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Modeling normal and dysbiotic subgingival microbiome: effect of nutrients

Divyashri Baraniya, Monika Naginyte, Tsute Chen, Jasim M. Albandar, Susan M. Chialastri, Deirdre Ann Devine, Philip D Marsh and Nezar Noor Al-hebshi

Appendix materials

Appendix Table 1: Composition of RTF and nutrient-rich media (for 100 ml)

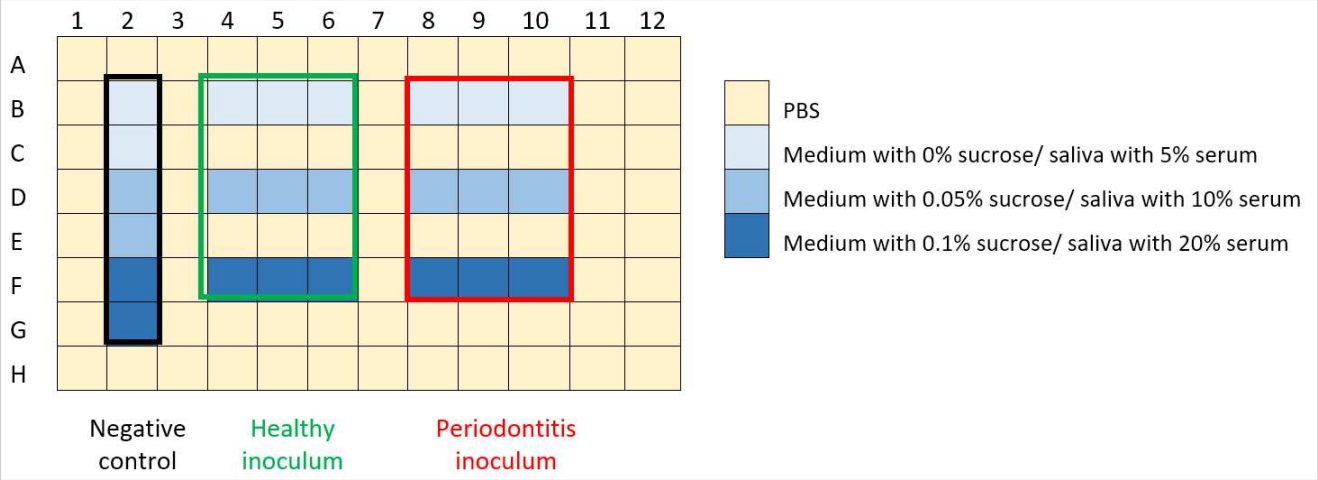
components	Quantity
Reduced transport fluid (RTF)	
K_2HPO_4	0.045 g
KH_2PO_4	0.045 g
NaCl	0.09 g
$(NH_4)_2\ SO_4$	0.09 g
$Mg\ SO_4$	0.018g
Na_2CO_3	0.04 g
EDTA	0.034 g
Cysteine	0.25 g
Water	100 ml
Supplemented BHI (sBHI)	
BHI (Difco)	3.7 g
Mucin	0.1 g
Hemin	0.5 mg
Vit. K	0.05 mg
Sucrose	0g, 0.05g or 0.1g
Cysteine	0.25 g
Water	100 ml
Modified SHI (mSHI)	
Protease peptone	1 g
Trypticase peptone	0.5 g
Yeast extract	0.5 g
Arginine	17.4 mg
Mucin	0.25 g
N-acetylmuramic acid	1 mg
Hemin	0.5 mg
Vitamin K	0.1 mg
Urea	6 mg
Sucrose	0g, 0.05g or 0.1g
Sheep's blood	5 ml
Cysteine	0.25 g
PBS*	95 ml
sBHI/mSHI blend (BSHI)	
BHI (Difco)	3.7 g
Trypticase peptone	0.5 g
Yeast extract	0.5 g
Arginine	17.4 mg
Mucin	0.25 g
N-acetylmuramic acid	1 mg
Hemin	0.5 mg
Vitamin K	0.1 mg
Urea	6 mg

Sucrose	0g, 0.05g or 0.1g
Sheep's blood	5 ml
Cysteine	0.25 g
Water	95 ml

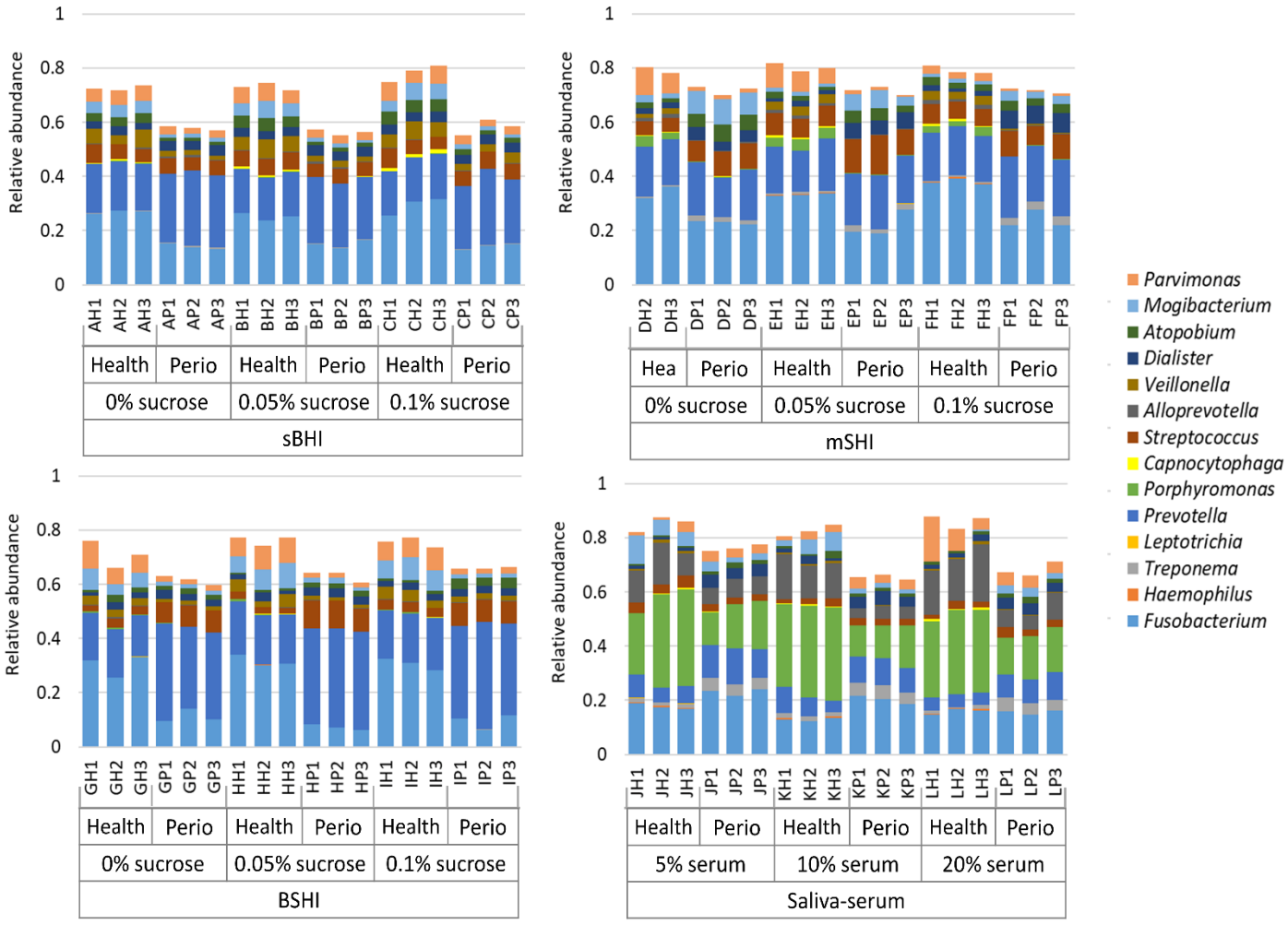
* In place of KCl in the original composition.

Appendix Table 2. Composition of the saliva-serum media

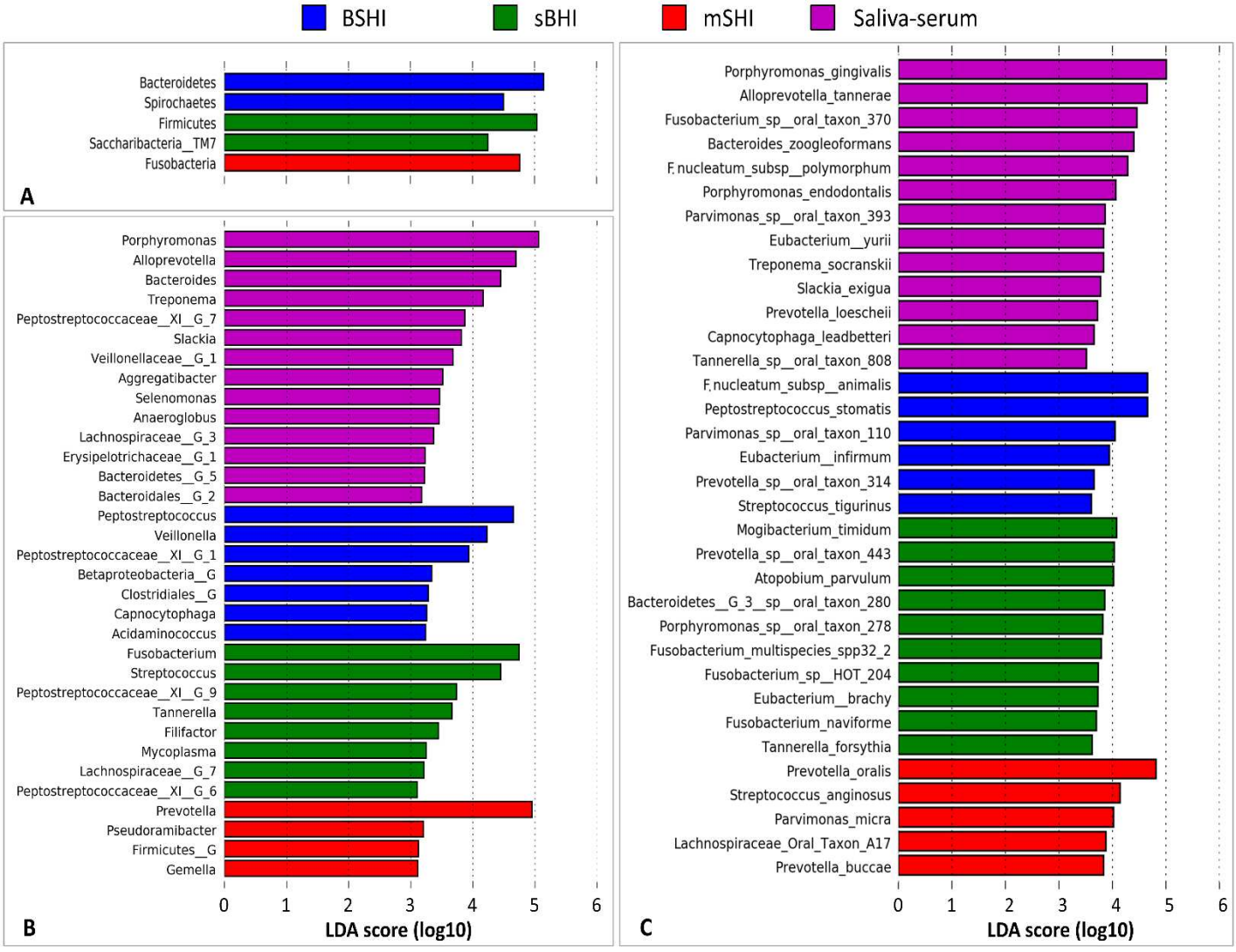
Medium	Filter-sterilized 50% saliva	Human serum, heat inactivated
Saliva with 5% serum	95 ml	5 ml
Saliva with 10% serum	90 ml	10 ml
Saliva with 20% serum	80 ml	20 ml



Appendix Figure 1. Layout of the plate used for growing the microbiomes



Appendix Figure 2. Microbiological profiles of individual microbiomes. The most abundant genera identified in the individual microbiomes grown in the different media, showing high reproducibility.



Appendix Figure 3. Differential enrichment. Phyla (A), genera (B) and species (C) that were enriched in the different media, as determined by LEfSe analysis. Showing results for features with FDR ≤ 0.05 and LDA scores ≥ 3 for phyla and genera and ≥ 3.5 for species.

Sequencing and data preprocessing statistics

A total of 9,198,185 paired sequences were obtained, of which 96.5% merged successfully. Quality filtration, alignment and chimera check removed ~60% of the merged reads. One sample with <5,000 reads was excluded. Of the remaining sequences, 89% could be assigned species-level taxonomy (mean of 44854± 17293 reads per sample). See Appendix Dataset 2 below for detailed statistics.

Appendix Dataset 1. List of species included in the calculation of the subgingival dysbiosis index (SDI) and their relative abundances in the health and periodontitis inocula.
http://www.homd.org/ftp/publication_data/20191023/List%20of%20species%20used%20for%20calculation%20of%20SDI.xlsx

Appendix Dataset 2. Sequencing and data processing statistics.
http://www.homd.org/ftp/publication_data/20191023/stats.xlsx

Appendix Dataset 3. Relative abundances of phyla identified in the individual microbiomes and respective clinical inoculum.
http://www.homd.org/ftp/publication_data/20191023/Relative%20abundances%20of%20phyla%20per%20sample.xlsx

Appendix Dataset 4. Relative abundances of genera identified in the individual microbiomes and respective clinical inoculum.
http://www.homd.org/ftp/publication_data/20191023/Relative%20abundances%20of%20genera%20per%20sample.xlsx

Appendix Dataset 5. Relative abundances of species identified in the individual microbiomes and respective clinical inoculum.
http://www.homd.org/ftp/publication_data/20191023/Relative%20abundances%20of%20species%20per%20sample.xlsx

Appendix Dataset 6. Species and genera identified in the in vitro microbiomes but not in the clinical inocula.
http://www.homd.org/ftp/publication_data/20191023/Species%20identified%20in%20the%20in%20vitro%20microbiomes%20only.xlsx

Appendix discussion

Pooled samples were used as inocula to mitigate the variations in microbial profiles between individuals and to maximize diversity. Nevertheless, pooling from five subjects cannot account for all of the variation in the composition of the subgingival microbiome between subjects, and different results may be obtained if the study was repeated with inocula from a different set of subjects, especially for the SDI. Therefore, not including biological replicates in addition to the technical replicates, is one of the study limitations.

To ensure the growing microbiomes were not disturbed during media replenishment on days 2, 4 and 6, the lid with pegs (on which the microbiomes are growing) was carefully, and without washing, moved to another base plate into which fresh medium had been pipetted.

We used DNA yield as a measure of biofilm quantity "biomass" since we found the crystal violet assay, which is the most commonly used biomass assay, to be incompatible with the hydroxyapatite-coated pegs (HA absorbs crystal violet and produces a very high background). Although DNA yield as a measure of biomass has some limitations, including the possibility of column saturation during DNA extraction and the presence of eDNA in oral biofilms, it demonstrated important differences between the health- and periodontitis-derived microbiomes as well as microbiomes grown in the different media.

It is important to note that measurement of DNA yield as well as sequencing does not differentiate between live and dead cells and, therefore, some of the bacterial taxa identified may have been already dead by the time the microbiomes were harvested for analysis. To address this limitation, we used the ATPase assay to assess viability, which we believe is one of the study strengths and provided another dimension of comparison between the media.

The first 500 bases of the 16S rRNA gene accounts for nearly half of the sequence variability of the entire gene (Dewhirst et al. 2010), which is why the V1-V3 region was targeted for sequencing. This region has been found to provide superior taxonomic resolution for oral bacterial species (and bacteria from aerodigestive tract in general) (Escapa et al. 2018). Our BLASTn-based algorithm exploits this hyper-variability to classify the majority of the reads to the species-level. This cannot be attained with QIIME that employs operational taxonomy unit (OTU) calling and a Bayesian classifier for taxonomy assignment, which results in classification of a significant fraction of the reads to the genus, and even family, level. To

maximize reliability of species-level taxonomy assignment, we have always implemented stringent quality-filtration parameters including a sliding 50-nucleotide window with average Qscore of 35, which we have found to reduce sequencing errors by 10 fold and minimize detection of spurious species (data not published). This, however, results in losing significant number of reads (~30%), while another 30% are removed at the chimera check step.

The reliability of relative abundance-based, microbiome differential abundance methods, including LEfSe, has been questioned recently, since they do not account for total microbial load and can result in false positives and negatives (Morton et al. 2019). LEfSe, however, employs Wilcoxon rank sum test, which has been shown to control well for false discovery rates with multiplicity correction methods (Hawinkel et al. 2019). We, therefore, corrected the p-values obtained from LEfSe using the Benjamini-Hochberg method, and limited reporting to features with FDR ≤ 0.05 and LDA score ≥ 3 (3.5 for species) to minimize false positives. The low sensitivity (i.e. potential false negatives) remains a limitation that could not be eliminated.

An interesting observation in connection with the SDI, is that mSHI media appeared to improve dysbiosis for the healthy microbiome, but negatively impacted the diseased microbiome. The only explanation is that it is possible that mSHI favors growth of the more abundant species in the inoculum, i.e the health-associated species in the healthy inoculum and the disease-associated species in the periodontitis inoculum.

Appendix references

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