Effect of Different Sweeteners on the Oral Microbiota and Immune System of Sprague Dawley Rats

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Abstract

Sucrose, xylose, and saccharin are common beverage additives used by humans. Long-term consumption of beverages containing these substances will inevitably affect the oral immune system and the composition of healthy oral microbiomes. In this study, we used 24 Sprague Dawley rats and divided them into four groups. Each group was fed water containing a specific dose of sucrose, saccharin, xylose, or pure water 12 hours a day for eight weeks. To determine the changes in composition, community structure, and functions of the oral microbiomes of rats, we collected oral microbiome samples and subjected them to high-throughput sequencing at the end of eight weeks. Meanwhile, ELISA was performed using the saliva samples from rats to estimate the concentrations of salivary immunoglobulin, to reveal the effect of sweetener on the oral immune system. Sequencing results showed that Firmicutes and Proteobacteria, were the predominant phyla in all the groups. In additions, we found that the oral microbial diversity of rats drinking sucrose water was significantly higher than that of rats in the other groups. Our results indicate that drinking water containing sweeteners could influence oral immunity as well as the composition, metabolic function, and diversity of the oral microbiota, thereby disrupting the original oral micro-ecosystem.

Introduction

The oral microbiota is an essential component of the human body. It has the potential to affect the health of the host by regulating immune functions, participating in food metabolism, and competing with exogenous pathogenic microbiota (Dewhirst et al. 2010). Changes in the oral microbiota may have unpredictable effects on host health. Sucrose, xylose, and saccharin are common beverage additives used by humans. These sweeteners, irrespective of their types, inevitably affect the delicate and intricate human oral microbiota (Wade et al. 2013). Many previous studies have focused on the effects of sweeteners on the intestinal microbiota (Suez et al. 2014), but little attention has been paid to oral microbiota. Oral immunoglobulins are vital components of the human oral immune system (Berglund et al. 1971). Some oral diseases are closely associated with the shift in oral immunoglobulin concentration (Kaufman et al. 2000; Sistig et al. 2002; Jensen et al. 2008; Helfand et al. 1996). Sucrose plays a role in increasing appetite and nutrition, which makes it a favorite beverage additive since ancient times. However, numerous studies have shown that long-term intake of sucrose can cause serious diseases, such as diabetes, obesity, and caries (Khiraoui et al. 2018). Other sweeteners, such as xylose and saccharin, cannot be broken down by the human’s digestive enzymes, are not absorbed by the gastrointestinal tract, hence, excreted directly through the kidneys and intestine (Chattopadhyay et al. 2014). Therefore, xylose and saccharin are being increasingly used in beverages as alternatives to sucrose. In addition, recent studies have reported that xylose and saccharin have destructive effect on the gut microbes and micro-ecological environment (Martínez-Carrillo et al.2019; Inan-Eroglu et al. 2019). Moreover, there are very few studies on the effects of sucrose, xylose, and saccharin on oral microbiota. Herein, we aimed to focus on this aspect in our research.
The aim of this study was to explore the effects of sucrose, xylose, and saccharin on oral microbiota and oral immunity. To this end, we fed SD rats with specific dosages of sucrose, xylose, and saccharin water for eight weeks (the rats were fed water containing additives 12 hours/day *ad libitum*). Rats were anesthetized after 8 weeks of feeding, and saliva and oral microbiome samples were collected. 16S rRNA analysis was performed to reveal the differences in community structure, metabolic function, and species composition of the oral microbiota in the four groups of rats and to identify common or unique bacterial species in all samples. ELISA was used to estimate the concentrations of IgG, IgE, IgM, and SIgA. We found that the oral immune system and microbiota were affected by sucrose, xylose, and saccharin. The sweeteners affected the oral microenvironment and disrupted the balance of oral micro-ecology. All these changes were inextricably linked to host oral health.

**Material And Methods**

**Animals and experimental design**

Six-week-old female SD rats were purchased from Dashuo Company (Chengdu, Sichuan Province, China) and maintained under the following conditions: temperature of the animal room controlled at 14-22 °C, humidity between 40-70 %, and light: dark cycle of 12:12 h. The rats were randomly divided into four groups: control, sucrose, xylose, and saccharin (six rats and one cage per group). The animals were fed *ad libitum* (Dashuo Company). With reference to previous experiments (Uebanso et al. 2017), according to the principle of low-dose sweetener consumption, rats in different groups were provided with drinking water containing 0.83 mg/mL xylose, 0.83 mg/mL sucrose, or 0.83 mg/mL saccharin for 12 h/d, and purified water was provided for another 12 h. After eight weeks of feeding, all rats were anesthetized, and saliva samples and oral microbiota samples were collected (from under the tongue, palate, and upper throat of the rats’ oral cavity). All samples were kept with dry ice under sterile conditions until they were shipped for testing. All experiments were approved by the Ethics Committee of Leshan People's Hospital (the approval reference number is IACUC Issue No. LH20191004). Throughout the experiment, all animals were handled according to the standard procedures, and no abuse occurred.

**ELISA**

Enzyme-linked immunosorbent assay (ELISA) was performed to measure the SIgA, IgE, IgM, and IgG contents in the saliva samples of the four groups of rats according to the manufacturer’s protocol (Tengfei Inc., Shenzhen, China). Four 24-well culture plates were used for the four saliva samples (Tengfei Inc.). Each experiment was repeated three times, in order to ensure accuracy of results. Saliva samples from the control, xylose, saccharin, and sucrose groups were added to sample wells at a volume of 10 µL, followed by the addition of 40 µL of diluent. Next, add 100uL of horseradish peroxidase (HRP)-labeled primary antibody to the wells, seal all reaction wells, and place them in an incubator at 37°C for 60 minutes. Discard the supernatant after 60 minutes, and absorb the excess liquid with absorbent paper. Finally, the washing solution was added to each well for 1 minute, and blotted dry. Repeat this step five
times. Then, the substrate 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well and incubated at 37 °C in the dark for 15 min. The reaction was terminated by adding 50 µL of stop solution to each well.

**DNA isolation, PCR amplification, and MiSeq sequencing analysis**

Following the manufacturer’s protocol, the E.Z.N.A@soil DNA Kit (Omega Bio-Tek, Norcross GA, USA) was used for microbial DNA isolation from the oral microbiome samples of rats. A NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, USA) was used to determine the final concentration and purity of DNA. Microbial DNA quality check was running on a 1% agarose gel. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene can be amplified with a thermocycler PCR system by using 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) as primers. PCR amplification conditions were shown as follows: initial denaturation at 95°C for 3 min; denaturation at 95°C for 30 s (27 cycles), temperature reduction at 55°C, annealing for 30 s, extension at 72°C for 45 s (27 cycles), finally extension at 72°C for 10 minutes. The total volume of PCR mix was 20 µL and consisted of several components: 0.4 µL of FastPfu DNA polymerase (TransGen Biotech Co., Beijing, China), 2.5 mM dNTPs (2 µL), template DNA (10 ng), 5 µM of each primer (0.8 µL), and 5× FastPfu buffer (4 µL). PCR products were separated on 2% agarose gel and then extracted using the AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, USA). Finally, the purified PCR products were quantified using QuantiFluor-ST (Promega, USA). Purified amplicons were pooled in equimolar concentrations and paired-end sequenced (2×300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocol of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). All data were uploaded to the National Center for Biotechnology Information official website (Accession Number: PRJNA607824).

Operational taxonomic units (OTUs) were clustered using software UPARSE (version 7.1 [http://drive5.com/uparse/) under condition 97% similarity cut off, and chimeric sequences were identified and removed by UCHIME. Using the RDP Classifier algorithm (version 11.5, http://rdp.cme.msu.edu/) to analysis the taxonomy of each 16S rRNA gene sequence. Against the Silva (SSU123) 16S rRNA database using a confidence threshold of 70%.

**Ecological and statistical analyses**

In order to accurately identify and describe the microbiota in the 24 samples and the corresponding sequencing data, we used the cloud platform (http://www.majorbio.com/) to draw a sparse curve for each sample. The mothur software (version 1.30.2) was used to calculate the depth (coverage) of the sequenced samples, as well as some microbial indices, such as sobs, Simpson, Shannon-Wiener, and alpha diversity, were used to describe the microbial diversity of the samples. The principal coordinate analysis of the microbial composition based on the OTU level was calculated and plotted using the R (programming language) software package (version 3.3.2). Venn diagrams were drawn using the R software package to reveal the unique and shared oral microbiota in all samples. Considering the statistical differences and biological relevance, linear discriminant analysis effect size (LEfSe) was used to identify the differential expression of OTUs of the oral microbiome from the four groups of rats. One-
way analysis of variance (ANOVA) was used to analyze the differences among the samples. $p < 0.05$ was considered statistically significant.

Functional prediction

We used PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved States) to predict the functional features of microbiomes in all the samples. PICRUSt was operating on the majorbio free cloud platform (www.majorbio.com) against the KEGG (Kyoto Encyclopedia of Genes and Genomes) database. The abundances of functional composition in each group were calculated. The results generated in a box diagram of COG functional classification statistics.

Results

ELISA

Immunoglobulins are a class of immune-active molecules that are the most critical components of the immune system. Oral immunoglobulins include varying concentrations of SIgA, IgG, IgM, IgD, and IgE. Herein, we performed ELISA only for SIgA, IgG, IgM, and IgE in rat saliva, to analyze the changes in immunoglobulins affected by different sweeteners. ELISA results are shown in Figs. 1A, 1B, and 1C. IgA, IgM, and IgG were detected in all saliva samples. IgE was not detected in any of the samples, probably due to its low concentration in saliva. The mean concentrations of SIgA and IgG were higher in the saliva of rats belonging to the sweetener feeding groups (saccharin group, xylose group, and sucrose group) than in the control group (Figs. 1A and 1B). For IgM, the mean concentrations in the saliva of the four groups were not significantly different (Fig. 1C). These results are closely resembling the characteristics of the three immunoglobulins. Particularly, SIgA and IgG are present at the highest concentrations in the saliva and are more vulnerable to external influences (Brandtzaeg et al. 2007). IgM increases mainly during the process of oral infection (Balmaseda et al. 2003). Our results indicate that xylose and sucrose may have analogous effects on the oral immune system.

Taxonomic composition

After denoising the denoisers of the 24 samples, an average of 39,987 sequences were obtained from the 24 samples after denoising. The Shannon curves, Simpson curves, Rarefaction curves, Coverage curves, and diversity index of each sample at a genetic distance of 3% are showed in Supplementary Materials (details, see Figs. S1-S4, and Table S1). Our sequencing data could be divided into 1,057 OTUs, and the content of each sample ranged from 410 to 739. In the saliva samples of the 24 rats, there were only Firmicutes and Proteobacteria found to exist in all saliva sample. Proteobacteria was the predominant phylum, accounting for 36.71-60.91 % of the 16S rRNA gene sequences (Fig. 1D). The composition of Firmicutes was between 20.42 % - 56.53 % (Fig. 1D). Investigating the genera of the total oral microbiota of all rats, the top four most abundant genera are listed in Table 1. Based on the bacterial genus data, detected OTUs were distributed 496 different genera (Fig. 1E). In the xylose group, 284 genera were detected, and 186 genera were detected in the saccharin group, while the most diverse community is the
sucrose group with 372 genera. For the relative abundance of oral microbiota at the phylum level, there were significant differences observed among the four groups of rats (Fig. 2A). The relative abundance of Rodentibacter, Streptococcus, and Rothia also showed obvious differences at the genus level (Fig. 2B). The sucrose group showed the highest bacterial community richness (measured by the total number of observed OTUs) among the four groups. (Fig. 2C, \( p < 0.005 \)). Bacterial community diversity was measured using the Shannon index. Consistently, the sucrose group showed significantly higher diversity compared to the control, xylose, and saccharin groups (Fig. 2D).

**Unique and shared bacterial taxa**

Next, we examined the unique and shared bacterial taxa between the oral microbiota of rats from the four groups (xylose, saccharin, sucrose, and control) using our sequencing data. The shared 257 OTUs from the 24 samples are shown in Fig. 3A. We identified differentially distributed OTUs among the rats from the four groups using LEfSe. Among the 257 shared OTUs, the top ten OTUs were 823, 796, 236, 915, 502, 650, 131, 293, 315, and 225 (Fig. 3B). Among them, OTU 225, which belongs to the phylum Actinobacteria and genus Rothia, was the dominant species in the control group (Fig. 3C). OTU 502, which belongs to the phylum Firmicutes and genus Streptococcus, was the major component of the sucrose group (Fig. 3D). In the saccharin group, OTU 236, which belongs to the genus Rodentibacter, was relatively more abundant (Fig. 3E). OTU 650, which is related to phylum Proteobacteria, genus Acinetobacter, was dominant in the xylose group (Fig. 3F).

**Community structures and functional predictions**

Principal coordinate analysis (PCoA) was used to detect the community structure of the rat oral microbiota. In Fig. 1F, each symbol represents a sample of the oral microbiota. Red, green, yellow, and blue dots represent the oral microbiota in the control, saccharin, xylose, and sucrose groups, respectively. Fig. 1F shows that bacterial communities in the control and sucrose groups were tightly clustered on the principal coordinate and separated from the oral bacterial community of rats in the xylose and saccharin groups represented along the central parallel axis 1 (PC1). This result was shown to have the most significant variable (35.25 %). Based on the results of 16S RNA sequencing, we mapped most detected microbial functional in all samples by PICRUS metagenome prediction (Fig. 4). It can be observed that the function profiles of control, sucrose, and xylose group were similar to each other. Amino acid transport and metabolism, Carbohydrate transport and metabolism, and Translation, ribosomal structure and biogenesis were the most abundant functions in all samples. Nevertheless, a few functional changes were observed in the saccharin group. For instance, compared with other three groups, in the saccharin group, the relative abundance of some function profiles was decreased, and these functional features included: Amino acid transport and metabolism, Translation, ribosomal structure and biogenesis, Carbohydrate transport and metabolism, Inorganic transport and metabolism, Cell wall/membrane/envelope biogenesis, Replication, recombination and repair, Transcription, and Energy production and conversion.
Discussion

Although xylose and saccharin have long been considered safe, research on intestinal microbiota reveals that they can cause glucose intolerance and interfere with the body’s energy homeostasis (Gerasimidis et al. 2019). In our study, at the phylum level, the number of Proteobacteria increased significantly in the saccharin group, and Firmicutes and Bacteroidetes were abundant in the xylose group among all samples. Previous studies have shown that consumption of xylose and saccharin leads to excessive growth of Bacteroidetes in the mouse gut, causing gut immune system disorders and eventually, systemic inflammation (Shin et al. 2015). In our study, we observed similar changes in the oral microbiota of the xylose group rats; the gut microbiota of obese people consisted of 20% more Firmicutes than lean people (Shin et al. 2015). At the genus level, we found that the level of Rodentibacter was significantly higher in the saccharin group than in other groups. Although Rodentibacter often colonizes the human oral cavity, as an opportunistic pathogen (Hurst et al. 2018). Additionally, the levels of Staphylococcus, Acinetobacter, and Lactobacillus, those species closely related to food fermentation, significantly increased in the xylose group rats. The fermentation process is often accompanied by acid production, and excessive acid formation can damage the enamel of the teeth (Bretz et al. 2005). According to the PCoA results, the sucrose group was very closely related to the control group. In contrast, the xylose and saccharin groups showed completely different compositions. This indicates that xylose and saccharin intake significantly changed the oral microbiota composition, increasing the risk of oral micro-ecological imbalance. In addition, sucrose group samples showed significantly higher community diversity and species richness compared to other groups, which consists with previous reports that sucrose provides metabolic substrates for bacterial growth (Etxeberria et al. 2015). In summary, the results on the relative abundance of oral microbiota among the four groups of rats suggest that all groups exhibited a variable and complex community structure and abundance of oral microbiota. Thus, long-term consumption of sucrose, xylose, and saccharin will change the oral micro-ecology balance, which may have a negative impact on the host.

The unique and shared bacterial taxa analysis results indicate that intake of sucrose, xylose, and saccharin interfered with the original balanced micro-ecological of the healthy oral cavity. For example, Streptococcus, which is often found in oral microbes of patients suffering from caries, is a dominant genus in the sucrose group. Acinetobacter, which is the primary pathogen of pneumonia, meningitis, peritonitis, endocarditis, urinary tract infections, and skin infections, is a core genus of the xylose group (Munoz-Price et al. 2008). Furthermore, based on the high-throughput sequencing data, rely on the PICRUSt, the functional composition of microbial community in the three groups were predicted by COG function classification. The PICRUSt predicted analysis indicated that, there was a decrease at functional abundance level in metabolic functional features, including amino acid and carbohydrate metabolism, which implied that the microbial metabolism in the saccharin group tended to be depressed.

Changes in oral immunoglobulin content can reflect the effects of sucrose, xylose, and saccharin on the oral immune system. In our experiment, although SIgA, IgG, and IgM were identified in all rat saliva samples, only SIgA and IgG predominated in the sucrose and xylose groups. IgM, which mainly appears
in oral cavity infections, did not show any differences in concentration among the groups. S IgA and IgG are major components of saliva, gingival crevicular fluid, and other secretions in the oral cavity (El-Gebaly et.al 2012). Changes in the oral immunoglobulin concentration reflect the effect of sweetener consumption on the oral immune system. This result is consistent with the previous microbiota composition analysis, indicating micro-ecological disorders.

In summary, our results revealed the effects of sucrose, xylose, and saccharin on the composition, diversity, and metabolic functions of oral microbiota. However, our study has certain limitations. For example, we have observed that these sweeteners interfered with the rat oral micro-environment and disrupted the oral micro-ecological balance, based on only 24 samples. Thus, further studies using samples from more animals and even humans are required. Likewise, we did not measure the rats’ specific water intake, weight gain and water loss throughout the entire study, which is another flaw of the study. More suitable animal models can provide more information on the effect of different sweeteners. Nevertheless, our results confirmed the effect of different sweeteners on the oral microbiota and immune system, indicating the need to control the use of sucrose, xylose, and saccharin as beverage additives.

Declarations

Ethics approval and consent to participate

All experiments were approved by the Ethics Committee of Leshan People's Hospital and the approval reference number is IACUC Issue No. LH20191004.

Consent for publication

All authors consent for the manuscript to be published.

Availability of data and material

All data were uploaded to the National Center for Biotechnology Information official website (Accession Number: PRJNA607824).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceived and designed the experiments: Can Song, Performed the experiments: Xi Cheng, Feihong Huang and Can Song, Contributed reagents/materials/analysis tools: Can Song, Xiurong Guo, Hui Lei and
Quan Zhou, Wrote the paper: Xi Cheng and Can Song. All authors reviewed the manuscript.

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**Table**

Table 1 The top 4 abundance bacteria genus among the four groups of rats oral microbiota.
| Sample group     | Top four abundant genera | Composition ratio |
|------------------|---------------------------|--------------------|
| Sucrose group    | *Streptococcus*           | 20.25%             |
|                  | *Rodentibacter*           | 18.34%             |
|                  | *Rothia*                  | 16.81%             |
|                  | *Pasteurellaceae*         | 10.82%             |
| Xylose group     | *Acinetobacter*           | 19.56%             |
|                  | *Staphylococcus*          | 13.20%             |
|                  | *Streptococcus*           | 8.92%              |
|                  | *Rodentibacter*           | 6.85%              |
| Control group    | *Rothia*                  | 20.47%             |
|                  | *Streptococcus*           | 19.11%             |
|                  | *Rodentibacter*           | 18.84%             |
|                  | *Pasteurellaceae*         | 10.69%             |
| Saccharin group  | *Rodentibacter*           | 36.05%             |
|                  | *Streptococcus*           | 21.70%             |
|                  | *Rothia*                  | 10.73%             |
|                  | *Pasteurellaceae*         | 10.54%             |
|                  | *Haemophilus*             | 6.70%              |