Sex-based diverse plaque microbiota in children with severe caries

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Total word count: 3,199

Number of figures and tables: 4 figures and 1 table

Number of references: 37

ABSTRACT

Severe early childhood caries (S-ECC) is a multifactorial disease that can lead to suffering and reduced oral health related quality of life in young children. The bacterial and fungal composition of dental plaque and how children`s sex is associated with S-ECC are largely unknown. In this study, V4-16S rRNA and ITS1 rRNA gene amplicon sequencing was used to compare the plaque bacteriome and mycobiome of children <72 months of age; 40 with S-ECC (15 males, 25 females) and 40 caries-free (19 males, 21 females). Health- and nutrition-related questionnaire data were also investigated. This study aimed to analyze potential sex-based differences in the supragingival plaque microbiota of young children with S-ECC and those caries-free. Behavioral and nutritional habit differences were observed between children with S-ECC and those caries-free and between male and female children. Overall, higher levels of *Veillonella dispar*, *Streptococcus mutans* and other bacterial species, were found in the S-ECC group compared to caries-free controls ($p < 0.05$). A significant difference in the abundance of *Neisseria* was observed between males and females with S-ECC ($p < 0.05$). Fungal taxonomic analysis showed significantly higher levels of *Candida dubliniensis* in the plaque of children with S-ECC compared to caries-free ($p < 0.05$), but no differences were observed with *Ca. albicans* ($p > 0.05$). Significant differences in the relative abundance of *Mycosphaerella, Cyberlindnera* and *Trichosporon* fungal species were also observed between the caries-free and S-ECC groups ($p < 0.05$). Machine learning analysis revealed the most important bacterial and fungal species for classifying S-ECC versus caries-free. Different patterns of crosstalk between microbial species were observed between male and female children. Our work demonstrates that plaque microbiota and sex may be important determinants for S-ECC and could be factors to consider for inclusion in caries risk assessment tools.

Keywords: Dental caries; child, preschool; human microbiome; fungi; bacteria; machine learning

Abstract word count: 293

INTRODUCTION

The oral cavity is composed of unique niches colonized by a variety of microorganisms, including bacteria, fungi and viruses. More than 700 species of bacteria and 100 species of fungi have been identified (Dewhirst et al., 2010; Peters et al., 2017). Oral microbial communities are highly dynamic and, often, exist in a homeostatic equilibrium with the host. Under certain conditions, this equilibrium is disturbed. A dysbiotic interaction between oral polymicrobial communities (including bacteria and fungi) and the host immune system, involving multiple mechanisms not yet fully characterized, is crucial for oral disease onset and progression (Lamont et al., 2018).

Recent studies using a rodent model or dental plaque samples from children and *in-vitro* analyses have shown that inter-kingdom interactions involving *Candida albicans* and cariogenic bacteria were associated with dysbiosis and subsequent onset of oral diseases (Falsetta, 2014; Xiao et al., 2018). However, little is known about the role of other fungal species in oral infections. Thus, it is imperative to include mycobiome analysis when studying oral diseases.

Early childhood caries (ECC) is a common pediatric disease defined as any caries experience comprising the primary dentition in children <72 months of age. Severe early childhood caries (S-ECC) is a more aggressive presentation of ECC (American Academy of Pediatric Dentistry, 2017). It affects more than 530 million children worldwide, with higher prevalence rates in developing countries (GBD, 2018; Johansson et al., 2016)

Risk factors for S-ECC include poor oral hygiene, inadequate fluoride exposure, limited access to care, high abundance of cariogenic microbes and high-sugar diet, among others. S-ECC can lead to long-term complications and can negatively impact childhood health and quality of life (Grant et al., 2019; Kirthiga et al., 2019; Schroth et al., 2009).

Due to the extent of the lesions and dental treatment required, frequently, children have to undergo rehabilitative dental surgery under general anesthesia, which is not free of risks (Lee et al., 2013). This surgical approach does not address causative factors and are a huge burden to the health care systems (Schroth et al., 2016).

There is an imperative need to identify how risk factors for S-ECC, such as sex and plaque microbiome, interact to comprehend why some children are at greater risk. Currently, few studies have investigated the role of sex in ECC (Ribeiro et al., 2017; Watson MR, 1999). Whether the bacterial and fungal composition of the plaque samples from young male children differs from females and how these different factors interact to determine S-ECC susceptibility remain to be characterized. The purpose of this study was to analyze potential sex-based differences in the plaque microbiota (including the mycobiota) of young children with S-ECC and those caries-free.

METHODS

Study Population, Design and Sample Collection

The study protocol was approved by the University of Manitoba`s Health Research Ethics Board (HREB #HS20961–H2017:250) and by the Misericordia Health Centre (MHC), Winnipeg, MB, Canada. Written informed consent was provided by the parents or legal caregivers. Eighty children <72 months of age were recruited. Overall, 40 children had S-ECC and 40 were caries-free (dmft index equal to 0; no decayed, missing, or filled primary tooth surface) (Agnello et al., 2017). Dental examinations to confirm the caries-free status of controls were conducted by an experienced dentist (R.J.S.) using artificial light and no radiographs. None

of the caries-free children had incipient (non-cavitated) caries lesions. All children included in the S-ECC were recruited at the MHC, on the day of their dental rehabilitative surgery. The caries-free control subjects were recruited in the community and seen at the Children's Hospital Research Institute of Manitoba in Winnipeg, Canada.

Supragingival plaque samples were collected by scrubbing a sterile interdental brush on all available tooth surfaces (Agnello et al., 2017). Samples were dislodged into 1 ml of RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) and immediately frozen at -80°C until further analysis. Children`s parents or legal caregivers completed an interviewed questionnaire on the child`s general and oral health, feeding practices, and oral hygiene habits.

DNA extraction, library preparation, 16S and ITS1 rRNA amplicon sequencing and analysis

Extracted DNA was sent on dry ice to McGill University - Génome Québec Innovation Center (Montreal, Canada) for library preparation and paired-end Illumina MiSeq PE250 sequencing of the V4 region of bacterial 16S rRNA and Internal Transcribed Spacer 1 (ITS1) fungal rRNA genes. Sequencing data were analyzed using QIIME2 2018.11 (Quantitative Insights into Microbial Ecology) (Bolyen, 2018).

Classification of caries status using machine learning approaches

LASSO (least absolute shrinkage and selection operator model), was used for the classification of S-ECC and caries-free groups (Tibshirani, 1996). To investigate how the bacteria and the fungi associate with each other, we calculated the correlation scores between them in different subgroups (males with S-ECC, caries-free males, females with S-ECC, caries-free females).

Statistical analysis

Questionnaire data were analyzed with Number Cruncher Statistical Software 9 (Kaysville, Utah) and Chi-square, Fisher`s exact, and T-tests were performed when appropriate. Differential abundance analyses were performed with linear discriminant analysis effect size (LEfSe) and DESeq2 negative binomial Wald test (Love et al., 2014; Segata et al., 2011). Adjusted p-value ≤0.05 was considered statistically significant.

Additional details about the methods used are described in the Appendix.

RESULTS

Eighty children with a mean age of 45.9 ± 12.8 months participated. Among children with S-ECC (N = 40), 25 (62.5%) were females. Whereas 21 (52.5%) of the caries-free children (N = 40) were females. Overall, children with S-ECC were more likely to be from rural areas, be bottle-fed, go to bed with a bottle, frequently snack before bedtime and prefer to eat sweet food compared to caries-free controls ($p < 0.05$). Additionally, they were less likely to be breastfed, have their teeth brushed twice a day, and use tooth paste than caries-free children ($p < 0.05$; Table 1). When the groups were stratified by sex, caries-free females were significantly less likely to snack frequently than caries-free males ($p = 0.05$). Whereas, male children with S-ECC had the first dental visit at an earlier age and had their mouths being cleaned at home at a younger age than females with S-ECC ($p < 0.01$; Table 1).

Supragingival plaque bacterial community analyses

The Illumina sequencing generated 8,035,685 16S rRNA sequences for 80 samples, with an average of 100,446 sequences per sample. After trimming and filtering, 4,367 ASVs were assigned to 11 bacterial phyla, 99 genera and 233 species. A total of 61 genera and 135 species had non-zero counts in at least 5% of the samples and the sequences had an average of 295 bp. The alpha diversity analysis (within samples), showed higher Pielou`s evenness ($p = 0.02$) in the caries-free group compared to the S-ECC group. No differences were observed when stratifying the groups by sex ($p > 0.05$; Fig. 1A.I) nor when comparing diversity with Shannon index (Appendix Figure 1A). The beta diversity analysis showed significant bacterial community differences for S-ECC status (pseudo-$F = 2.12$, $R^2 = 0.025$, $p = 0.004$; permutational multivariate analysis of variance (PERMANOVA)) but not for place of residence (pseudo-$F = 0.67$, $R^2 = 0.008$, $p = 0.87$; PERMANOVA). Principal coordinates analysis (PCoA) of bacterial species also showed a separation of the S-ECC samples from the caries-free when the data from females were analyzed separately, adjusting for place of residence (Fig. 1B.I, pseudo-$F = 2.22$, $R^2 = 0.045$, $p = 0.004$; PERMANOVA).

Taxonomic assignment showed that *Veillonella*, *Neisseria* and *Streptococcus* were the most abundant genera in the S-ECC group. *Actinomyces* was the most abundant genus in the caries-free group, followed by *Neisseria* and *Corynebacterium*, respectively. Among the 20% most abundant bacterial genera detected, higher relative abundance of *Streptococcus*, *Veillonella,* *Prevotella* and *Selenomonas* and lower relative abundance of *Actinomyces* and *Leptotrichia* were observed in the S-ECC group compared to the caries-free ($p<0.05$, LEfSe, Fig. 1C.I).

Among the 20% most abundant species, significantly higher levels of *Veillonella* sp. oral taxon 780, *V. dispar, S. mutans* and others were identified in the S-ECC group compared to caries-free control ($p < 0.05$). However, higher levels of *Corynebacterium durum* and *Lautropia*

188 *mirabilis* were found in caries-free children ($p < 0.05$, LEfSe, Fig. 1D.I). Within the S-ECC
189 group, *Neisseria* was identified as significantly more abundant in males than in females ($p <$
190 0.05, LEfSe, Fig. 1C.I).
191
192 **Supragingival plaque fungal community analysis**
193 The Illumina sequencing generated 7,053,886 ITS1 rRNA sequences for 80 plaque samples, with
194 an average of 88,174 sequences per sample. After filtering and trimming 1,091 ASVs were
195 classified into 4 fungal phyla, 27 genera and 30 species. Eleven genera and ten species were
196 present in at least 5% of the samples. Significant differences in alpha diversity were observed
197 between the S-ECC and caries-free groups ($p < 0.01$; Fig. 1A.II and Appendix Figure 1D). Beta
198 diversity analysis revealed that plaque fungal communities between children with S-ECC vs
199 caries-free significantly differed (pseudo-$F = 4.84$, $R^2 = 0.055$, $p = 0.002$; PERMANOVA). The
200 same was not true for rural vs urban (pseudo-$F = 0.62$, $R^2 = 0.007$, $p = 0.62$; PERMANOVA). A
201 separation of samples from caries-free and S-ECC groups were observed in the PCoA plot when
202 only data from males were analyzed (Fig. 1B.II, pseudo-$F = 3.53$, $R^2 = 0.09$, $p = 0.02$;
203 PERMANOVA).
204 No sex-based differences were observed with alpha and beta diversity analyses ($p > 0.05$;
205 Fig. 1A.II-B.II, Appendix Figure 1B). *Candida* was the most abundant fungal genus in the S-
206 ECC group, regardless of sex. However, in the caries-free group, *Malassezia* was the most
207 abundant genus identified in females with 6.5-fold higher counts compared to males (Fig. 1C.II).
208 They also had 4.5-fold lower counts of *Candida* than males. Interestingly, *Ca. dubliniensis* was
209 significantly more abundant in the dental plaque of children with S-ECC compared those caries-
210 free ($p < 0.05$, LEfSe, Fig. 1D.II). Whereas, no significant differences in the abundance of *Ca.*

albicans was observed among S-ECC and caries-free groups. Within the S-ECC group, *Ca. dubliniensis* and *Ca. albicans* had an average of 30,088 and 4,091 counts per sample, respectively. *Mycosphaerella, Cyberlindnera and Trichosporon* species were differentially abundant between the caries-free and S-ECC groups ($p < 0.05$, LEfSe, Fig. 1C.II – D.II).

To evaluate whether place of residence and other variables described in Table 1 were responsible for all the differences observed between the plaque microbiota of caries-free and S-ECC groups, we performed additional tests using the LEfSe and DESeq2 methods. The results confirmed that the composition of the plaque microbiota differed between caries-free and S-ECC groups (Appendix Figures 2 and 3).

Classification of S-ECC status using machine learning approaches

To evaluate the performance of the supragingival plaque bacterial and fungal species in the classification of S-ECC status, the LASSO-based classification models were used. Data from males and females were used separately to understand whether there were any sex-based differences. A 10-fold cross validation strategy was used to measure the performances of the LASSO models. Fig. 2A and Fig. 2B illustrate the area under the receiver operating characteristics (ROC) curves (AUC) of the classification model performance using bacterial and fungal microbiome data, respectively. From the AUC values, it was observed that females were better classified (AUC: 0.94) with bacterial data, whereas males were better classified (AUC: 0.91) using fungal data. Combining data from males and females together resulted in relatively poorer classification performances (0.88 AUC for bacterial species and 0.77 AUC for fungal species) than treating them separately.

Among bacterial species, *R. aeria*, *L. mirabilis*, *Kingella oralis*, and *Co. durum* were found to be the most important features for classification in females, whereas *Treponema socranskii* and *S. mutans* were the most important in males. While considering all children, *Veillonella* sp., *S. mutans*, *Prevotella melaninogenica* and *K. oralis* were the most important species. Classifying using the fungal species information showed *Ca. dubliniensis* to be the most important feature in all three groups (males, females, and both). Besides, *Malassezia restricta* was also found to be important when both males and females were considered together (Fig. 2C-D).

Potential crosstalk between bacteria and fungi in the supragingival plaque

Following the identification of the important species of bacteria and fungi, based on the LASSO model, we analyzed how they were correlated with each other. Fig. 3A-D shows the correlations in the four subgroups. The results demonstrate noticeable differences in the correlation between fungal and bacterial species among the subgroups. For example, the fungus *Ca. dubliniensis* showed a negative correlation with *Prevotella histicola* only in the "males with S-ECC" subgroup (Fig. 3A). Next, we analyzed the significant correlations from all groups (Fig. 4). Interesting correlations were observed among fungal and bacterial species and are shown in Fig. 4.

DISCUSSION

To our knowledge, this is the first study to investigate the differences between the fungal composition of the supragingival plaque mycobiome of preschool male and female children with S-ECC and those caries-free using current molecular techniques. We also evaluated whether sex

associates with the plaque microbiome to influence caries risk in young children using machine learning approaches.

The significant differences in dietary and oral hygiene habits observed between children with S-ECC and those caries-free has been reported in previous studies (Agnello et al., 2017). Within these two groups, sex-based differences were also observed. Our findings indicate no differences in tooth brushing frequency between the sexes. However, males with S-ECC had their first dental visit at a younger age and were younger when their teeth started to be cleaned. These findings could indicate that parents may give more attention to the oral health of males than females. Nonetheless, a cross-sectional study from China reported the opposite; female children possibly receive more attention from caregivers than males, which suggests that in addition to other factors such as gender, cultural differences might also play a role in oral-health related behavioural differences between males and females (Qiu et al., 2016). Other possible factors might include genetic variations, social factors, chronology of tooth eruption, and hormonal differences, which could affect the composition of their plaque microbiome, explaining the differences observed in our study (Ferraro and Vieira, 2010; Shaffer et al., 2015).

Alpha diversity analyses of the bacterial and fungal data showed significant differences between S-ECC and caries-free groups but not between males and females. Higher alpha diversity in the caries-free group has been reported in previous studies, demonstrating that higher alpha diversity is related to health (Gross et al., 2012; Xiao et al., 2016). Overall, the beta diversity analysis showed that both the plaque bacterial and fungal communities significantly differed between S-ECC and caries-free children. Interestingly, when stratifying the data by sex, a statistically significant separation between the bacterial and fungal microbiomes by S-ECC status was observed only in females or males, respectively. This agrees with the machine

learning results, as the LASSO models using data from females performed better with 16S data for the classification of caries-free vs S-ECC, whereas, the models using data from males performed better with ITS data.

We observed higher relative abundance of *Veillonella* species and *S. mutans* in the S-ECC group compared to the caries-free controls. A possible explanation is the mutual relationship that has been proposed between *Veillonella* species and acidogenic *Streptococcus.* The former facilitates the growth of the latter, while benefiting from their production of lactate (Becker et al., 2002; Distler and Kroncke, 1980). For example, *V. parvula* has been shown to stimulate glucose fermentation by *Streptococcus salivarius* by decreasing the external concentrations of lactate, which is an inhibitor of glycolysis. However, the consumption of lactate by *Veillonella* may lead to a less acidic or non-cariogenic environment, suggesting its dual nature (Hamilton and Ng, 1983; Kara et al., 2006). Our machine learning analysis suggested that *Veillonella* species was important for S-ECC classification.

Bacteria found to be more abundant in caries-free groups compared to S-ECC groups, such as *Leptotrichia, Actinomyces* and *Corynebacterium* species, have been reported as positively associated with oral health (Xiao et al., 2016; Xu et al., 2014). Similar findings were described in our previous study with Canadian First Nations children (Agnello et al., 2017). Significant differences were also observed in the plaque microbiota of males and females within the S-ECC group, demonstrating that sex may also influence the composition of the plaque microbiota.

Ca. dubliniensis was surprisingly the most abundant fungal species detected in the S-ECC group. Previously, *Ca. dubliniensis* has been detected in plaque samples of children with caries using culture-based approaches (Al-Ahmad et al., 2016). Another study also showed isolation of

Ca. dubliniensis in carious dentine samples (Kneist et al., 2015). The high similarity between *Ca. albicans* and *Ca. dubliniensis,* the dearth of efficient techniques for distinguishing them and very few studies using NGS sequencing of the oral mycobiome, may be the reasons for the poor characterization of *Ca. dubliniensis* until now (Al-Ahmad et al., 2016). *Ca. albicans* is the most studied and well characterized oral fungal species. Other *Candida* species, however, are consistently isolated in oral samples (Peters et al., 2017). *Malassezia*, which was mostly identified in our caries-free group, has been reported to be a prominent commensal in the oral cavity (Diaz et al., 2017; Dupuy et al., 2014; Ward et al., 2018). Further studies are required to elucidate whether the detected fungal species are resident or transient members of the oral mycobiota.

The use of machine learning methods for biological data analysis is an emerging field and has the advantage of extracting important features in a high-dimensional and sparse setting. LASSO-based model is one of these methods which offers feature selection with both higher prediction accuracy and simpler interpretation (Ma and Huang, 2008). Our results showed unique characteristics for each subgroup (males with S-ECC, caries-free males, females with S-ECC and caries-free females), demonstrating that sex-related factors may play a role in microbial interactions.

The strength of this study is that it provides a holistic view of S-ECC using a trans-disciplinary approach. It has, however, some limitations. The presence of retrospective questions in the questionnaire might have introduced recall bias and response bias is also possible on the part of parents. It also lacked the individual dmft/dmfs scores for the S-ECC group. Convenient sampling was used for recruitment, meaning that the groups were not matched by sex, age, and socioeconomic status, during recruitment. Other confounding factors for the association between

S-ECC and the plaque microbiota could be place of residence, oral hygiene and feeding habits (Agnello et al., 2017; Willis et al., 2018). However, these variables were evenly distributed among the subgroups ($p > 0.05$, males vs females within S-ECC and caries-free groups). We also demonstrated that after adjusting for possible confounding effects, the composition of the plaque microbiota of S-ECC children still differed from those caries-free. Future studies with larger cohorts will be performed to confirm our findings.

CONCLUSION

Overall, our findings indicate that sex may be a differentiating factor in the microbial composition of the supragingival plaque of caries-free children and those with S-ECC. This supports the idea that there are biomarkers for S-ECC that are unique for male and female children. We are, however, still far from fully understanding the role of sex and microbiota in S-ECC risk. Our results could guide further trans-disciplinary studies aiming to create better tools for determining S-ECC risk, which will allow a more personalized dental diagnosis, treatment and prognosis for young males and females. Oral microbiome and sex may be important determinants for S-ECC and could be factors to consider for inclusion in caries risk assessment tools.

ACKNOWLEDGEMENTS

The authors thank the Misericordia Health Centre (Winnipeg, MB, Canada), the parents/caregivers and children. Additional thanks to Dr. Anjali Yadav for comments on the manuscript and Melina Sturym for assisting with the recruitment of participants.

348 This study was funded by operating grants from the Children's Hospital Research Institute of
349 Manitoba (CHRIM) and the Canadian Institute of Health Research (CIHR). V.C.J. is supported
350 by a Doctoral Fellowship from Research Manitoba/CHRIM and the University of Manitoba
351 Graduate Fellowship. R.J.S. holds a CIHR Embedded Clinician Researcher salary award in
352 "Improving access to oral health care and oral health care delivery for at-risk young children in
353 Manitoba".
354
355 The authors declare no potential conflicts of interest with respect to the authorship and/or
356 publication of this article.
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358 **LIST OF ABBREVIATIONS**
359 ECC: Early childhood caries; S-ECC: Severe early childhood caries; dmft: decayed, caries-
360 related missing, or filled primary teeth; NGS: Next generation sequencing; GA: General
361 anesthesia; ITS1: Internal Transcribe Spacer 1; QIIME2: Quantitative insights into microbial
362 ecology; ASV: amplicon sequence variants; PCoA: Principle coordinate analysis; LASSO: Least
363 absolute shrinkage and selection operator; AUC: Area under the receiver operating
364 characteristics curve; ROC: Receiver operating characteristics; SVM: Support vector machine;
365 RF: Random forest; NCSS: Number cruncher statistical software; FDR: False discovery rate;
366 PERMANOVA, permutational analysis of variance; LEfSe, linear discriminant analysis effect
367 size.
368
369 **AUTHOR CONTRIBUTIONS**

P.C. and R.J.S. designed and directed the study; R.J.S., K.M, D.O. and B.A.M. contributed to participant recruitment and sample collection; V.C.J. and A.A. designed and conducted the sample processing for sequencing; V.C.J., D.O. and R.J.S. contributed to the analysis of the questionnaire data; V.C.J. conducted the 16S and ITS1 rRNA sequencing data analysis and interpretation; R.S. and P.H. designed and performed the machine learning classifications; R.S., K.D. and P.H. contributed to data interpretation and critically revised the manuscript; V.C.J., R.S., B.A.M. R.J.S. and P.C. drafted and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

AVAILABILITY OF DATA

The datasets supporting the conclusions based on the microbial community analyses were submitted to the NCBI Sequencing Read Archive Repository [PRJNA555320].

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FIGURES

Figure 1. Bacterial and fungal diversity and taxonomic profile of supragingival plaque of children with S-ECC and those caries-free. Alpha diversity analysis. Boxplot of Pielou's evenness index for (A.I) bacterial taxa and (A.II) fungal taxa in S-ECC and caries-free groups, and subgroup analysis by sex. The line inside the box represents the median. Whiskers represent the lowest and highest values within the 1.5 interquartile range. Beta diversity analysis. Principal coordinates analysis (PCoA) plots of Bray-Curtis dissimilarities for (B.I) bacterial and (B.II) fungal species, according to caries status and sex. A separation of samples by caries-status was observed among the females 16S data and males ITS1 data when stratifying by sex ($p < 0.05$, permutational multivariate analysis of variance). Taxonomic profiles of children`s dental plaque according to caries status and sex at bacterial (C.I) genus and (D.I) species level and fungal (C.II) genus and (D.II) species level. (C.I and D.I) Colours were assigned only to the 20% most abundant taxa. Taxonomic profiles include only taxa assigned up to genus or species level. S-ECC, severe early childhood caries. $^{*}p < 0.05$ between caries-free and S-ECC; $^{\#}p < 0.05$ between males and females with S-ECC, according to LefSE analysis.

Figure 2. Classification performance and relative feature importance of bacteria and fungi. (A) Receiver operating characteristics (ROC) curves of classification using bacterial data only. (B) ROC curves of classification using fungal data only. Lines of different colors represent ROC curves of different groups: males, females, and both (all children). The area under the curve (AUC) values are shown in the legend. The x-axis represents the false-positive rate, and the y-axis represents the true-positive rate. (C) Heatmap of relative feature importance of the top most

important bacteria for the three groups (both or all children, males and females) while classifying S-ECC and caries-free groups. (D) Heatmap of relative feature importance of the top most important fungi for the three groups (both or all children, males and females) while classifying S-ECC and caries-free groups. The color with the highest intensity represents the most important bacteria or fungi. Bacterial and fungal species that are important in at least one of the three groups have been included in the figures. S-ECC, severe early childhood caries.

Figure 3. Correlation plots of the most important bacteria and fungi in different groups. A prefix "B_" denotes bacterium, whereas a prefix "F_" denotes fungus. Blue and red color shades represent positive and negative correlations, respectively. Color intensity and area of the circles are proportionate to the absolute value of the correlations. White color represents that there is no correlation. (A) males with S-ECC. (B) caries-free males. (C) females with S-ECC. (D) caries-free females. S-ECC, severe early childhood caries.

Figure 4. Correlation network of the significantly correlated bacterial and fungal species among different groups. Different colors represent different groups (males with S-ECC, caries-free males, females with S-ECC, and caries-free females). Groups are also shaded with the same color for visual clarity. Solid and dashed lines denote positive and negative correlations, respectively. The thickness of the lines is proportional to the absolute values of the correlations which ranges from 0.31 to 0.77. S-ECC, severe early childhood caries.

TABLE

Table 1. Demographics and behavioral characteristics of study participants.

Variable	Caries Status		Caries Status and Sex			
	Caries-Free	S-ECC	Caries-Free		S-ECC	
	(*n* = 40)	(*n* = 40)	Females (*n* = 21)	Males (*n* = 19)	Females (*n* = 25)	Males (*n* = 15)
Age (months)	46.2 ± 14.2	45.6 ± 11.4[a]	47.9 ± 15.1	44.3 ± 13.2[a]	45.7 ± 11.5	45.4 ±11.6[a]
Place of residence						
Urban	39 (97.5)	8 (20.0)[b**]	20 (95.2)	19 (100.0)[b]	4 (16.0)	4 (26.7)[b]
Rural	1 (2.5)	32 (80.0)	1 (4.8)	0 (0.0)	21 (84.0)	11 (73.3)
Overall health						
Very good	32 (80.0)	24 (60.0)[c*]	16 (76.2)	16 (84.2)[b]	14 (56.0)	10 (66.7)[c]
Good/Fair	8 (20)	16 (40.0)	5 (23.8)	3 (15.8)	11 (44.0)	5 (33.3)
Ever breast-fed						
Yes	33 (82.5)	21 (52.5)[c**]	18 (85.7)	15 (79.0)[b]	14 (56.0)	7 (46.7)[c]
No	7 (17.5)	19 (47.5)	3 (14.3)	4 (21.0)	11 (44.0)	8 (53.3)
Ever bottle-fed						
Yes	30 (75.0)	40 (100.0)[b**]	14 (66.7)	16 (84.2)[b]	25 (100.0)	15 (100)[b]
No	10 (25.0)	0 (0.0)	7 (33.3)	3 (15.8)	0 (0.0)	0 (0.0)
Put to bed with bottle						
Yes	7 (17.5)	25 (62.5)[c**]	2 (9.5)	5 (26.3)[b]	17 (68.0)	8 (53.3)[c]
No	33 (82.5)	15 (37.5)	19 (90.5)	14 (73.7)	8 (32.0)	7 (46.7)
Snacks before bedtime						
Yes	16 (40.0)	30 (75.0)[c**]	8 (38.1)	8 (42.1)[c]	17 (68.0)	13 (86.7)[b]
No	24 (60.0)	10 (10.0)	13 (61.9)	11 (57.9)	8 (32.0)	2 (13.3)
Sweet preference						
Do not prefer	12 (30.0)	3 (7.5)[b*]	5 (23.8)	7 (36.8)[b]	2 (8.0)	1 (6.7)[b]
Prefers occasionally[1]	24 (60.0)	28 (70.0)	14 (41.7)	10 (52.6)	18 (72.0)	10 (66.7)
Prefers frequently[2]	4 (10.0)	9 (22.5)	2 (9.52)	2 (10.5)	5 (20.0)	4 (26.7)
Snacking frequency (times per day)	3.3 ± 1.9	3.9 ± 1.9[a]	3.05 ± 1.4	4.11 ± 1.8[a*]	3.99 ± 1.9	3.53 ± 2.4[a]
Uses toothpaste						
Yes	39 (97.5)	32 (80.0)[b*]	21 (100.0)	18 (94.7)[b]	21 (84.0)	11 (73.3)[b]
No	1 (2.5)	8 (20.0)	0 (0.0)	1 (5.3)	4 (16.0)	4 (26.7)
Oral health						
Very good/Good	39 (97.5)	10 (25.0)[b**]	20 (95.2)	19 (100)[b]	6 (24.0)	4 (26.7)[b]
Fair/Poor/Very poor	1 (2.5)	30 (75.0)	1 (4.8)	0 (0.0)	19 (76.0)	11 (73.3)
Use of fluoridated toothpaste						
Yes	30 (75.0)	31 (77.5)[c]	17 (81.0)	13 (68.4)[b]	21 (84.0)	10 (66.7)[b]
No	10 (25.0)	9 (22.5)	4 (19.0)	6 (31.6)	4 (16.0)	5 (33.3)
Tooth brushing frequency						
≥ twice/day	27 (67.5)	11 (27.5)[c**]	14 (66.7)	11 (57.9)[c]	7 (28.0)	4 (26.7)[b]
< twice/day	13 (32.5)	29 (72.5)	7 (33.3)	8 (42.1)	18 (72.0)	11 (73.3)
Age at the first dental visit[3]	20.5 ± 10.4	21.4 ± 13.2[a]	21.8 ± 11.7	19 ± 8.8[a]	26.3 ± 14.4	13.5 ± 4.9[a**]
Age when mouth started to be cleaned[3]	10.7 ± 9.9	13.3 ± 7.2[a]	12.7 ± 8.17	10.7 ± 5.4[a]	13.26 ± 7.3	6.86 ± 3.7[a**]

Values are presented as mean ± SD or n (%). S-ECC, severe early childhood caries.
[a] *T*-test; [b] Fisher's exact test; [c] Chi-square analysis; *$p \leq 0.05$; **$p \leq 0.01$; [1]Weekly; [2]Daily; [3]In months.

FIGURE 1.

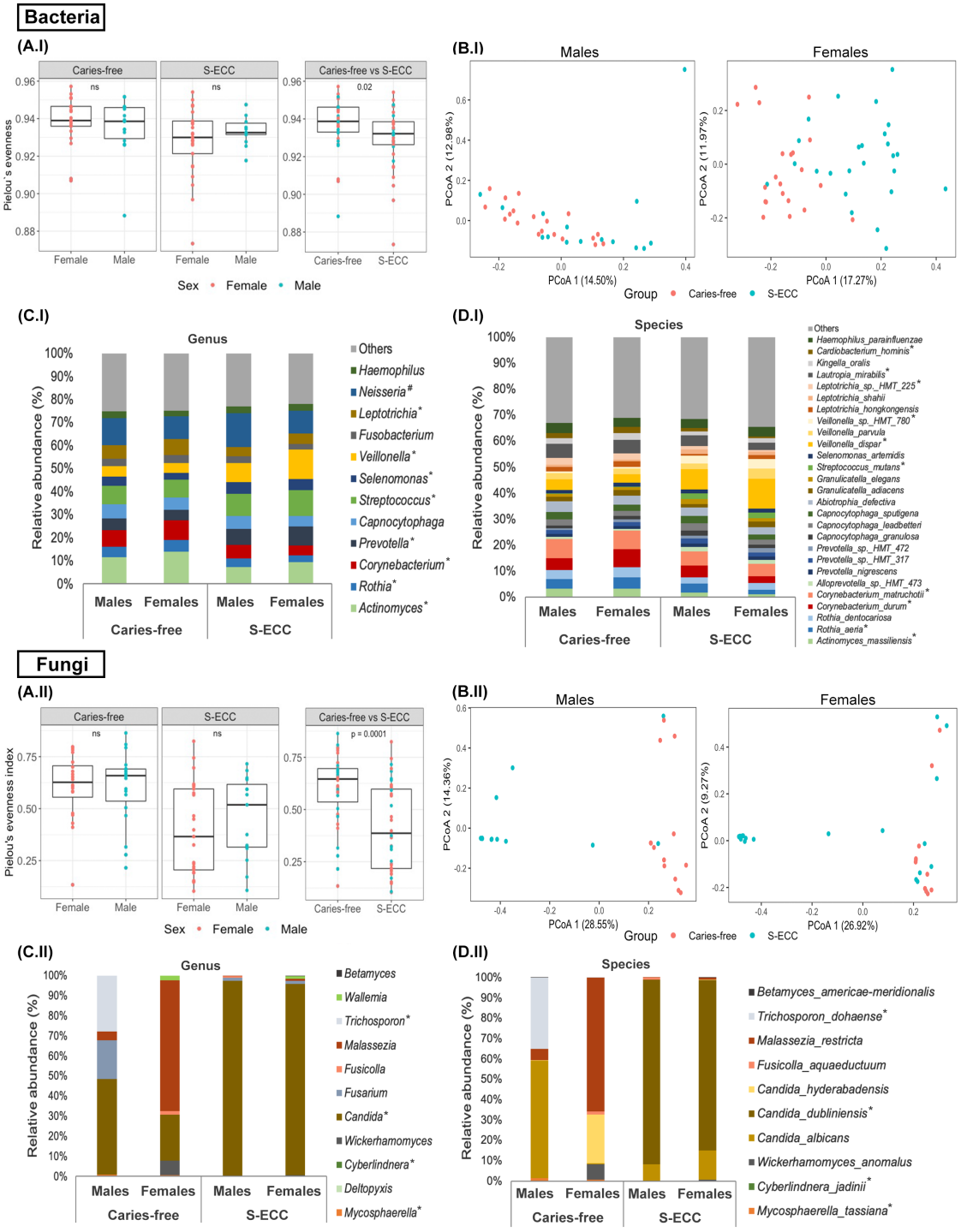

Bacteria
(A.I)
Caries-free
S-ECC
Caries-free vs S-ECC
ns
ns
0.02
Pielou`s evenness
Female
Male
Caries-free
S-ECC
Sex • Female • Male
(B.I)
Males
Females
PCoA 2 (12.98%)
PCoA 1 (14.50%)
PCoA 2 (11.97%)
PCoA 1 (17.27%)
Group • Caries-free • S-ECC
(C.I)
Genus
Relative abundance (%)
Others
Haemophilus
Neisseria#
Leptotrichia*
Fusobacterium
Veillonella*
Selenomonas*
Streptococcus*
Capnocytophaga
Prevotella*
Corynebacterium*
Rothia*
Actinomyces*
Males
Females
Caries-free
S-ECC
(D.I)
Species
Others
Haemophilus_parainfluenzae
Cardiobacterium_hominis*
Kingella_oralis
Lautropia_mirabilis*
Leptotrichia_sp._HMT_225*
Leptotrichia_shahii
Leptotrichia_hongkongensis
Veillonella_sp._HMT_780*
Veillonella_parvula
Veillonella_dispar*
Selenomonas_artemidis
Streptococcus_mutans*
Granulicatella_elegans
Granulicatella_adiacens
Abiotrophia_defectiva
Capnocytophaga_sputigena
Capnocytophaga_leadbetteri
Capnocytophaga_granulosa
Prevotella_sp._HMT_472
Prevotella_sp._HMT_317
Prevotella_nigrescens
Alloprevotella_sp._HMT_473
Corynebacterium_matruchotii*
Corynebacterium_durum*
Rothia_dentocariosa
Rothia_aeria*
Actinomyces_massiliensis*
Fungi
(A.II)
Caries-free
S-ECC
Caries-free vs S-ECC
ns
ns
p = 0.0001
Pielou's evenness index
Sex • Female • Male
(B.II)
Males
Females
PCoA 2 (14.36%)
PCoA 1 (28.55%)
PCoA 2 (9.27%)
PCoA 1 (26.92%)
Group • Caries-free • S-ECC
(C.II)
Genus
Relative abundance (%)
Betamyces
Wallemia
Trichosporon*
Malassezia
Fusicolla
Fusarium
Candida*
Wickerhamomyces
Cyberlindnera*
Deltopyxis
Mycosphaerella*
(D.II)
Species
Betamyces_americae-meridionalis
Trichosporon_dohaense*
Malassezia_restricta
Fusicolla_aquaeductuum
Candida_hyderabadensis
Candida_dubliniensis*
Candida_albicans
Wickerhamomyces_anomalus
Cyberlindnera_jadinii*
Mycosphaerella_tassiana*

FIGURE 2.

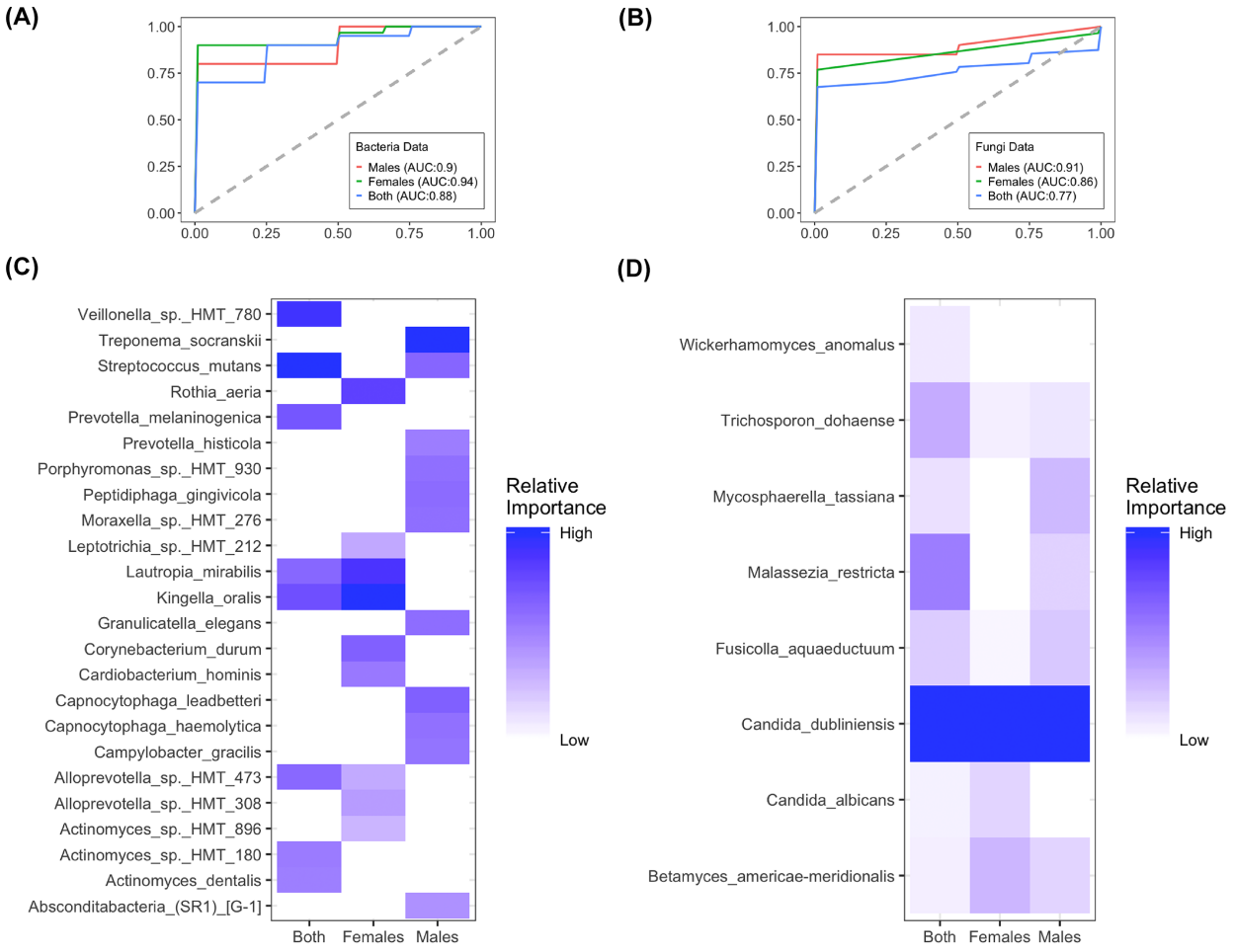

(A)
Bacteria Data
Males (AUC:0.9)
Females (AUC:0.94)
Both (AUC:0.88)
(B)
Fungi Data
Males (AUC:0.91)
Females (AUC:0.86)
Both (AUC:0.77)
(C)
Veillonella_sp._HMT_780
Treponema_socranskii
Streptococcus_mutans
Rothia_aeria
Prevotella_melaninogenica
Prevotella_histicola
Porphyromonas_sp._HMT_930
Peptidiphaga_gingivicola
Moraxella_sp._HMT_276
Leptotrichia_sp._HMT_212
Lautropia_mirabilis
Kingella_oralis
Granulicatella_elegans
Corynebacterium_durum
Cardiobacterium_hominis
Capnocytophaga_leadbetteri
Capnocytophaga_haemolytica
Campylobacter_gracilis
Alloprevotella_sp._HMT_473
Alloprevotella_sp._HMT_308
Actinomyces_sp._HMT_896
Actinomyces_sp._HMT_180
Actinomyces_dentalis
Absconditabacteria_(SR1)_[G-1]
Both Females Males
Relative Importance
High
Low
(D)
Wickerhamomyces_anomalus
Trichosporon_dohaense
Mycosphaerella_tassiana
Malassezia_restricta
Fusicolla_aquaeductuum
Candida_dubliniensis
Candida_albicans
Betamyces_americae-meridionalis
Both Females Males
Relative Importance
High
Low

FIGURE 3.

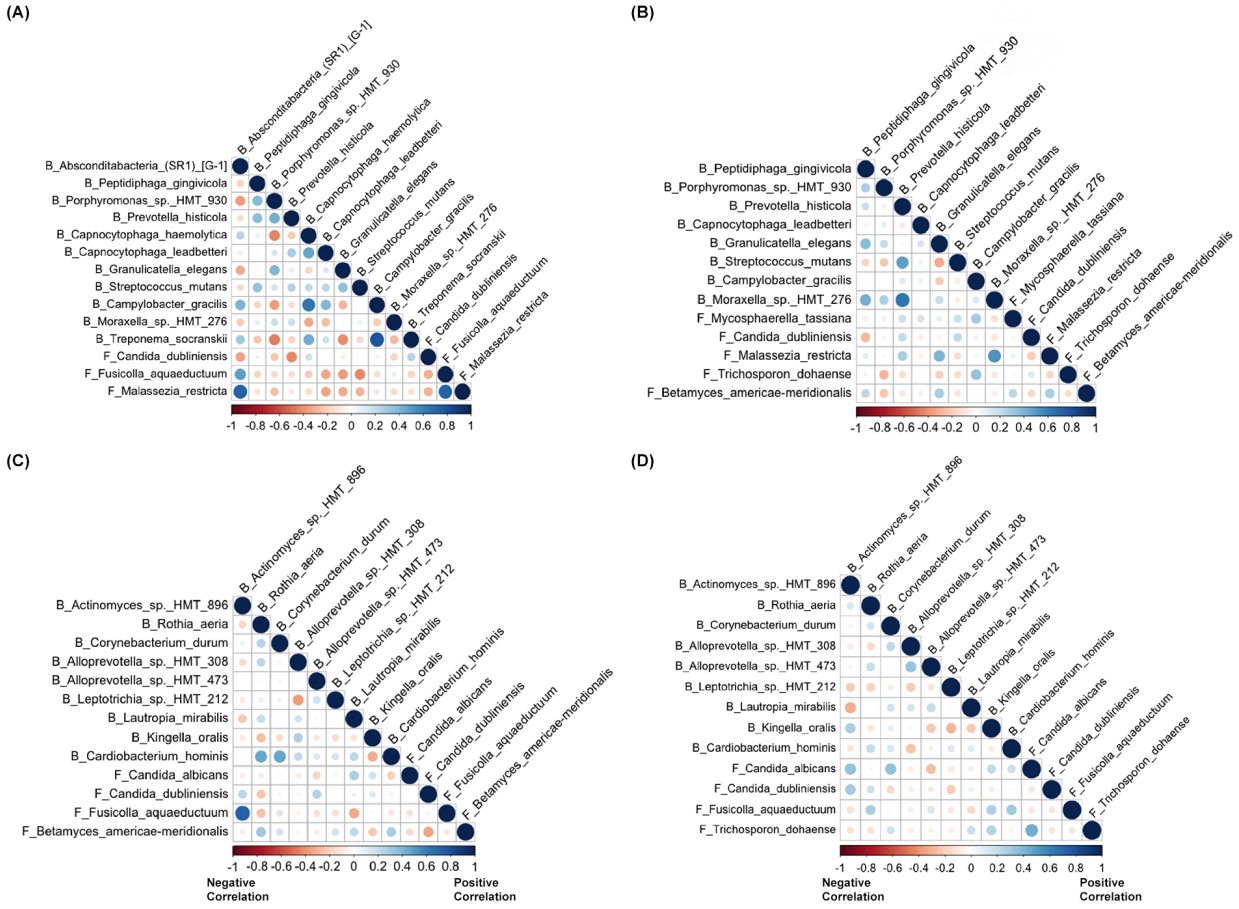
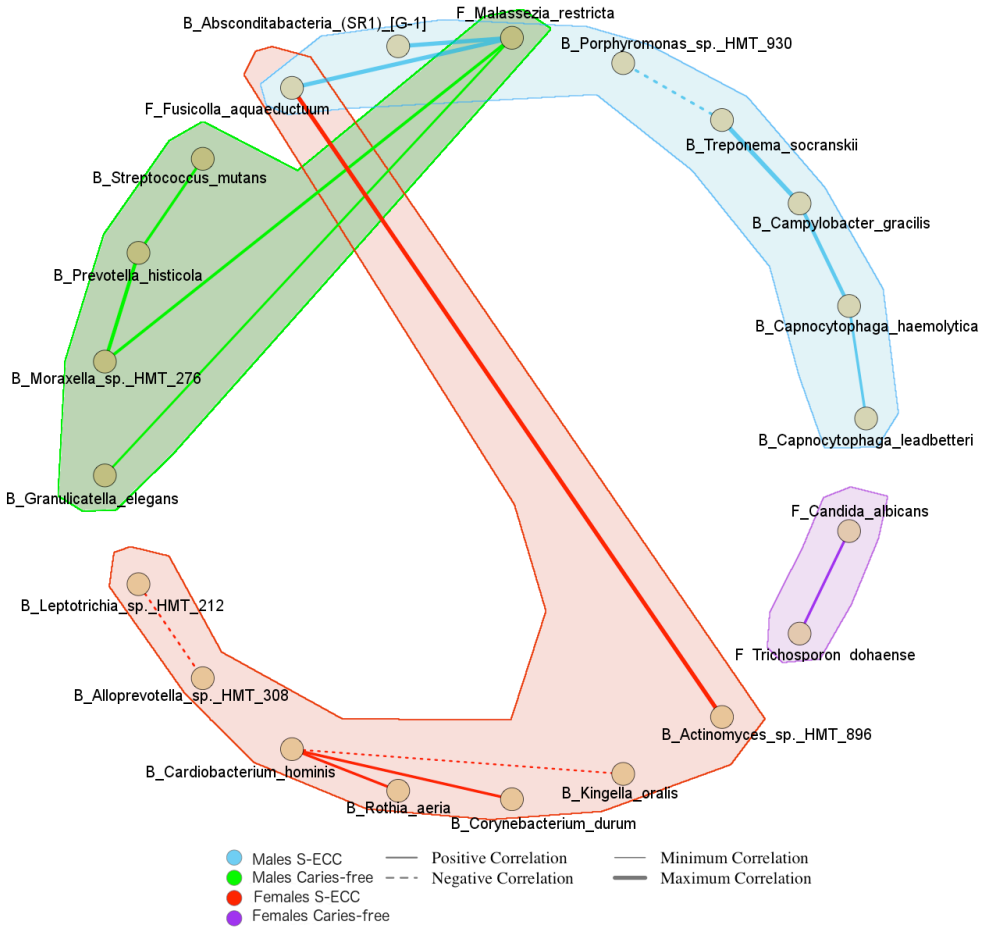

(A)
B_Absconditabacteria_(SR1)_[G-1]
B_Peptidiphaga_gingivicola
B_Porphyromonas_sp._HMT_930
B_Prevotella_histicola
B_Capnocytophaga_haemolytica
B_Capnocytophaga_leadbetteri
B_Granulicatella_elegans
B_Streptococcus_mutans
B_Campylobacter_gracilis
B_Moraxella_sp._HMT_276
B_Treponema_socranskii
F_Candida_dubliniensis
F_Fusicolla_aquaeductuum
F_Malassezia_restricta
-1 -0.8 -0.6 -0.4 -0.2 0 0.2 0.4 0.6 0.8 1
(B)
B_Peptidiphaga_gingivicola
B_Porphyromonas_sp._HMT_930
B_Prevotella_histicola
B_Capnocytophaga_leadbetteri
B_Granulicatella_elegans
B_Streptococcus_mutans
B_Campylobacter_gracilis
B_Moraxella_sp._HMT_276
F_Mycosphaerella_tassiana
F_Candida_dubliniensis
F_Malassezia_restricta
F_Trichosporon_dohaense
F_Betamyces_americae-meridionalis
-1 -0.8 -0.6 -0.4 -0.2 0 0.2 0.4 0.6 0.8 1
(C)
B_Actinomyces_sp._HMT_896
B_Rothia_aeria
B_Corynebacterium_durum
B_Alloprevotella_sp._HMT_308
B_Alloprevotella_sp._HMT_473
B_Leptotrichia_sp._HMT_212
B_Lautropia_mirabilis
B_Kingella_oralis
B_Cardiobacterium_hominis
F_Candida_albicans
F_Candida_dubliniensis
F_Fusicolla_aquaeductuum
F_Betamyces_americae-meridionalis
-1 -0.8 -0.6 -0.4 -0.2 0 0.2 0.4 0.6 0.8 1
Negative Correlation
Positive Correlation
(D)
B_Actinomyces_sp._HMT_896
B_Rothia_aeria
B_Corynebacterium_durum
B_Alloprevotella_sp._HMT_308
B_Alloprevotella_sp._HMT_473
B_Leptotrichia_sp._HMT_212
B_Lautropia_mirabilis
B_Kingella_oralis
B_Cardiobacterium_hominis
F_Candida_albicans
F_Candida_dubliniensis
F_Fusicolla_aquaeductuum
F_Trichosporon_dohaense
-1 -0.8 -0.6 -0.4 -0.2 0 0.2 0.4 0.6 0.8 1
Negative Correlation
Positive Correlation

FIGURE 4.


B_Absconditabacteria_(SR1)_[G-1]
F_Malassezia_restricta
B_Porphyromonas_sp._HMT_930
F_Fusicolla_aquaeductuum
B_Treponema_socranskii
B_Streptococcus_mutans
B_Campylobacter_gracilis
B_Prevotella_histicola
B_Capnocytophaga_haemolytica
B_Moraxella_sp._HMT_276
B_Capnocytophaga_leadbetteri
B_Granulicatella_elegans
F_Candida_albicans
B_Leptotrichia_sp._HMT_212
F_Trichosporon_dohaense
B_Alloprevotella_sp._HMT_308
B_Actinomyces_sp._HMT_896
B_Cardiobacterium_hominis
B_Kingella_oralis
B_Rothia_aeria
B_Corynebacterium_durum
Males S-ECC
Males Caries-free
Females S-ECC
Females Caries-free
Positive Correlation
Negative Correlation
Minimum Correlation
Maximum Correlation