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Smokeless tobacco impacts oral microbiota in a Syrian Golden hamster cheek pouch carcinogenesis model

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Abstract

The use of smokeless tobacco products (STPs) can cause many serious health problems. The oral microbiota plays important roles in oral and systemic health, and the disruption in the oral microbial population is linked to periodontal disease and other health problems. To assess the impact of smokeless tobacco on oral microbiota *in vivo*, high-throughput sequencing was used to examine the oral microbiota present in Syrian Golden hamster cheek pouches. Sixteen hamsters were divided into four groups and treated with the STP Grizzly snuff (0, 2.5, 25, or 250 mg) twice daily for 4 weeks. After 0, 1, 2, 3, and 4 weeks of treatment, bacterial genomic DNA was extracted from oral swabs sampled from the cheek pouches of the hamsters. The oral bacterial communities present in different hamster groups were characterized by sequencing the hypervariable regions V1-V2 and V4 of 16S rRNA using the Illumina MiSeq platform. Fifteen phyla, 27 classes, 59 orders, 123 families, and 250 genera were identified from 4,962,673 sequence reads from the cheek pouch samples. The bacterial diversity and taxonomic abundances for the different treatment groups were compared to the non-treated hamsters. Bacterial diversity was significantly decreased after 4 weeks of exposure to 2.5 mg, and significantly increased by exposure to 250 mg STP. Treatment with 250 mg STP significantly increased Firmicutes, transiently increased Cyanobacteria and TM7, and decreased Bacteroidetes and Fusobacteria compared to the control group. At the genus level, 4 weeks of administration of 250 mg STP significantly increased *Granulicatella*, *Streptococcus*, *Oribacterium*, *Anaerococcus*, *Acidaminococcus*, *Actinomyces*, *Eubacterium*, *Negativicoccus*, and *Staphylococcus*, and decreased *Bacteroides*, *Buleidia*, *Dialister*, and *Leptotrichia*, and transiently decreased *Arcanobacterium* compared to the control group. For the first time, an animal model was used for evaluating the effects of STP on oral microbiota by metagenomic sequencing. Our results provide a view of the shift of the oral microbiota in response to STP exposure in Syrian Golden hamster. Our findings indicate that the use of smokeless tobacco significantly disrupts the oral microbiota.

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Keywords

Smokeless tobacco; Oral microbiota; Toxicity; Bacterial diversity

1. Introduction

Smokeless tobacco products (STPs) are typically used by chewing, dipping (placing the product in the oral cavity), or less frequently by sniffing. There are more than 300 million STP users worldwide [1]. In the United States, about 3% adults [2] and 6% of high school students [3] are STP users. STPs are highly addictive, since nicotine is absorbed directly through oral or nasal mucosa. Besides nicotine, STPs contain thousands of chemicals, including at least 30 carcinogens, that can cause oral leukoplakias, oral cancer, and esophageal cancer [4]. STP usage also can cause gum disease and tooth decay, and increases the risk of heart disease, stroke, and premature birth [5]. Worldwide, in 2010, STPs caused more than 62,000 deaths due to cancer and more than 200,000 deaths due to ischemic heart disease [6], making it also a global economic burden. Understanding the toxicity and pathogenesis of STPs in those adverse health consequences is important.

Oral health is very important for overall health of an individual [7]. More than 700 species of bacteria colonize the oral cavity and form a complex ecological community that plays important roles in oral and systemic health [8]. The composition of the oral microbial community is highly variable from person to person and is affected by many factors, including diet, personal oral hygiene, stress, and medicinal drug exposure. Perturbations in the indigenous oral microbial population can cause oral infections, which have been linked to bacterial endocarditis, kidney disease, and diabetes [9–11]. In consideration of the importance of the oral microbiota to the overall health of an individual, disturbing the oral microbiota by exposure to STPs could be a critical step for initiating those adverse health consequences.

The aim of this study was to assess the impact of smokeless tobacco on the oral microbiota in a Syrian Golden hamster cheek pouch carcinogenesis model used in a parallel study at the National Center for Toxicological Research on the toxicological effects of STPs. The hamster cheek pouch stores partially-chewed food and the stored food can be pressed back to mouth when needed. When introduced into the cheek pouch, smokeless tobacco can remain for several hours and is periodically extruded in small amounts. In addition, the absorption of compounds from the cheek pouch into the systemic circulation is slow, which simulates the use of smokeless tobacco in human [12]. The hamster cheek pouch model has been widely used in oral cancer studies [13,14], but to our knowledge there are no reports about the microbiota of the cheek pouch and its application in studying the effects of toxicants on the oral microbiota. To this end, metagenomics sequencing, using the Illumina MiSeq platform and QIIME pipeline tools, was used to identify and classify bacterial microbiota present in the cheek pouches of hamsters upon smokeless tobacco exposure.

2. Methods and materials

2.1. Smokeless tobacco administration and oral swab collection

Male Golden Syrian hamsters (3–4 weeks) were purchased from Harlan Laboratories (Haslett, MI). All animal experimental procedures were performed in accordance with the animal study protocol approved by the Animal Care and Use Committee of the National Center for Toxicological Research, U.S. Food and Drug Administration. Because moist snuff is the most popular form of STPs used in the United States [15], Grizzly snuff, one of the major brands of STP, was chosen in our study as a representative of STPs. Grizzly snuff (American Snuff Co.) was purchased at a retail store in Little Rock, Arkansas in October 2013, and stored in sealed plastic bag at 4 °C until use. STP was used to represent Grizzly snuff in following text of this paper unless otherwise specified. Hamsters (n = 16) were randomly divided into 4 groups (n = 4 per group) according to the dosage of STP (0, 2.5, 25, or 250 mg). STP was administered into the hamsters' right cheek pouch twice per day (a.m. and p.m.) for 28 consecutive days. Two swabs (Dacron swab) were collected per hamster from the inside of the hamster's right cheek pouch on the day prior to the beginning dose (week 0) and then on day 7 (week 1), 14 (week 2), 21 (week 3), and 28 (week 4) prior to administering the first dose of the day. The swabs were immediately placed into tubes containing 300 µl of 20 mM Tris-HCl (pH 8.0) and then stored at –80 °C. A total of 160 swabs were collected throughout the 28-day experiment.

2.2. DNA extraction

Dacron swabs were separated from the stick with sterile scissor and placed in a 2 ml microcentrifuge tube. 300 µl Tris buffer from swab stock tube was transferred to the same microcentrifuge tube, then 300 µl 2 X lysis buffer (40 mg/ml lysozyme, 200 µg/ml lysostaphin, 40 mM Tris-HCl (pH 8.0), 4 mM EDTA, and 2.4% Triton) was added. Swabs were incubated in lysis buffer for overnight at 37 °C, then bacterial genomic DNA was extracted from swabs using QIAamp DNA mini kits (QIAGEN, Germantown, MD) following the manufacturer's instruction for isolation genomic DNA from Gram-positive bacteria. All extracted DNA samples were stored at –20 °C until further use.

2.3. 16S rRNA library preparation and sequencing

16S rRNA library preparation and sequencing were conducted at the Genomic Sequencing and Analysis Facility of the University of Texas at Austin. All genomic DNA samples were normalized to an equal concentration (10 ng/µl), and then used for bacterial 16S rRNA library construction using primers for the V1-2 region and the V4 region in separate PCR amplifications. Each PCR reaction contained 1 µl 5 µM specified 16 s F primer (Table 1), 1 µl 5 µM specified 16 s R primer (Table 1), 1 µl gDNA, 10 µl NEBNext High-Fidelity 2X PCR Master Mix (New England BioLabs Inc., Ipswich, MA), and 7 µl deionized H₂O. All reactions were run in triplicate with an initial denaturation step of 30 s at 98 °C, followed by 12 cycles of 30 s denaturation at 98 °C, 30 s of annealing at 62 °C, and 30 s extension at 72 °C, and 1 cycle of final extension for 5 min at 72 °C. Triplicate PCR reactions were combined and purified using the Agencourt AMPure XP system (Beckman Coulter Life Sciences, Indianapolis, IN). Illumina-specific barcoded primers (Table 1) (Integrated DNA Technologies, Coralville, IA) were then added in a second PCR reaction that consisted of 15

µl NEBNext High-Fidelity 2X Master Mix, 5 µl primers, and 10 µl DNA from the first amplification. Different barcodes were used for each sample and variable region, allowing for pooling samples for sequencing. The parameters for the second PCR were as follows: initial denaturation step of 30 s at 98 °C, followed by 7 cycles of 30 s denaturation at 98 °C, 30 s of annealing at 62 °C, and 30 s extension at 72 °C, and 1 cycle of final extension for 5 min at 72 °C. All samples were pooled and sequenced on one lane of an Illumina MiSeq sequencer (Illumina, San Diego, CA) at the University of Texas at Austin.

2.4. Bioinformatics analysis of sequence reads

The bioinformatics analysis was conducted using FLASH version (1.2.11) to merge paired-end reads followed by running a Quantitative Insights into Microbial Ecology (QIIME) (version 1.9.1)-based workflow. Reads were filtered to retain only Phred quality scores ≥ 20. A total of 4,962,714 sequence reads were obtained from 160 samples. One sample with low counts was eliminated from further analysis, resulting in 4,962,673 reads with maximum reads of 78,861, minimum of 7,740, median of 31,311, mean of 31,212, and a standard deviation of 10,889. Operational taxonomic units (OTUs) were assigned at 97% similarity using the Greengenes 13_8 database for reference-based picking and UCLUST (version 1.2.22q) algorithm for de novo clustering. Alpha rarefaction and beta diversity analyses were conducted using core_diversity_analyses.py script in QIIME. OTU abundance data of 159 samples were grouped according to the treatment time points and dosages of STP, and further analyzed at different taxonomical levels (phylum, class, order, family, and genus) separately. Two-way analysis of variance (ANOVA) was used to determine significant differences in diversity between groups, and mixed repeated ANOVA was used to identify significant differences in relative abundance of individual OTUs between different treatment groups according to smokeless tobacco dosage and exposure time.

3. Results

3.1. Comparison of data from V1-2 and V4 variable regions of 16S rRNA oral microbiota from control and STP treated Syrian Golden hamsters

In this study, hamster's cheek pouch was used as an *in vivo* model system to evaluate the impact of smokeless tobacco on the oral microbiota. 16S rRNA gene contains hypervariable regions (V1-V9), which are flanked by highly conserved regions and used to design primers to amplify hypervariable regions and as molecular fingerprints to profile complex microbial community. Due to specificity of primers, no single primer pair is universal to cover all bacteria and no single hypervariable region can identify all bacteria. Previous studies showed that V1, V2, and V4 regions contain great intra-species diversity. To maximize OTU identification in this study, sequencing data from V1-V2 region and V4 region were combined to profile bacterial community in the cheek pouch of hamster [16]. Fifteen phyla, 27 classes, 59 orders, 123 families, and 250 genera were identified from all samples. OTUs were grouped according to the STP dosage and were further analyzed at different taxonomical levels (phylum, class, order, family, and genus) separately. The bacterial diversity was determined by sequencing two different hypervariable regions of 16S rRNA. A total of 2,498,216 sequence reads were derived from the V4 region and 2,464,457 from V1-2 region. The mean number of sequences per sample was $31,623 \pm 12,161$ for V4 region, and

30,806 $\pm$ 9528 for V1-2 region. Alpha diversity analysis was performed by computing rarefaction curves. The rarefaction curves of the V1-2 region and V4 region were compared in Fig. 1A. Bacteria diversity identified from V4 region sequence reads was significantly higher than that identified from V1-2 region (Fig. 1A) (p value < 0.001) which is consistent with an earlier report [16]. Fig. 1B shows the number of taxa identified in the sequence data of V4 and V1-2 regions at the levels of genus and phylum. Most of the OTUs that were identified in region V1-2 overlapped with that identified in region V4; however, there are some unique OTUs found in either V1-2 or V4 (Fig. 1B). To maximize OTU identification, OTUs of V4 and V1-2 were combined for further analysis unless otherwise specified.

3.2. Commensal oral microbiota in cheek pouch of hamster

We initially characterized the composition of commensal oral microbiota in the cheek pouches of the hamsters. Sequence reads of the 16 samples collected at week 0 were combined and analyzed; and this is defined as the basal level of commensal oral microbiota. 91.76% of OTUs were classified in 14 phyla, 24 classes, 50 orders, 100 families, and 192 genera, leaving 8.24% sequences without classification at phylum level. At the phylum level, Firmicutes represented a mean of 39.73%, Bacteroidetes 30.40%, Actinobacteria 18.45%, Proteobacteria 2.79%, Fusobacteria 0.18%, and TM7 0.17%, while the other 9 phyla were found in relatively low proportions (0.04% of total relative abundance) (Fig. 2A, Table S1). The abundance of bacteria in the hamster cheek pouch is similar to that found in humans at the phylum level [17]. An earlier study showed that in the core microbiota of healthy human oral microbial communities, Firmicutes represents 28% of the population, Actinobacteria 24%, and Bacteroidetes 16% [17]; however, the proportion of Proteobacteria is much higher in humans (22%) [17] than in hamster (2.79%). A previous study has reported that the overall composition of mouse oral microbiota is significantly different from that of human and hamster, with Firmicutes representing over 80% of the population [18].

Twenty-four bacterial classes were identified from the samples collected at week 0 (Table S2). The 8 most prevalent classes were Bacteroidia (30.40%), Clostridia (21.62%), Actinobacteria (18.45%), Bacilli (16.70%), Erysipelotrichi (1.41%), Gammaproteobacteria (1.94%), Betaproteobacteria (0.77%), and TM7-3 (0.17%). Among the 50 orders identified from the samples collected at week 0, 45 were assigned with a specific order name (Table S3). The most predominant order was Bacteroidales (30.40%), followed by Clostridiales (19.02%), Lactobacillales (16.19%), Actinomycetales (12.15%), Bifidobacteriales (6.30%), Coriobacteriales (2.61%), Erysipelotrichales (1.41%), Pseudomonadales (1.23%), Burkholderiales (0.76%), and Pasteurellales (0.56%). The remaining 40 identified orders accounted for only 1.13% of the total OTUs. A total of 100 families was identified from week 0 samples, with 78 being assigned with a specific family name (Table S4). The 10 most abundant families were Bacteroidaceae (21.37%), Streptococcaceae (13.27%), Actinomycetaceae (10.88%), Veillonellaceae (10.15%), Clostridiaceae (8.15%), Prevotellaceae (7.98%), Bifidobacteriaceae (6.30%), Carnobacteriaceae (2.74%), Coriobacteriaceae (2.59%), and Erysipelotrichaceae (1.35%).

At the genus level, 104 OTUs were assigned with a specific genus name, with abundances totaling 72.70% of the microbiota sequences, while 5 were named to the class level (0.55%),

18 named to the order level (0.14%), and 65 named to the family level (18.37%) (Table S5). The most prevalent genus found in commensal oral microbiota of hamster was *Bacteroides* (21.4%), followed by *Parascardovia* (9.6%), *Prevotella* (8.0%), *Megasphaera* (5.4%), *Streptococcus* (5.4%), and *Bifidobacterium* (4.9%) (Fig. 2B). A difference in the composition ratio of bacteria was observed between the cheek pouch of hamster and the human oral cavity. According to the Human Oral Microbiota Database (HOMD), the six most prevalent genera in the human oral microbiota are *Streptococcus* (26.9%), *Veillonella* (9.8%), *Selenomonas* (3.5%), *Gemella* (3.3%), *Fusobacterium* (3.0%), and *Prevotella* (2.7%) [19,20]. The abundance of *Streptococcus* is higher in the human oral cavity (26.9%) than that in the hamster (5.4%) at the genus level; however, in the hamster cheek pouch there were two unclassified genera under the family Streptococcaceae with the abundance of 4.26 and 3.63%, respectively. Therefore, the difference in the abundance of Streptococcaceae may not be significant at the family level. The abundance of Veillonella is higher in the human oral cavity (9.8%) than in hamster (2.15%). In the hamster cheek pouch there was an unclassified genus under the family Veillonellaceae with 2.18% abundance. Thus, at the family level, the abundance of Veillonellaceae in hamster cheek pouches might be closer to that of the human oral cavity. Therefore, despite of the difference in composition ratio of genera, cheek pouch of hamster may be used as a model system to study microbiota of human oral cavity.

3.3. Effect of smokeless tobacco on the diversity of the oral microbiota

In order to obtain a general understanding of the dose response to the selected STP, 0, 2.5, 25, or 250 mg of STP were used for insertion into the hamster cheek pouch. The dose of the STP was chosen according to the previous publication and based on 10-fold reductions of the highest value of 250 mg [21]. The administration of STP did not cause any significant changes in the growth of the tested hamsters. We also did not observe pathologic change inside the hamsters' mouths during the experiment.

To evaluate whether smokeless tobacco could affect the diversity of oral microbiota, we compared alpha diversities before the treatment at week 0 to that after the four weeks treatment with STP. The species richness (number of OTUs per sample) in the control group (0 mg STP) did not change after four weeks (Fig. 3A). Four week exposure to 2.5 mg STP resulted in a statistically significantly decrease bacterial diversity (p value < 0.05 between week 0 and week 4) (Fig. 3B). Bacterial diversity was decreased at week 2 when 25 mg STP was administered; but at week 3, the diversity reverted to the level of week 0 and week 1, then was further increased at week 4 (Fig. 3C). Administration of 250 mg STP started to increase the diversity of hamster oral microbiota from week 1, and reached the highest at week 4 ($p < 0.001$ between week 0 and week 4) (Fig. 3D). Previous studies indicated that increased diversity and richness of oral microbiota are associated with infectious oral diseases in human [19,22]. Thus, significant increase in the diversity of oral microbiota of the hamster suggested that exposure to 250 mg STP might have possible adverse effect on oral health, although no disease was observed in this experiment.

3.4. Impact of smokeless tobacco product to the composition abundance of oral microbiota

To determine whether STPs influenced the composition of oral microbiota, we analyzed the relative abundance of OTUs by grouping samples according to the dosage of STP. Fig. 4 shows the averaged relative abundance of the four treatment groups at the phylum level over four weeks. Comparing STP-treated samples with the control group, there were no significant changes in the relative abundance of different phyla in 2.5 and 25 mg STP treatment groups. However, 250 mg STP caused significant changes in the composition ratio of phyla (Fig. 4). The relative abundance of the seven most abundant phyla was all changed by exposure to 250 mg STP (Fig. 5A). The relative abundance of Firmicutes, TM7 and Cyanobacteria was significantly increased by the exposure of 250 mg STP compared with control group (Fig. 5B–D). The abundance of Bacteroidetes and Fusobacteria was significantly decreased compared to the control group (Fig. 5A, E, and 5F). Exposure to 250 mg STP increased the abundance of Firmicutes 9.1–22.5% (Fig. 5B), and decreased abundance of Bacteroidetes 18.1–20.8% (Fig. 5E). Because Firmicutes and Bacteroidetes are the two most abundant phyla of commensal microbiota identified in the hamster cheek pouch, our data indicate that exposure of 250 mg STP dramatically altered the composition ratio of the oral microbiota.

Fig. 6A shows the impact of exposure to 250 mg STP on the relative abundance of the 13 most abundant bacterial classes. There was a significant increase in the relative abundance of Bacilli, TM7-3, and Chloroplast (class) (Fig. 6A–D), and significant decrease in the abundance of Bacteroidia and Fusobacteria (Fig. 6A, E, and 6F). Bacilli accounted for the principal change in the Firmicutes. The relative abundance of Bacilli was increased 13.9–25.0% by in the 250 mg STP exposure group (Fig. 6B). In contrast to the increased Bacilli, the abundance of Bacteroidia was decreased 18.1–20.8% by 250 mg STP (Fig. 6E). Fig. 7A showed the impact of exposure to 250 mg STP on the relative abundance of 17 most abundant bacterial orders. Significant increases in the relative abundance were observed in Lactobacillales, Streptophyta, Bacillales_Other, Pasteurellales, I025, and Bacillales throughout the four-week treatment with 250 mg STP (Fig. 7B–G), compared with control group. The relative abundance of Bacteroidales and Fusobacteriales was significantly decreased by administration of 250 mg STP (Fig. 7H and I). Fig. 8A shows the impact of exposure to 250 mg STP on the relative abundance of the 33 most abundant bacterial families. The relative abundance of Carnobacteriaceae and the other nine families was significantly increased by exposure to 250 mg of the STP (Fig. 8B–K). Carnobacteriaceae is the prevalent family under phylum Firmicutes in the 250 mg STP treatment group, and its abundance 7–18% over the control (Fig. 8B). Bacteroidaceae is the major family from the Bacteroidetes phylum, and its abundance decreased 10–22% after 250 mg STP exposure (Fig. 8L). Fig. 9A shows the impact of 250 mg STP on the relative abundance of bacterial genera. The relative abundance of 18 genera was significantly increased, including *Granulicatella*, *Streptococcus*, *Oribacterium*, *Anaerococcus*, *Acidaminococcus*, *Actinomyces*, *Eubacterium*, *Negativococcus*, *Staphylococcus*, and six unclassified genera, and transiently increased *Lactobacillus* (Fig. 9B–R). The relative abundance of five genera was significantly decreased by exposure to 250 mg STP, including *Bacteroides*, *Buleidia*, *Dialister*, and *Leptotrichia*, and transiently decreased *Arcanobacterium* (Fig. 9S–Y). Because

the reliable sequencing result was not obtained using both V4 region and V1-V2 region primers for some taxa, the calculation for Carnobacteriaceae and Granulicatella were based on the sequencing results of V1-V2 region, while the calculation for Bacilli_other_other, Lactobacillales_other, Oribacterium, Bulleidia, Dialister, and an unclassified genus from Bacilli, was on the sequencing results of V4 region. Unfortunately, not all sequences could be confidently assigned to a specific taxonomic name, which might represent unknown bacteria and may need further studies to characterize. Among these unclassified OTUs, the relative abundance of 1 order, 3 families, and 5 genera was significantly increased (Table S6).

Fig. 10 is a summary of the impact of 250 mg STP on the oral microbiota in the hamster cheek pouch. Exposure to 250 mg STP significantly increased the relative abundance of 3 phyla, 3 classes, 6 orders, 10 families, and 17 genera, and decreased the abundance of 2 phyla, 2 classes, 2 orders, 2 families and 7 genera. These changes showed statistical significance ($p < 0.05$) between the control group and 250 mg STP treatment group (Table S6). Besides these significant changes, administration of 250 mg STP also impacted the relative abundance of some other taxa. There were increased tendency in 2 phyla, 3 classes, 2 orders, 6 families, and 15 genera (Figure S1–S3, Fig. 10 (taxa name in red)) and decreased tendency in 1 orders, 3 families, and 5 genera (Figure S1–S3, Fig. 10 (taxa name in blue)). Although these changes did not have statistical significance ($p > 0.05$) between control group and 250 mg STP group (Table S6), they might also provide useful information for the impact of 250 mg STP on the oral microbiota. Treatment of 250 mg Grizzly snuff changed the balance of bacteria community under 7 phyla (Fig. 10). These 7 phyla are the most prevalent phyla in cheek pouch of hamster, accounting for 91.75% of total abundance in untreated hamster (Fig. 2). Among those phyla, Firmicutes showed the prominent abundance increasing, while Bacteroidetes showed the prominent abundance decreasing.

4. Discussion

Oral use of STPs is linked to oral infections, oral cancer, and other systemic diseases [4]. It has been reported that nicotine affected the growth of *Streptococcus mutans* [23,24]. High sugar content in STPs enhanced the growth of *Lactobacillus casei* and led to dental caries [25]. Our previous study showed that extracts from STPs affected the *in vitro* growth of some species of oral bacteria [26]. Taken together, these results suggest that STP may disrupt the ecological balance of oral microbiota; however, the impact of STPs on diversity and abundance of oral microbiota has not been well studied. To understand better the toxicity of STPs, we investigated the impact of STP on oral microbiota in a Syrian Golden hamster cheek pouch *in vivo* model by metagenomics sequencing, which enable us to observe both culturable and unculturable bacteria.

The human oral cavity is a very complex microbial habitat with the presence of a shedding surface (mucosa) and solid surfaces (teeth or dentures) for microbial colonization, and nutrients from saliva and dietary food [17]. The hamster cheek pouch is similar to the shedding surface. Our results showed that at the phylum level, the composition of commensal microbiota present in cheek pouch of hamster was similar to that of human oral microbiota, since Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria are the four

most prevalent phyla in both hamster cheek pouch and human oral tissue [17]. At the genus level, the composition of the bacteria community found in the hamster cheek pouch was different from human oral microbiota reported in HOMD [19,20]. However, even in human subjects, the composition of microbiota is highly variable from person to person and differs greatly according to different habitats in oral cavity [8,19]. Many factors could affect the oral microbial population, such as diet, personal oral hygiene, stress, and medical treatments, and it might be difficult to choose exactly the same background human subjects as controls [17]. Moreover, STPs contain at least 30 carcinogens, thus ethical concerns have limited using human subjects in *in vivo* toxicological studies of STPs. Hamster cheek pouches can serve as temporary storage for STPs, and it is simple to keep hamsters under the same living conditions and choose the same background for different treatment groups. Also, microbiota changes of the same group of hamsters can be observed at different time points. Comparing before and after data for the same STP treatment group can minimize individual differences and more accurately assess STP impact. Therefore, the hamster cheek pouch is a good *in vivo* model system to assess the overall impact of smokeless tobacco on oral microbiota.

The alpha diversity results indicated that exposure to STP had dose-dependent differential effects on the diversity of oral bacterial community. Exposure to low dose (2.5 mg) levels of STP significantly decreased the diversity, while exposure to high dose (250 mg) significantly increased the bacterial diversity. The middle dose (25 mg) slightly decreased diversity in the first week, then increased diversity afterwards. Our previous *in vitro* study showed similar results. Extracts from STPs could stimulate the growth of some bacteria at low concentration and inhibit the growth of some bacteria at high concentration [26]. These dose dependent effects might be due to the components of STPs, which contain high sugar content (added as sweetener) [27] and toxic compounds as well. When exposed to a low concentration of STP, the sugar content might promote the growth of prevalent bacteria, so the diversity of the bacterial community decreased. However, in exposure to a high concentration of STP, toxic compounds might inhibit growth of some bacteria, disturbing the balance of oral microbiota, and increasing diversity of oral bacterial community. Fig. 10 showed that the number of taxa with increased abundance was more than that with decreased abundance after exposure to 250 mg STP, supporting that the exposure to STPs can increase diversity of oral microbiota. Increased diversity of oral microbiota can initiate infectious oral diseases [19,22].

STPs usage has been linked to increased dental caries and periodontitis [25,28,29]. Previous studies demonstrated that cariogenic streptococci and acidogenic lactobacilli contributed to carious lesions by converting sugar to acid and demineralizing teeth. In this study, we demonstrated that the relative abundance of *Streptococcus*, *Lactobacillus*, and *Veillonella* was significantly increased by exposure to 250 mg STP. Periodontitis is an infectious disease with a complex polymicrobial etiology. Previous studies have observed the increases of *Eubacterium*, *Actinomyces*, *Aggregatibacter*, *Streptococcus*, and *Staphylococcus* in periodontitis [22,30,31]. The abundance of these genera was increased by exposure to 250 mg STP. *Eubacterium*, *Streptococcus*, and *Lactobacillus* are also identified as pathogens causing Endodontic infection [7]. The abundance of *Granulicatella*, *Staphylococcus*, and *Streptococcus* was significantly increased by exposure to 250 mg STP. *Granulicatella* has been reported as an agent of endocarditis [32]. *Staphylococcus aureus* and *Streptococcus pneumoniae* are common pathogens for aspiration pneumonia [33]. The relative abundances

of these disease-related bacteria were increased by exposure to 250 mg STP, suggesting that exposure to smokeless tobacco might lead to potential adverse effect on oral health.

Exposure to 250 mg STP variably disrupted the balance of oral microbiota present in cheek pouch of hamster (Fig. 10), although exposure to 2.5 and 25 mg STP did not show similar effects. Different taxa showed different responses to 250 mg STP. Some taxa showed the same tendency (either increased or decreased) from week 1 to week 4. Some taxa exhibited a transient increase or decrease, and then returned to same level as control group. Some taxa started to increase abundance at week 4. Over 700 bacteria grow in the oral cavity, interacting with each other and forming a complex bacterial community [17]. Our previous study showed that different species of bacteria had different responses to extracts of STPs. Some bacteria were inhibited or killed by STPs, while other bacteria could tolerate extracts of STPs [26]. Therefore, part of the changes in bacterial abundance in the 250 mg STP exposure group could be due to direct effects on specific bacteria in the microbial consortia of the oral microbiota and secondary responses due to the structural community changes in the oral microbiota caused by this STP.

5. Conclusion

For the first time, an animal model was used for evaluating the effects of smokeless tobacco on oral microbiota by metagenomic sequencing. Our results provide a view of the shift of the oral microbiota in response to STP exposure in Syrian Golden hamster. Our findings indicate that the use of smokeless tobacco significantly disrupts the oral microbiota, and the outcome of our study is informative in understanding the potential pathogenesis of health problems caused by STPs. The information from this study will extend the FDA's knowledge about the toxicity of STPs, which could aid in the risk assessment of smokeless tobacco. Since various STPs might have different toxic effects due to their different chemical compositions, further studies on the pathological effects caused by the known toxic components of STPs and the toxic effects of different types of STPs from different manufactures using hamster's cheek pouch model would be informative.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Acknowledgments and Disclaimer

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References

1. Tyx RE, Stanfill SB, Keong LM, Rivera AJ, Satten GA, Watson CH. Characterization of bacterial communities in selected smokeless tobacco products using 16S rDNA analysis. *PLoS One*. 2016; 11(1):e0146939. [PubMed: 26784944]
2. Center for behavioral health statistics and quality. 2014 National Survey on drug use and health: detailed tables. Substance Abuse and Mental Health Services Administration; Rockville, MD: 2015.
3. Centers for disease control and prevention. tobacco use among middle and high school Students- United States, 2011–2015. *Morbidity and Mortality Weekly Report*. 2016; 65(14):361–367. [PubMed: 27077789]
4. Kallischnigg G, Weitkunat R, Lee PN. Systematic review of the relation between smokeless tobacco and non-neoplastic oral diseases in Europe and the United States. *BMC Oral Health*. 2008; 8:13. [PubMed: 18452601]
5. Critchley JA, Unal B. Health effects associated with smokeless tobacco: a systematic review. *Thorax*. 2003; 58(5):435–443. [PubMed: 12728167]
6. Siddiqi K, Shah S, Abbas SM, Vidyasagaran A, Jawad M, Dogar O, Sheikh A. Global burden of disease due to smokeless tobacco consumption in adults: analysis of data from 113 countries. *BMC Med*. 2015; 13:194. [PubMed: 26278072]
7. Scannapieco FA. The oral microbiome: its role in health and in oral and systemic infections. *Clin Microbiol Newsl*. 2013; 35(20):7.
8. Kilian M, Chapple IL, Hannig M, Marsh PD, Meuric V, Pedersen AM, Tonetti MS, Wade WG, Zaura E. The oral microbiome-an update for oral healthcare professionals. *Br Dent J*. 2016; 221(10):657–666. [PubMed: 27857087]
9. Chan S, Pasternak GM, West MJ. The place of periodontal examination and referral in general medicine. *Periodontol 2000*. 2017; 74(1):194–199. [PubMed: 28429485]
10. Winning L, Patterson CC, Neville CE, Kee F, Linden GJ. Periodontitis and incident type 2 diabetes: a prospective cohort study. *J Clin Periodontol*. 2017; 44(3):266–274. [PubMed: 28036104]
11. Michaud DS, Izard J. Microbiota, oral microbiome, and pancreatic cancer. *Cancer J*. 2014; 20(3): 203–206. [PubMed: 24855008]
12. Grasso P, Mann AH. Smokeless tobacco and oral cancer: an assessment of evidence derived from laboratory animals. *Food Chem Toxicol*. 1998; 36(11):1015–1029. [PubMed: 9771564]
13. Kreimann EL, Itoiz ME, Dagrosa A, Garavaglia R, Farias S, Batistoni D, Schwint AE. The hamster cheek pouch as a model of oral cancer for boron neutron capture therapy studies: selective delivery of boron by boronophenylalanine. *Cancer Res*. 2001; 61(24):8775–8781. [PubMed: 11751398]
14. Monti-Hughes A, Aromando RF, Perez MA, Schwint AE, Itoiz ME. The hamster cheek pouch model for field cancerization studies. *Periodontol 2000*. 2015; 67(1):292–311. [PubMed: 25494606]
15. Delnevo CD, Wackowski OA, Giovenco DP, Manderski MT, Hrywna M, Ling PM. Examining market trends in the United States smokeless tobacco use: 2005–2011. *Tob Control*. 2014; 23(2): 107–112. [PubMed: 23117999]
16. Cai L, Ye L, Tong AH, Lok S, Zhang T. Biased diversity metrics revealed by bacterial 16S pyrotags derived from different primer sets. *PLoS One*. 2013; 8(1):e53649. [PubMed: 23341963]
17. Zaura E, Keijser BJ, Huse SM, Crielaard W. Defining the healthy “core microbiome” of oral microbial communities. *BMC Microbiol*. 2009; 9:259. [PubMed: 20003481]
18. Chun J, Kim KY, Lee JH, Choi Y. The analysis of oral microbial communities of wild-type and toll-like receptor 2-deficient mice using a 454 GS FLX Titanium pyrosequencer. *BMC Microbiol*. 2010; 10:101. [PubMed: 20370919]
19. Costalonga M, Herzberg MC. The oral microbiome and the immunobiology of periodontal disease and caries. *Immunol Lett*. 2014; 162(2 Pt A):22–38. [PubMed: 25447398]
20. Palmer RJ Jr. composition and development of oral bacterial communities. *Periodontol 2000*. 2014; 64(1):20–39. [PubMed: 24320954]

21. Summerlin DJ, Dunipace A, Potter R. Histologic effects of smokeless tobacco and alcohol on the pouch mucosa and organs of the Syrian hamster. *J Oral Pathol Med*. 1992; 21(3):105–108. [PubMed: 1583594]
22. Griffen AL, Beall CJ, Campbell JH, Firestone ND, Kumar PS, Yang ZK, Podar M, Leys EJ. Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME J*. 2012; 6(6):1176–1185. [PubMed: 22170420]
23. Keene K, Johnson RB. The effect of nicotine on growth of *Streptococcus mutans*. *Miss Dent Assoc J*. 1999; 55(4):38–39. [PubMed: 10823053]
24. Huang R, Li M, Gregory RL. Effect of nicotine on growth and metabolism of *Streptococcus mutans*. *Eur J Oral Sci*. 2012; 120(4):319–325. [PubMed: 22813222]
25. Vellappally S, Fiala Z, Smejkalova J, Jacob V, Shriharsha P. Influence of tobacco use in dental caries development. *Cent Eur J Public Health*. 2007; 15(3):116–121. [PubMed: 17958204]
26. Liu M, Jin J, Pan H, Feng J, Cerniglia CE, Yang M, Chen H. Effect of smokeless tobacco products on human oral bacteria growth and viability. *Anaerobe*. 2016; 42:152–161. [PubMed: 27756619]
27. Going RE, Hsu SC, Pollack RL, Haugh LD. Sugar and fluoride content of various forms of tobacco. *J Am Dent Assoc*. 1980; 100(1):27–33. [PubMed: 6927883]
28. Katuri KK, Alluri JK, Chintagunta C, Tadiboina N, Borugadda R, Loya M, Marella Y, Bollepalli AC. Assessment of periodontal health status in smokers and smokeless tobacco users: a cross-sectional study. *J Clin Diagn Res*. 2016; (10):ZC143–ZC146.
29. Weintraub JA, Burt BA. Periodontal effects and dental caries associated with smokeless tobacco use. *Public Health Rep*. 1987; 102(1):30–35. [PubMed: 3101120]
30. Kumar PS, Griffen AL, Barton JA, Paster BJ, Moeschberger ML, Leys EJ. New bacterial species associated with chronic periodontitis. *J Dent Res*. 2003; 82(5):338–344. [PubMed: 12709498]
31. Abusleme L, Dupuy AK, Dutzan N, Silva N, Burleson JA, Strausbaugh LD, Gamonal J, Diaz PI. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J*. 2013; 7(5):1016–1025. [PubMed: 23303375]
32. Ruoff KL. Miscellaneous catalase-negative, gram-positive cocci: emerging opportunists. *J Clin Microbiol*. 2002; 40(4):1129–1133. [PubMed: 11923320]
33. Dasaraju PV, Liu C. Infections of the respiratory system. In: Baron S, editor *Medical Microbiology*. 4. Galveston (TX): 1996.
34. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*. 2013; 8(4):e61217. [PubMed: 23630581]

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.anaerobe.2018.05.010>.

Fig. 1. Comparison of data derived from the V1-V2 and V4 variable regions

A) Alpha diversity was compared for V1-V2 and V4 sequence data. Error bars indicate standard errors of the means. B) The total number of named taxa with found in each of the data sets at the levels of genus and phylum.

Fig. 2. Abundance of commensal microbiota present in the hamster cheek pouch
 A) Relative abundance of phyla. B) Relative Abundance of genera.

Fig. 3. The impact of STP on the alpha diversity of microbiota present in the hamster cheek pouch

A) Diversity of the control group (0 mg STP). B) Diversity of the 2.5 mg STP exposure group. C) Diversity of the 25 mg STP exposure group. D) Diversity of the 250 mg STP exposure group. The combined data from V1-V2 region and V4 region were applied (compared to the combined data, the separated data from V1-V2 region or V4 region did not change the trends of diversity caused by STP).

Fig. 4. The impact of STP on the relative abundance of phyla

The mean relative abundance (%) of OTUs found in oral swab of hamster is presented. Samples were grouped based on the treatment dose and treatment time of STP.

Fig. 5. The impact of STP (250 mg) on the relative abundance of phyla
 5A: Heatmap analysis of the most abundant 7 phyla. The heatmap was clustered by phyloseq [34] and the heatmap colors represent the relative abundance of the bacterial phylum assignments within each sample. 5B–5F: Significant impact of STP on the relative abundance of bacterial classes. The blue lines represent control group (no exposure to STP) and the red lines represent the exposure group (exposure to 250 mg STP). Error bars indicate standard errors of the means. The p value between the 250 mg STP exposure group and the control group was less than 0.05 for all taxa. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 6. The impact of STP (250 mg) on the relative abundance of bacterial classes

6A: Heatmap analysis of the most abundant 13 bacterial classes. The heatmap was clustered by phyloseq [34] and the heatmap colors represent the relative abundance of the bacterial class assignments within each sample. 6B–6F: Significant impact of STP on the relative abundance of bacterial classes. The blue lines represent the control group (no exposure to STP), and the red lines represent the exposure group (exposure to 250 mg STP). Error bars indicate standard errors of the means. The p value between 250 mg STP exposure group and control group was less than 0.05 for all taxa. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 7. The impact of STP on the relative abundance of bacterial orders

7A: Heatmap analysis of the most abundant 17 bacterial orders. The heatmap was clustered by phyloseq [34] and the heatmap colors represent the relative abundance of the bacterial order assignments within each sample. 7B–7I: Significant impact of STP on the relative abundance of bacterial orders. The blue lines represent the control group (no exposure to STP), and the red lines represent the exposure group (exposure to 250 mg STP). Error bars indicate standard errors of the means. The p value between 250 mg STP exposure group and control group was less than 0.05 for all taxa. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 8. The impact of STP (250 mg) on the relative abundance of bacterial families
 8A: Heatmap analysis of the most abundant 33 bacterial families. The heatmap was clustered by phyloseq [34]. And the heatmap colors represent the relative abundance of the microbial family assignments within each sample. 8B–8M: Significant impact of STP on the relative abundance of bacterial families. The blue lines represent the control group (no exposure to STP), and the red lines represent the exposure group (exposure to 250 mg STP). Error bars indicate standard errors of the means. # highlighted abundance from V1-V2 data only and * highlighted abundance from V4 data only. The p value between 250 mg STP exposure group and control group was less than 0.05 for all families. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 9. The impact of STP (250 mg) on the relative abundance of bacterial genera
 9A: Heatmap analysis of the most abundant 67 bacterial genera. The heatmap was clustered by phyloseq [34] and the heatmap colors represent the relative abundance of the microbial genus assignments within each sample. 9B–9Y: Significant impact of STP on the relative abundance of bacterial genera. The blue lines represent the control group (no exposure to STP), and the red lines represent the exposure group (exposure to 250 mg STP). Error bars indicate standard errors of the means. # highlighted abundance from V1–V2 data only and *highlighted abundance from V4 data only. The p value between 250 mg STP exposure group and control group was less than 0.05 for all genera. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 10. The overall impact of 250 mg STP on the oral microbiome in hamster's cheek pouch
The taxonomic name in red indicates that its abundance was increased by exposure to 250 mg STP. The taxonomic name in blue indicates that its abundance was decreased by exposure to 250 mg STP. * means the abundance change caused by STP had statistical significance. 'g_-' and 'Other' were unclassified taxa. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Primers used for 16S rRNA gene sequencing.

Primer		Sequence of primers
1 st round PCR	V1/V2 ^a	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTTTGATCMTGGCTCAG -3' 3'- TGAGGATGCCCTCCGT GACAGAGAATATGTGTAGAGGCTCGGGTGCTCTG-5'
	V4 ^a	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTGYCAGCMGCCGCGGTA -3' 3'- TAATCTWTGGGVHCATCAGG GACAGAGAATATGTGTAGAGGCTCGGGTGCTCTG-5'
2 nd round PCR	Barcoded primer ^b	5'-AATGATACGGCGACCACCGAGATCTACAC ATCACG TCGTCGGCAGCGTC-3' 5'-CAAGCAGAAGACGCATACGAGAT CGAAAC GTCTCGTGGGCTCGG-3'

^aFor primer of V/V2 and V4 region, underline highlight gene-specific primer sequences.

^bFor barcoded primer, underline highlight dual-barcode sequences, which was specific for each sample.