.037	0.082	0.070068	0.313341	0.089	0.125	0.001385	0.015384
<i>Lachnospiraceae</i> _ [G-2]_sp._oral_taxon_096_OTU_227	0.007	0.011	0.019	0.613616	0.830885	0.079	0.090	0.003203	0.031053
<i>Parvimonas</i> _sp._oral_taxon_110_OTU_287	0.080	0.011	0.090	0.003986	0.046839	0.023	0.033	0.084822	0.301722
<i>Fusobacterium naviforme</i> <i>nucleatum</i> _ss_vincentii_OTU_193	0.013	0.001	0.014	1.07E-07	6.4E-06	0.020	0.021	4.12E-13	3.66E-11
<i>Actinomyces</i> _sp._oral_taxon_181_OTU_512	0.007	0.002	0.009	0.00362	0.044467	0.017	0.019	5.23E-07	1.39E-05
<i>Veillonella dispar</i> <i>parvula</i> sp._str._3144 sp._str._6127_OTU_245	0.015	0.019	0.034	0.000331	0.007167	0.016	0.036	0.000196	0.002832
<i>Streptococcus</i> _sp._Oral_Taxon_71_OTU_178	0.010	0.002	0.012	0.007353	0.076435	0.014	0.016	0.001509	0.01642
<i>Haemophilus haemolyticus</i> _OTU_269	-0.024	0.028	0.005	0.000549	0.009886	-0.024	0.004	0.003947	0.037585
<i>Haemophilus haemolyticus</i> sp._oral_taxon_036_OTU_488	-0.037	0.042	0.005	1.22E-05	0.000471	-0.036	0.006	0.005075	0.047482
TM7_[G-2]_sp._oral_taxon_350_OTU_368	-0.037	0.044	0.007	0.002811	0.037061	-0.041	0.003	2.20E-06	5.34E-05
<i>Porphyromonas</i> _sp._oral_taxon_275_OTU_396	-0.052	0.062	0.010	0.00012	0.003603	-0.056	0.006	1.93E-05	0.00043
<i>Alloprevotella</i> _sp._oral_taxon_473_OTU_240	-0.073	0.089	0.017	0.008521	0.085292	-0.083	0.006	7.82E-07	1.99E-05
<i>Aggregatibacter segnis</i> sp._oral_taxon_513_OTU_165	-0.136	0.177	0.041	0.000709	0.012369	-0.100	0.078	0.201136	0.490373
<i>Porphyromonas pasteri</i> _OTU_180	-0.186	0.384	0.199	0.129418	0.45721	-0.242	0.142	0.005261	0.048099
<i>Staphylococcus epidermidis</i> _OTU_404	-0.370	0.374	0.004	0	0	-0.367	0.007	1.05E-10	6.24E-09
<i>Streptococcus dentisani</i> <i>mitis</i> _OTU_143	-0.585	1.096	0.511	0.000284	0.00692	-0.021	1.075	0.944756	0.923424
<i>Neisseria elongata</i> _OTU_72	-1.194	2.006	0.812	0.003506	0.044076	-0.887	1.119	0.105215	0.34686
<i>Fusobacterium nucleatum</i> _ss_polymorphum_OTU_139	-1.074	3.143	2.069	0.013295	0.114067	-1.385	1.757	0.000679	0.008234

Table S2 - Changes in mean relative abundance observed for the significantly different taxa following use of the control toothpaste at 2 and 6 weeks compared to baseline (p<0.05 and q<0.05).

Taxa	Change in mean relative abundance baseline - 2 weeks (%)	Mean relative abundance at baseline (%)	Mean relative abundance after 2 weeks (%)	p values	q values	Change in mean relative abundance baseline - 6 weeks (%)	Mean relative abundance after 6 weeks (%)	p values	q values
<i>Staphylococcus_epidermidis_OTU_404</i>	0.0091	0.001	0.010	8.57E-08	6.18E-06	0.003	0.004	0.007922	0.115601
<i>Treponema_denticola_OTU_453</i>	-0.0251	0.029	0.004	0.001643	0.047391	-0.012	0.017	0.50162	0.907451
<i>Filifactor_alocis_OTU_425</i>	-0.0017	0.007	0.005	0.460093	0.96431	0.031	0.038	0.000911	0.021241
<i>Veillonella_sp._oral_taxon_780_OTU_291</i>	0.0005	0.006	0.006	0.940854	1	-0.005	0.001	0.000123	0.003398
<i>Granulicatella_elegans_OTU_266</i>	0.0236	0.032	0.055	0.483451	0.965249	-0.026	0.005	4.86E-05	0.001562
<i>Haemophilus_haemolyticus_OTU_269</i>	-0.0225	0.033	0.011	0.045405	0.363872	-0.027	0.006	0.000917	0.021241
<i>Haemophilus_parahaemolyticus_OTU_440</i>	-0.0141	0.035	0.020	0.504652	0.965252	-0.032	0.003	2.22E-07	9.16E-06
<i>Aggregatibacter_aphrophilus_OTU_350</i>	-0.0353	0.048	0.000	0.028673	0.298221	-0.046	0.002	6.16E-10	5.94E-08

Table S3 - Bacterial pathways with significant differences between the zinc and control toothpaste compared at baseline, 2 and 6 weeks using LefSe, the values are the LDA values from the analysis, green are associated with the zinc toothpaste and red with the control

Pathway	Zinc			Control		
	Baseline	2wks	6wks	Baseline	2wks	6wks
Arginineandprolinemetabolism_ko00330			2.376667			
Biotinmetabolism_ko00780		2.15328	2.203195			
Carbonfixationpathwaysinprokaryotes_ko00720		2.265513	2.381425			
Citratecycle_TCAcycle_ko00020			2.177695			
Cysteineandmethioninemetabolism_ko00270			2.393635			
Folatebiosynthesis_ko00790			2.012742			
Glyoxylateanddicarboxylatemetabolism_ko00630			2.38831			
Nitrogenmetabolism_ko00910			2.750432			
Nitrotoluenedegradation_ko00633			2.205684			
Pertussis_ko05133	2.562744		2.684277			
Phenylalanine_tyrosineandtryptophanbiosynthesis_ko00400			2.374109			
Porphyrinandchlorophyllmetabolism_ko00860		2.809919	2.925532			
Pyruvatemetabolism_ko00620			2.171136			
Ubiquinoneandotherterpenoid_quinonebiosynthesis_ko00130		2.195491	2.25654			
Valine_leucineandisoleucinebiosynthesis_ko00290			2.107305			
Aminosugarandnucleotidesugarmetabolism_ko00520						2.60904
D_Alanimetabolism_ko00473						2.154925
Fructoseandmannosemetabolism_ko00051						2.583552
Galactosemetabolism_ko00052						2.625874
Glycerophospholipidmetabolism_ko00564						2.272171
Glycolysis_Gluconeogenesis_ko00010					2.486691	2.570259
Insulinsignalingpathway_ko04910						2.001751
Nucleotideexcisionrepair_ko03420				2.093471		
Pentoseandglucuronateinterconversions_ko00040					2.340842	2.383589
Phosphotransferasesystem_PTS_ko02060						2.807879
Sphingolipidmetabolism_ko00600						2.055858
Starchandsucrosemetabolism_ko00500						2.860972

Table S4 - Bacterial pathways with significant differences between timepoints Base-2wks and Base-6wks for the zinc and control toothpaste using LefSe, the values are the LDA values from the analysis coloured by product (orange and green for zinc toothpaste, yellow and blue for control toothpaste) and timepoint (orange and yellow for baseline, green and blue for 2 or 6 wks).

Pathway	Zinc				Control			
	Base-2wks		Base-6wks		Base-2wks		Base-6wks	
	Base	2wks	Base	6wks	Base	2wks	Base	6wks
ABCtransporters_ko02010				2.97762				
Alanine_aspartateandglutamatemetabolism_ko00250		2.098065		2.220879				
Aminoacyl_tRNAbiosynthesis_ko00970		2.717592		2.755099				2.575509
Aminobenzoatedegradation_ko00627	2.246641		2.347817		2.208723			
Aminosugarandnucleotidesugarmetabolism_ko00520			2.427373					
Arginineandprolinemetabolism_ko00330				2.285717				
Ascorbateandaldaratemetabolism_ko00053			2.04415					
Bacterialchemotaxis_ko02030	2.219643							
Benzoatedegradation_ko00362	2.022122		2.130042					
Biosynthesisof12_14_and16_memberedmacrolides_ko00522			2.090628					
Biosynthesisofansamycins_ko01051			2.183434					
Biotinmetabolism_ko00780		2.146218		2.22397				
Bisphenoldegradation_ko00363			2.06499					
Butanoatemetabolism_ko00650	2.271076		2.322616					
Carbonfixationpathwaysinprokaryotes_ko00720		2.416135		2.456932				
Citratecycle_TCAcycle_ko00020		2.157195		2.232848				
Cysteineandmethioninemetabolism_ko00270		2.558129		2.570685				
Fattyacidbiosynthesis_ko00061					2.246673			
Fattyacidmetabolism_ko00071	2.170191		2.303981				2.157757	
Folatebiosynthesis_ko00790		2.160686		2.220927				
Fructoseandmannosemetabolism_ko00051	2.558557		2.648927					
Galactosemetabolism_ko00052	2.556921		2.627005					
Geranioldegradation_ko00281	2.192017		2.339693		2.257095		2.246686	
Glycerolipidmetabolism_ko00561	2.117732		2.320961					
Glycerophospholipidmetabolism_ko00564	2.4583		2.451224					
Glycolysis_Gluconeogenesis_ko00010	2.485846		2.568226					
Histidinemetabolism_ko00340		2.008519		2.016263				
Homologousrecombination_ko03440		2.082128						
Limoneneandpinenedegradation_ko00903			2.114043		2.07436			
Lipopolysaccharidebiosynthesis_ko00540		2.493845						
Lysinebiosynthesis_ko00300		2.111039		2.162122				
Lysinedegradation_ko00310	2.236201		2.265194		2.147815			
MetabolismofxenobioticsbycytochromeP450_ko00980							2.005211	
Methanemetabolism_ko00680		2.286446						
Nitrogenmetabolism_ko00910		2.796681		2.847563				
Nitrotoluenedegradation_ko00633		2.238315						
Nonribosomalpeptidestructures_ko01054	2.189519		2.309273				2.271498	
Nucleotideexcisionrepair_ko03420		2.276798						
PantothenateandCoAbiosynthesis_ko00770				2.026059				
Pentoseandglucuronateinterconversions_ko00040	2.387231		2.471376					
Phenylalanine_tyrosineandtryptophanbiosynthesis_ko00400		2.596714						
Phosphotransferasesystem_PTS_ko02060			2.809412					
Porphyrinandchlorophyllmetabolism_ko00860		3.152023		3.168803				
Purinemetabolism_ko00230		2.800878		2.826018				2.551414
Pyrimidinemetabolism_ko00240		2.456479		2.512123				2.424709
Pyruvatemetabolism_ko00620				2.07894				
Ribosome_ko03010		2.533469		2.573737				2.378935
RNAdegradation_ko03018				2.148655				
Selenocompoundmetabolism_ko00450		2.227814		2.252188				
Sphingolipidmetabolism_ko00600	2.02905		2.073594					
Starchandsucrosemetabolism_ko00500	2.868128		2.934203					
Steroiddegradation_ko00984			2.062861					
Terpenoidbackbonebiosynthesis_ko00900		2.071899		2.084648				
Thiaminemetabolism_ko00730		2.509824		2.549548				
Two_componentsystem_ko02020	3.025789		2.752958					
Tyrosinemetabolism_ko00350	2.032017		2.086177					
Valine_leucineandisoleucinebiosynthesis_ko00290		2.13824		2.13137				
Valine_leucineandisoleucinedegradation_ko00280			2.152442		2.258636			
Vibriocholeeraepathogeniccycle_ko05111	2.278024		2.165451					

Oral Metataxonomic Bacterial Processing Pipeline (Illumina) report

Raw sequencing reads were processed simultaneously as follows. PCR primers used for initial 16S rRNA gene amplification were removed from each fragment using Cutadapt [1] version 1.14 due to the presence of degenerate bases that may impact downstream taxonomic assessment. Sickle version 1.33 [2] was used to quality trim DNA reads using a minimum quality value of 28. Reads less than 100 bp following quality trimming were discarded. If a single read was discarded during this process its read pair was also discarded. Reads passing filtering were merged using Pandaseq version 2.9 [3] to generate overlapping contigs with a minimum overlap of 20 bp and a minimum amplicon length of 200 bp. The resulting overlapped reads were de-replicated using Vsearch version v1.9.6 linux x86 64 [4] and searched against a BLAST database composed of the HOMD, HOMD extended and Greenegenes sequences (HOMDXTGG) described in [5]. Taxonomic classification was then performed as previously described [6] at 99 % identity across 98 % of the read length. Reads not classified by this process were discarded. This process resulted in 908 taxonomically classified OTUs. The resulting classification table and associated representative sequences, selected as the most abundant sequence for each classified taxa, were used as inputs for QIIME [7] (Quantitate Insights into Microbial Ecology) version 1.9.1. To perform the functional predictions, the OTUs were assigned taxonomies using QIIME's assign taxonomy script, with the default settings and using SILVA release 123 as the reference database. Samples with less than 1000 counts were filtered from the resulting OTU table, which was then rarefied to the 5th percentile to account for variable sequencing depths. OTUs representing less than 0.5 % of the total sequence count were then removed. The OTU table was then analysed with Tax4Fun [8] version 0.3.1, using the default settings and metabolic profiles calculated against SILVA release 123.

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Supplementary information

Shotgun Metagenomics Pipeline Report

Metatranscriptomic reads were processed simultaneously as follows. FastQC version v0.11.5 was used to analyse the quality of each sample [1]. Only samples with a gc content between 25 and 75 percent (inclusive) and an average base quality of at least 20 were analysed further. Trimmomatic version 0.36 [2] was used to quality trim DNA reads using a minimum average quality value of 28 over a sliding window of length 100. Reads less than 100 bp following quality trimming were discarded. If a single read was discarded during this process its read pair was also discarded. Reads passing this filter were then run through Kneaddata v0.5.1 [3] to remove human sequences. Reads were aligned to Kneaddata's default human database using Bowtie2 version 2.2.9, and any aligned reads were discarded from the dataset. Unmapped reads were then classified taxonomically and functionally. Taxonomic classification was performed using Kraken version 1.0 [4] against an in-house generated database based on HOMD (Human Oral Microbial Database) [5], with a custom *Neisseria flava* species added. The Kraken classifications were then analysed by Bracken version 0.1 [6] to produce species-level abundance estimates. To minimise false positives during Bracken estimation, a minimum of 10 reads were required for a classification to be subject to re-estimation. Taxonomic output was visualised using the GraPhlAn (version 1.1) and Krona (version v2.7) visualisation tools for shotgun metagenomics data [7], [8]. Functional classification was performed with HUMAnN2 version 0.9.3 [9], using the default databases for pathway coverage and abundance, and UniRef90 for the gene families. HUMAnN2 used MetaPhlAn2 version 2.6.0 [10] for an initial taxonomic assignment, Bowtie2 version 2.2.9 for nucleotide alignments and DIAMOND version 0.8.22 [11] for translated read alignments. The gene family results from HUMAnN2 were mapped to a GO slim designed specifically for metagenomics (http://www.geneontology.org/GO slims/goslim_metagenomics.obo).

References

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Any changes to trial outcomes after the trial commenced, with reasons.

Samples were to be collected by a single clinician across the study to ensure consistency in sample collection. Due to illness, the study clinician was unavailable for sample collection at week 12. Samples were collected by multiple other clinicians. Initial data analysis indicated that samples at week 12 were not consistent with weeks 0, 2 and 6 [increased levels of DNA] and were subsequently removed from analysis.