Original Article

Impact of Refined and Unrefined Sugar and Starch on the Microbiota in Dental Biofilm

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Aims and Objective: Sugar is not only associated with dental diseases but also, along with carbohydrates, is linked to various health issues including obesity, cancer, diabetes, heart, liver, and kidney-related diseases. At the same time, a polyphenol present in unrefined sugar and starch (UReSS) is shown to inhibit microbial growth and prevent biofilms and dental plaque. The question arises, “is sugar the causative agent for dental diseases, or is its refined form the cause?” The objective of this study is to conduct in-vivo studies of the impact of refined and unrefined sugar and starch on the microbiota of dental biofilm.

Materials and Methods: An in-vivo study was performed using saliva and dental biofilm samples collected from 75 healthy subjects. For this study, healthy volunteers (n = 75) were randomly divided into five groups and were given sweet meals either made with refined white sugar and white rice (ReSS) or with unrefined brown sugar and red rice (UReSS). This was followed by using or not using a polyphenolic mouthwash. Before and after 4 h of eating a sweet meal, the saliva and dental plaque were collected and the DNA was analyzed by 16S metagenomic sequencing. The results were expressed in fold change of bacteria from 0 to 4 h. Statistical analyses have been performed by logarithmic linear discriminant analysis (LDA), Student’s t-test, and Wilcoxon signed-rank test.

Results: Upon LEfSe and statistical analysis, in-vivo experiments clearly showed that UReSS significantly decreased bacteria associated with dental diseases. In contrast, ReSS showed a significant increase in Actinomyces, Streptococcus, and Selenomonas with a high LDA score (Log 4.2) and statistical significance (P < 0.003). Mouthwash significantly decreased bacterial taxa associated with diseases in both the ReSS and UReSS groups. The in-vivo study showed a significant increase and decrease in Streptococcus levels in refined and unrefined sugar groups, respectively.

Conclusion: In conclusion, polyphenols aid in the prevention of dental caries. This study recommends using polyphenol-rich unrefined sugars and carbohydrates for both oral and general health. This study is the first of its kind to bring awareness to the effects of refined and unrefined starch and sugars on the oral microbiota.

Keywords: Hygiene, jaggery, microbiota, mouth wash, oral health, polyphenols, refined sugars, sugars

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INTRODUCTION

The oral microbiome is comprised hundreds of microbial species that co-inhabit and functionally interact in oral biofilms, which later matures into dental plaque accompanied by inflammation, periodontitis, and systemic diseases.[11-2]

Polyphenols may exhibit its protective effect either by stimulating a salivary flow, inhibiting bacterial mechanisms, interfering with sugars fermentation, inducing bacterial competition within the dental biofilms, or by changing the biofilm pH by the release of alkali.[3-6]

This brings up the important question, “Are refined kinds of sugar and starch the culprit in tooth decay?”[7] Current research has proven that the obesity epidemic and major health issues had occurred during the industrial food era which had encouraged the increased intake of refined carbohydrates and sugars. Conversely, the consumption of whole grains (unrefined carbohydrates) is associated with a decreased risk of diseases mentioned earlier.[8]

Research has been done regarding daily sugar exposure and frequency, which has influence on dental plaque composition and dental caries.[9,10] However, a lower caries rate was found in hamsters eating unrefined sugar than in those eating refined sugar[11] that have phenol compounds[12] with inhibitory properties against the cariogenic bacteria Streptococcus mutans and S. sobrinus.[13] This is comparable to commercial antibacterial agents[14] and shows the potential importance of caries prevention by polyphenols.

Phenolic compounds from sugarcane obtained during sugar production include juice, molasses, and jaggery, which possess antibacterial activity against carcinogenic bacteria but not refined white sugar.[15-17]

Thus, the purpose of this investigation is to observe whether the microbiota (bacteria and fungi) is changed significantly by refined sugar and refined starch (ReSS) in comparison to unrefined sugar and starch (UReSS). The public cannot avoid sugar and the happiness derived from experiencing sweetness to prevent oral and other health diseases. Sugar in the form of glucose is also needed as an energy source. Therefore, it will be interesting to see whether UReSS is the way to use sugar and starch wisely without causing oral dysbiosis.

MATERIALS AND METHODS

Quercetin hydrate and all other reagents were obtained from Sigma-Aldrich. Red rice, jaggery, and white sugar were purchased online (www.gorganics.in and everybodywholefoods.com). A mouthwash was freshly prepared with 20% finely powdered dried sugarcane bagasse in water.

ETHIC STATEMENT

Though this is not a clinical trial but a preliminary research study, human consent approval was done. Before the beginning of the study, each participant and participant’s parent signed a human informed consent form for study participation and provided saliva and dental biofilm samples and also any personal data needed for the study. The parents and participants were informed about the consumption of sugar and starch meal and the potential damage caused by using the mouthwash immediately. Ethical approval was received from the School SRC Committee, and all experiments were performed following relevant guidelines and regulations (www.societyforscience.org/isef). Besides, subjects and their parents were advised on the way to perform the Institutional Review Board (IRB)-approved procedures.

STUDY DESIGN AND ORAL-CLEANING METHODS

All the 75 healthy subjects (40 boys and 35 girls, 10–12-year old) were randomly divided into groups (N = 15 for each group). To reduce variations due to dietary factors, all subjects were given the same breakfast (25% protein, 10% fat, and 65% refined carbohydrate) on the day of the experiment [Figure 1]. Except for the control group, all the subjects were served one of the two sugary-starch sweet meals: (A) 70% refined starchy rice + 25% refined white sugar and 5% clarified butter and (B) 70% unrefined red rice + 25% unrefined brown sugar (jaggery) and 5% clarified butter, at the end of the breakfast. Another two groups are (A) + mouthwash and (B) + mouthwash. The control group is served breakfast without a sweet meal. Saliva samples and dental biofilm samples were collected at time 0 from all the volunteered subjects before the sweet meal was served. The subjects were refrained from cleaning their mouth nor eating or drinking water or any food for 4 h till the experiment was done. Subjects of groups 3 and 5 cleaned their mouth with a 15 mL volume of mouthwash (for 1 min). After 4 h again the saliva and dental biofilm samples were collected from all the group members. The faces of the teeth used for the study were incisal and facial. The dental biofilm was collected from canine teeth. The subjects were asked to spit approximately 1 mL of saliva into a vial containing lysis buffer (Fast ID DNA Extraction Kit, Genetic-ID, Fairfield, IA, USA). Dental biofilm samples were collected by using a toothpick and then dropped into a vial containing lysis buffer (Fast ID DNA Extraction Kit, Genetic-ID, Fairfield, IA, USA).
DNA Extraction Kit). The samples were frozen until DNA extraction was performed.

**Inclusion factors**
The volunteers (10–12 years old) were checked by a dentist and those having healthy teeth were chosen for the study.

**Exclusion factors**
Any volunteers possessing problems with teeth were eliminated from the study.

**Oral hygiene procedure**
Toothpaste brushing and tongue cleaning were done twice a day. Volunteers were instructed to brush with 100 mg of sodium fluoride-containing toothpaste. Then, tongue cleaning was done by a curved, stainless steel scraper for gently cleaning the tongue five times and thorough rinsing of the oral cavity three times with water.

**Method of preparation of sweet meal**
The sugar-to-rice ratio of 25% was the same for making the ReSS and UReSS sweet meals. White or red rice was soaked and cooked. Sugar was melted by heating in water and mixed with cooked rice and stirred well.

**DNA extraction**
The saliva tubes were homogenized with 0.1 mm zirconium beads (Research Products International Corp.) using the BioSpec Mini-Bead Beater for 30 s at a minimal speed. The samples were then subjected to a Fast ID Magnetic DNA Extraction Kit. About 200 ng of DNA was used for real-Time PCR (qPCR) and bacterial profile analysis. qPCR was performed via Taqman Universal PCR Master Mix (Applied Biosystems).

**Genomic analysis**
A metagenomic sequencing library was endowed via amplification of variable regions 3 and 4 (V3-V4) of the 16S rRNA gene. Illumina MiSeq was used for sequencing the V3-V4 amplicon from both ends. Randomly 20 samples from five individuals were used for sequencing. QIIME (v1.9.1) and Green-genes databases were used to assign bacterial taxa. Sequencing libraries for *in-vivo* samples were prepared by UBiota. Amplification of the variable regions V3 and V4 of the bacterial small ribosomal subunit (16S) was done and sequenced using Illumina MiSeq from both the ends.

Percent change was calculated from the change in the abundance levels of bacteria before and at the end of the 4 h after eating the sweet meal, made either from the ReSS or the UReSS.

**Statistics**
LEfSe (version 1.0) was used to detect differentially abundant bacteria using the online Galaxy (http://huttenhower.sph.harvard.edu/galaxy/). The threshold

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**Figure 1:** Experimental design. The process flow of the sample collection.
on the logarithmic linear discriminant analysis (LDA) score for discriminative features was set to 2.0 along with effect size measurements (LEfSe). LEfSe was used to identify the microbial components whose sequences were more abundant in the refined sugar and carbohydrates than in those of the unrefined group and the control group. Student’s t-test was used to identify significant differences between the different groups (P < 0.05).\[21\] Fold change (FC) of bacterial taxa from zero time to 4 h after eating the unrefined and refined sweet meal was calculated in Excel. In the initial stage of mature biofilm, the doubling of the bacterial population happens in 1–2 h.\[22\] Hence, 4 h is sufficient to detect significant variation in the dental biofilm microbiota. Statistical analysis using Kruskal–Wallis or Wilcoxon signed-rank test was performed using Prism GraphPad (GraphPad Software, Inc., USA).

**RESULTS**

This study characterizes the change in the microbial flora present in the saliva and dental biofilm of subjects within 4 h of consuming ReSS or UReSS [Figure 1]. The bacterial distribution was characterized in respect of the relative taxonomic abundances. A total of 14 phyla and 4500 genera and species (class, order, and family were studied) were detected in the saliva and dental biofilm samples.

**DENTAL BIOFILM**

To determine the variations in dental microbiota composition between the ReSS and UReSS, LDA coupled with effect size measurements (LEfSe) was applied to determine which taxa were enriched in the different groups according to the metagenomic analysis of the microbiome.

After 4 h, the dental biofilm samples of the control group had a healthy normal flora with an LDA LOG of 3–4 in Enterobacteriales, Enterobacteriaceae, Radyrhizobiaceae, Pseudomonadaceae, Oxalobacteraceae, Solirubrobacterales, Sinobacteriales, Gaiellales, Gaiellaceae, Comamonadaceae, Kselleaceae, Rhizobiaceae, Nakamurellaceae, Terobacteriaceae, Micrococcaceae, and Peptostreptococcaceae [Figure 2(a) and (b)].

**Figure 2:** The microbial communities of dental biofilm samples. 1—Control, 2—refined group (ReSS), 3—ReSS+mouthwash, and 4—unrefined group (UReSS) were analyzed using LDA Effect Size (LEfSe) algorithm to determine the optimal characteristic taxa and rank them according to the effect size (a). Only group 5 (UReSS + mouthwash) did not have a significant LDA score of more than log 2.
The group that did not use polyphenolic-containing mouthwash after eating a sweet meal containing refined sugar had bacteria that are involved in caries and periodontitis with an LDA score of LOG of $>4$ in *Actinomyces* ($P < 0.003$) and more than 1000-fold increase. *Selenomonas* had more than a log score of 3.8 with $P < 0.01$ and 159-fold differences. In the ReSS group, *Streptococcus* had a 9-fold increase ($P < 0.03$) and *Fusobacterium* had more than a 1000-fold increase ($P < 0.005$) [Table 1]. *Prevotella*, Gemellaceae, Leptotrichia, Campylobacter, Clostridiales, and Neisseriaceae were others, which are statistically increased taxa in the ReSS group. *Acidobacteria* showed a significant decrease [Table 1].

The group that ate the UReSS dessert had bacteria with an LDA score of LOG of $>4$ in *Corynebacterium*, Campylobacteria, and Clostridiales, but none of them was statistically significant. In the UReSS group, *Weeksellaceae*, *SRI*, *Prevotella*, *s__nanseensis*, *Actinobacteria*, *Streptococcus*, *Porphyromonas*, *Prevotella*, Propionibacteriaceae, *Selenomonas*, *Noxia*, *Moxarella*, *Granulicatella*, Mogibacteriaceae, Aggregatibacter, and *Rothia* all showed a significant decrease. There was no substantial growth of any bacteria [Table 2].

Veillonellaceae, Bacteriaceae, Cardiobacteriaceae, Cardiobacterales, Staphylococcaceae, Bacteroidaceae, Lachnospiraceae, Coriobacteriaceae, Bacillaceae, Moryella, Bacteroides, TM7, Schwartzia showed log scores of more than 2 but was not statistically significant [Tables 1 and 2].

**Dental biofilm with mouth wash**

*Betaproteobacteria*, *Gemella*, and *Granulicatella* showed a statistically significant increase in the ReSS group after mouth wash [Table 3].

The group that used the polyphenolic-based mouthwash after eating a sweet meal containing unrefined sugar had no bacterial species. This could be due to mere cleanup or due to the polyphenol in the mouthwash. In the UReSS group after mouth wash, Enterobacteriaceae decreased and *Blautia* increased significantly [Table 4].

**Saliva**

No statistically significant differences were found when comparing the five groups in saliva [Figure 3]. In saliva samples, the LDA scores did not show much difference between the ReSS and UReSS groups compared with the dental biofilm samples. In saliva, the groups that ate the UReSS dessert had bacteria with an LDA score of LOG of $>3$ and were Carnobacteriaceae and *Granulicatella* (41% increase), whereas the control had Proteobacteria. Irrespective of ReSS or UReSS, there was no significance with and without mouthwash in saliva samples.

### Table 1: Changes in taxa in the refined group (ReSS)

| OTU*          | P-value | FC | LDA | Status |
|---------------|---------|----|-----|--------|
| Acidobacteria; o__Sva0725 | 0.003   | 259 |     | H      |
| Firmicutes; f__Ruminococcaceae | 0.3     | 2   |     | H      |
| Proteobacteria; g__Haemophilus; s__parainfluenzae | 0.3     | 2   |     | H      |
| Proteobacteria; f__Neisseriaceae | 0.003   | 10,559 | 4.3 | H      |
| Actinobacteria; g__Actinomyces | 0.003   | 3,184 |     | CP, EC |
| Fusobacteria; g__Fusobacterium | 0.005   | 2,890 |     | P      |
| Bacteroidetes; g__Prevotella | 0.03    | 519 |     | P      |
| Firmicutes; g__Selenomonas | 0.01    | 159 | 3.8 | CP     |
| Firmicutes; c__Bacilli; o__Gemellales; f__Gemellaceae | 0.005   | 115 |     | H      |
| p__Proteobacteria; g__Cardiobacterium | 0.3     | 32  | 3.2 | H      |
| Fusobacterium; g__Leptotrichia | 0.02    | 30  |     | P      |
| Proteobacteria; g__Moxarella | 0.1     | 24  |     | CP     |
| TM7; c__TM7-3; o__CW040 | 0.1     | 20  | 3   | P      |
| Proteobacteria; g__Campylobacter | 0.02    | 11  |     | G      |
| Firmicutes; g__Megasphaera | 0.1     | 10  |     | EC     |
| Firmicutes; o__Clostridiales | 0.05    | 10  |     | P      |
| Firmicutes; g__Streptococcus | 0.03    | 9   |     | P      |
| Bacteroidetes; o__Bacteroidales | 0.2     | 8   | 3   | P      |
| Proteobacteria; g__Neisseria | 0.1     | 5   |     | H      |
| Actinobacteria; g__Corynebacterium; s__durum | 0.1    | 3   |     | H      |

H = health, EC = early caries, G = gingivitis, CP = caries progression, P = periodontitis

*OTUs are analytical units that represent individual strains or species, as such more than one can be assigned to the same taxonomy. Here, FC is the fold change of bacterial taxa from 0 to 4 h after eating the unrefined sweet meal. A statistically significant increase is shown by the bold interface
Emerging microbiota data show that the consumption of processed carbohydrates affects the ecosystem of the mouth leading to dental caries. Conversely, consumption of whole grains (unrefined carbohydrates) is associated with a decreased risk of the diseases mentioned earlier. This is because, during the refinement of foods, the polyphenols are lost in the refining process. Polyphenols influence microbial signaling pathways which alter the interactions among the members of the ecosystem, leading to a healthy oral cavity, especially biofilm.

Ignoring these research studies, sugar is charged guilty of causing dental caries and periodontal diseases. Most of these studies are focussed on the effect of refined sucrose and starch and do not acknowledge that the

### Table 2: Changes in taxa in the unrefined group (UReSS)

| bacterial taxa | P-value | FC  | LDA | Status |
|----------------|---------|-----|-----|--------|
| Bacteroidetes; Weeksellaceae | 0.05 | 255 |     | P      |
| Fusobacteria; g__Fusobacterium | 0.1  | 53  |     |        |
| Proteobacteria; g__Actinobacillus; s__parahaemolyticus | 0.1  | 45  |     | H      |
| Proteobacteria; f__Neisseriaceae | 0.1  | 41  |     | H      |
| SR1; | 0.01  | 38  |     | P      |
| Actinobacteria; g__Actinomyces | 0.1  | 38  |     | CP, EC |
| Bacteroidetes; g__Prevotella; s__nanceiensis | 0.04  | 33  |     | H      |
| Proteobacteria; g__Acinetobacter | 0.001 | 18  |     |        |
| Fusobacteria; f__Leptotrichiaceae | 0.2  | 17  |     | P      |
| Firmicutes; g__Streptococcus | 0.01  | 17  |     | P      |
| Actinobacteria; g__Rothia; s__mucilaginosa | 0.1  | 13  |     | EC     |
| Bacteroidetes; o__Bacteroidales | 0.1  | 13  |     | P      |
| Proteobacteria; g__Eikenella | 0.1  | 12  |     | H      |
| Proteobacteria; g__Haemophilus | 0.2  | 12  |     | H      |
| Bacteroidetes; g__Porphyromonas | 0.04  | 12  |     | P      |
| Bacteroidetes; g__Prevotella | 0.02  | 10  |     | P      |
| Proteobacteria; f__Comamonadaceae | 0.1  | 9   |     | H      |
| Actinobacteria; g__Rothia; s__dentocariosa | 0.1  | 8   |     | P      |
| Actinobacteria; f__Propionibacteriaceae | 0.04  | 8   |     | CP     |
| Firmicutes; g__Selenomonas; s__noxia | 0.14  | 7   |     | P      |
| Proteobacteria; g__Enhydrobacter | 0.1  | 7   |     | H      |
| Proteobacteria; g__Moraxella | 0.03  | 6   |     | CP     |
| Firmicutes; g__Granulicatella | 0.03  | 6   |     | EC     |
| Proteobacteria; g__Cardiobacterium | 0.1  | 6   |     | P      |
| Proteobacteria; f__Rhodospirillaceae | 0.2  | 5   |     | H      |
| Firmicutes; f__Mogibacteriaceae | 0.04  | 5   |     | P      |
| Firmicutes; g__Selenomonas | 0.03  | 5   |     | P      |
| Proteobacteria; g__Lautropia | 0.1  | 5   |     | H      |
| Proteobacteria; g__Aggregatibacter | 0.05  | 5   |     | P      |
| Actinobacteria; g__Rothia; s__aeria | 0.01  | 5   |     | P      |
| Bacteroidetes; g__Prevotella; s__intermedia | 0.2  | 43  |     | P      |
| Bacteroidetes; g__Prevotella; s__melaninogenica | 0.2  | 23  |     | P      |
| Proteobacteria; g__Campylobacter | 0.2  | 15  | 3.8 | G      |
| Bacteroidetes; g__Capnocytophaga | 0.2  | 15  |     | EC     |
| Fusobacteria; g__Leptotrichia; s__ | 0.2  | 14  |     | P      |
| Firmicutes; g__Veillonella; s__dispar | 0.2  | 11  |     | EC     |
| Firmicutes; f__Lachnospiraceae | 0.2  | 6   |     | EC     |
| Bacteroidetes; g__Capnocytophaga; s__ochracea | 0.3  | 5   |     | P      |
| Actinobacteria; g__Corynebacterium; s__durum | 0.2  | 5   |     | H      |
| Firmicutes; o__Clostridiales | 0.3  | 2   | 3   | P      |

H = health, EC = early caries, G = gingivitis, CP = caries progression, P = periodontitis

*OTUs are analytical units that represent individual strains or species, as such more than one can be assigned to the same taxonomy.

Here, FC is the fold change of bacterial taxa from 0 to 4 h after eating the unrefined sweet meal. A statistically significant increase is shown by the bold interface.
difference is due to the frequent or increased use of refined sugar rather than the use of UReSS. Thus, the purpose of our investigation was to find whether the presence of refined (ReSS) and unrefined (UReSS) sugar and starch influence any significant change in oral microflora.

As most of the dental studies were done over long periods about disease progression, this study focussed on the effect of refined and unrefined sugars in a relatively short time of 4 h. This will help understand the initial changes in the microbiota in biofilms formation. We have used healthy subjects in this study that showed no signs of dental diseases but may have some established biofilms. This will give research data on how refined and unrefined sugars cause dysbiosis in microbiota in biofilm, leading later to oral diseases.

We have used LEfSe, a strict tool to identify genera that are differentially distributed in the five groups who had consumed a sweet meal that was either made of the refined or unrefined form of starch and sugar. The statistically significant differentially abundant taxa (LDA >2) can be considered as potential biomarkers of enriched bacteria in a group [Figure 2 and Tables 1–4]. All these analyses showed a pattern that in the UReSS group and the control group, there were fewer carcinogenic taxa than in the ReSS groups.

LDA score was very high (Log 4.3) for Actinomycetes in the ReSS group and showed 1000-fold differences (P < 0.003) [Figure 2 and Table 1], which are significantly increased in dental caries. However, the Actinomyces were not significantly increased.

### Table 3: Changes in taxa in the refined group (ReSS) and mouthwash

| OTU*                                      | P-value | FC | STATUS |
|-------------------------------------------|---------|----|--------|
| Proteobacteria; g__Kingella;s__           | 0.2     | 20 | H      |
| Firmicutes; f__Tissierellaceae            | 0.3     | 15 |        |
| Proteobacteria; g__Neisseria;s__cinerea   | 0.2     | 11 | H      |
| Proteobacteria; f__Pseudomonadaceae       | 0.1     | 11 | EC     |
| Proteobacteria; f__Comamonadaceae         | 0.3     | 9  | H      |
| Actinobacteria; g__Rothia;s__mucilaginosa  | 0.2     | 7  | H      |
| Firmicutes; g__Dorea                      | 0.1     | 7  |        |
| Bacteroidetes; g__Prevotella;s__nigrescens| 0.2     | 7  | P      |
| Actinobacteria; g__Rothia                 | 0.2     | 6  | H      |
| Firmicutes; g__Oscillospira               | 0.3     | 5  |        |
| Actinobacteria; f__Coriobacteriaceae      | 0.2     | 5  | H      |
| Proteobacteria; g__Desulfovibulus         | 0.3     | 2  | P      |
| Firmicutes; g__Lachnospira                | 0.3     | 2  | EC     |
| Proteobacteria; g__Neisseria;s__subflava  | 0.2     | 2  | H      |
| Firmicutes; f__Aerococcaceae              | 0.2     | 2  |        |
| Proteobacteria; c__Betaproteobacteria     | 0.03    | 2  |        |
| Firmicutes; g__Gemella                    | 0.04    | 6  | H      |
| Proteobacteria; f__Sphingomonadaceae      | 0.1     | 6  |        |
| Firmicutes; f__Ruminococcaceae;g__Ruminococcus| 0.1    | 6  | H      |
| Proteobacteria; g__Actinobacillus;s__porcinus| 0.1   | 4  |        |
| Firmicutes; g__Peptostreptococcus         | 0.1     | 4  | P      |
| Firmicutes; f__Acidaminobacteraceae       | 0.2     | 3  |        |
| Bacteroidetes; g__Prevotella;s__pallens   | 0.1     | 3  | P      |
| Bacteroidetes; g__Prevotella;s__copri     | 0.1     | 3  | P      |
| Firmicutes; f__Veillonellaceae;g__        | 0.1     | 3  | P      |
| Firmicutes; f__Streptococcaceae           | 0.2     | 3  | P      |
| Proteobacteria; g__Moraxella              | 0.2     | 2  | CP     |
| Firmicutes; _Faecalibacterium             | 0.3     | 2  |        |
| Firmicutes; g__Coprococci                 | 0.3     | 2  |        |
| Firmicutes; g__Faecalibacterium;s__praunziitii| 0.3  | 2  |        |
| Proteobacteria; o__Pseudomonadales        | 0.2     | 2  |        |
| Firmicutes; g__Granulicatella             | 0.05    | 2  | EC     |

H = health, EC = early caries, CP = caries progression, P = periodontitis

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in the UReSS group ($P < 0.1$). Many studies report that *Actinomyces* and *Streptococci* are the two predominant bacteria in biofilms collected within the first 6 h of biofilms formation. In the UReSS group, *Streptococcus* significantly decreased 17-fold differences ($P < 0.01$), whereas there was a 9-fold significant increase ($P < 0.03$) in the ReSS group. Both *Streptococcus* and *Actinomyces* are the early colonizers of the tooth surface and also frequently co-aggregate with each other as well as with other oral bacteria in the development and maturation of plaques.\(^{[26]}\) These bacteria then provide the substratum for the later colonizing bacteria.\(^{[27]}\)

**Table 4: Changes in taxa in the unrefined group (UReSS) and mouthwash**

| Taxa                                      | $P$-value | FC  | Status |
|-------------------------------------------|-----------|-----|--------|
| Fusobacteria                              | 0.2       | 9   | P      |
| Firmicutes; Ruminococcus                  | 0.2       | 8   | H      |
| Bacteroidetes; g__Bacteroides;s__uniformis | 0.2       | 7   |        |
| Fusobacteria; f__Leptotrichiaceae         | 0.2       | 7   | P      |
| Firmicutes; g__Coprococcus                | 0.2       | 5   |        |
| Proteobacteria; g__Neisseria              | 0.2       | 5   |        |
| Actinobacteria; g__Bifidobacterium        | 0.1       | 5   |        |
| Proteobacteria; f__Enterobacteriaceae     | 0.02      | 4   |        |
| Actinobacteria; g__Bifidobacterium;s__longum | 0.3     | 4   |        |
| Firmicutes; g__Faecalibacterium;s__prausnitzii | 0.3     | 2   |        |
| Actinobacteria;c__Actinobacteria           | 0.3       | 2   |        |
| Proteobacteria                            | 0.3       | 2   |        |
| Firmicutes; g__Blautia                    | 0.02      | 25  | H      |
| Actinobacteria; g__Collinsella            | 0.1       | 8   |        |
| Actinobacteria; g__Collinsella;s__aerofaciens | 0.1     | 7   |        |
| Firmicutes;c__ Bacilli                    | 0.1       | 6   |        |
| Firmicutes; Lactobacillales               | 0.1       | 5   |        |
| Proteobacteria; o__Burkholderiales        | 0.2       | 3   |        |
| Bacteroidetes                             | 0.1       | 3   |        |
| Firmicutes; f__Lachnospiraceae            | 0.2       | 2   |        |
| Bacteroidetes;c__Bacteroidia;o__Bacteroidales | 0.1   | 2   |        |

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**Figure 3:** The microbial communities of saliva samples. 1—Control and 4—UReSS were analyzed using LDA Effect Size (LEfSe) algorithm to determine the optimal characteristic taxa and rank them according to the effect size (a). Only groups 1 and 4 had significant LDA scores of more than log 2.

**Saliva**

In saliva samples, no distinguishable differences were found when comparing the five groups. This may be due to the fact that the highly diverse bacterial population is rapidly moving and constantly swallowed, and the change is not noticed in a short time. However, the plaque and biofilm are static and so the change in them is more prominent in a short time of 4 h.

**Effect of polyphenolic mouthwash unrefined sugar and starch on microbiota**

There were no significant bacteria taxa in the UReSS group, both in the LEFSE and statistical analysis [Figure 2 and Table 4]. This shows that mouthwash was more effective in the UReSS group than in the ReSS group. This could be due to the additive effect on the inhibition of bacterial growth due to polyphenols present both in sweet-meal and in mouthwash and or due to the oral cleaning effect or both.

The use of 4 h to give a sugar pulse may sound like a limitation of this study; however, it shows preliminary data that dysbiosis can still happen even in a single meal. This prompts to assess the potential risks of consuming processed and hydrolyzed starches vs. the preventive aspect of polyphenolics-rich UReSS. This vision will force to redirect preventive efforts toward modifying dietary patterns in the global...
population at large. Another limitation of this study is the involvement of a small group of 75 subjects. Therefore, a larger group with longer sugar exposure time is proposed in the future.

The strength of this study is the experimental design which took advantage of evaluating the effect of polyphenol on both the causation (eating) and the prevention (polyphenolic-mouthwash) in a short period of 4 h on biofilm microbiota. Therefore, oral cleaning is very important for oral microbiota apart from dietary factors such as sugar. Several research studies conclude that frequent disruption of biofilm is the key to preventing and treating caries and periodontal diseases along with other systemic diseases.\(^{(28,29)}\)

To the best of our knowledge, this is the first study that uses the methodological benefits of high-throughput sequencing to analyze the change in dental biofilms microbiota \textit{in vivo}, having the impact on oral microbiota in the presence of refined and unrefined sugars. Besides, this report has a clinical and political significance by providing a scientific basis for advising a new dietary recommendation of using UReSS to prevent oral caries. It is inevitable for the public to avoid sugar. Therefore, instead of a ban on sugars to curb obesity and dental and systemic diseases, this study will help policymakers for a practical approach to allow the enjoyment of eating wisely with the unrefined form of sugar.

**CONCLUSION**

In conclusion, this study showed that the microbiota from dental biofilm samples was distinct in the ReSS than in the UReSS groups. Therefore, we recommend using phytochemical-rich unrefined sugars and carbohydrates for both oral and general health. Unrefined sugar should still be consumed in moderation from the calorie point of view, but it is still a healthy choice from the microbiota point of view, and the polyphenol present in it has anticaresis activity. This study is a paradigm shift in oral health research and is the first in bringing awareness to the effects of ReSS and UReSS on the oral microbiota. The practical approach to help control caries is to enjoy eating wisely in the unrefined form of sugar.

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**CONFLICTS OF INTEREST**

There are no conflicts of interest.

**AUTHORS CONTRIBUTIONS**

Not applicable.

**ETHICAL POLICY AND INSTITUTIONAL REVIEW BOARD STATEMENT**

Not applicable.

**PATIENT DECLARATION OF CONSENT**

Not applicable.

**DATA AVAILABILITY STATEMENT**

Not applicable.

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