Effect of smokeless tobacco products on human oral bacteria growth and viability

Min Liu^{a, b}, Jinshan Jin^a, Hongmiao Pan^{a, c}, Jinhui Feng^{a, d}, Carl E. Cerniglia^a, Maocheng Yang^{e*},
Huizhong Chen^{a*}

Running title: Impact of smokeless tobacco exposure on oral bacteria

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22 **Abstract**

23 To evaluate the toxicity of smokeless tobacco products (STPs) on oral bacteria, seven smokeless
24 tobacco aqueous extracts (STAEs) from major brands of STPs and three tobacco-specific N-
25 nitrosamines (TSNAs) were used in a growth and viability test against 38 oral bacterial species or
26 subspecies. All seven STAEs showed concentration-dependent effects on the growth and viability of
27 tested oral bacteria under anaerobic culture conditions, although there were strain-to-strain variations.
28 In the presence of 1 mg/ml STAEs, the growth of 4 strains decreased over 0.32-2.14 log₁₀ fold, while
29 14 strains demonstrated enhanced growth of 0.3-1.76 log₁₀ fold, and the growth of 21 strains was not
30 significantly affected. In the presence of 10 mg/ml STAEs, the growth of 17 strains was inhibited 0.3-
31 2.11 log₁₀ fold, 18 strains showed enhanced growth of 0.3-0.97 log₁₀ fold, and 4 strains were not
32 significantly affected. In the presence of 50 mg/ml STAEs, the growth of 32 strains was inhibited 0.3-
33 2.96 log₁₀ fold, 8 strains showed enhanced growth of 0.3-1.0 log₁₀ fold, and 2 strains were not
34 significantly affected. All seven STAEs could promote the growth of 4 bacterial strains, including
35 *Eubacterium nodatum*, *Peptostreptococcus micros*, *Streptococcus anginosus*, and *Streptococcus*
36 *constellatus*. Exposure to STAEs modulated the viability of some bacterial strains, with 21.1-66.5%
37 decrease for 4 strains at 1 mg/ml, 20.3-85.7% decrease for 10 strains at 10 mg/ml, 20.0-93.3% decrease
38 for 27 strains at 50 mg/ml, and no significant effect for 11 strains at up to 50 mg/ml. STAEs from
39 snuffs inhibited more tested bacterial strains than those from snus indicating that the snuffs may be
40 more toxic to the oral bacteria than snus. For TSNAs, cell growth and viability of 34 tested strains were
41 not significantly affected at up to 100 µg/ml; while the growth of *P. micros* was enhanced 0.31-0.54
42 log₁₀ fold; the growth of *Veillonella parvula* was repressed 0.33-0.36 log₁₀ fold; and the cell viabilities
43 of 2 strains decreased 56.6-69.9%. The results demonstrate that STAEs affected the growth of some
44 types of oral bacteria, which may affect the healthy ecological balance of oral bacteria in humans. On
45 the other hand, TSNAs did not significantly affect the growth of the oral bacteria.

46 **1. Introduction**

47 The prevalence of smokeless tobacco products (STPs) is a public health issue especially among youth
48 and young adult males in the United States. It is estimated that 2.6% of US adults are current users of
49 smokeless tobacco products [1]. Moreover, a separate survey reports that 9.9% high school males
50 were current users of smokeless tobacco products in 2014.
51 (www.cdc.gov/tobacco/data_statistics/fact_sheets/smokeless/use_us/index.htm). Major types of
52 smokeless tobacco products in the US market include snuff, snus, chewing tobacco, and some
53 dissolvables (<http://www.cancer.org/cancer/cancercauses/tobaccocancer/smokeless-tobacco>) [2]. Use of
54 STPs can cause numerous health problems, including gingival and periodontal inflammation [3], oral,
55 esophageal and pancreatic cancer [4, 5], and cardiovascular disease associated with hematogenous
56 spread of oral bacteria [2]. Nicotine is one of the major alkaloids in STPs and exposure of nicotine
57 leads to addiction. Nicotine can be absorbed and enter the bloodstream through oral exposure, thus use
58 of STPs can cause nicotine addiction similar to smoking tobacco [4, 6, 7].

59 In the human oral cavity, there are more than 700 bacterial species or phylotypes, as identified by
60 cultivation or culture-independent molecular approaches [8, 9]. The commensal bacteria play an
61 important role in maintaining oral and systemic health [10-12]. Disruption of the ecology of oral
62 bacteria might cause oral infectious and systemic diseases [13, 14]. An important question is whether
63 oral bacteria can convert constituents in smokeless tobacco to more or less genotoxic and
64 carcinogenetic substances. A few studies have investigated the effects of nicotine on growth of oral
65 bacteria. Keene and Johnson [15] indicated a biphasic, dosage-dependent effect of nicotine on the
66 growth of *Streptococcus mutans*. In addition, the concentration of nicotine (10^{-3} M) in the saliva of
67 smokeless tobacco users could stimulate growth of *S. mutans* and possibly place the user at increased
68 risk for dental caries. Recently, Huang et al. [16] found that addition of nicotine enhanced biofilm
69 formation and biofilm metabolic activity of *S. mutans*, which suggested that smoking can increase the

development of caries by fostering increased formation of *S. mutans* biofilms on tooth surfaces. An *in vitro* study provided evidence of stimulated growth of *S. mutans* and *S. sanguinis* in the presence of smokeless tobacco extracts [17, 18]. Extracts from chewing tobacco with high sugar content can increase the growth of *Lactobacillus casei* [18, 19]. Another study indicated that extracts of STPs may serve as a growth substrate for *S. mutans*, *S. salivarius* and *S. sanguinis* [20]. Ayo-Yusuf et al. found that low concentrations of STPs on the South African market didn't inhibit oral bacterial flora [21]. All these results suggest that STPs might affect the growth of oral bacteria. However, the data on the effects of STPs on oral bacterial growth are limited, and few oral bacteria were investigated in these studies.

STPs contain more than 30 carcinogens [22], such as tobacco-specific N-nitrosamines (TSNAs), polycyclic aromatic hydrocarbons (PAHs), aldehydes, metals, and a variety of other chemical constituents [6]. Among these carcinogens, TSNAs are considered some of the most important due to the combination of abundance and strong carcinogenicity [23, 24]. Two major TSNAs, *N'*-nitrosonornicotine (NNN) and 4-(methylnitrosoamino)-1-(3-pyridinyl)-1-butanone (NNK) are known risk factors for human cancer [23]. Some studies report that the production of TSNAs increased during the fermentation process [25], indicating that oral bacteria might affect the genotoxicity and carcinogenesis of STPs.

In this study, we investigated the effects of STAEs from seven major brands of STPs in the US market and three major TSNAs, including NNN, NNK and 4-(methylnitrosoamino)-1-(3-pyridinyl)-1-butanol (NNAL, a major metabolite of NNK) on 38 oral bacterial species/subspecies.

2. Methods and Materials

2.1 Materials

About 50 cans of each of seven most popular brands of STPs were purchased from local stores

94 in Little Rock, Arkansas between 2012 and 2014, including two major brands of Snus named as Snus 1
95 (Camel Snus Large Robust) and 2 (Marlboro Snus Mint) [26], and five major brands of moist snuff
96 named as Snuff 3 (Copenhagen Snuff Original Fine Cut), 4 (Copenhagen Long Cut Wintergreen), 5
97 (Grizzly Long Cut Premium Wintergreen), 6 (Skoal Long Cut Classic Wintergreen), and 7 (Skoal
98 Banditis Wintergreen) [26, 27]. The most popular brands of snus and moist snuff were selected in order
99 to ensure that the results described herein covered a significant portion of the market for these kinds of
100 STPs. NNN, NNK and NNAL were purchased from Sigma-Aldrich Company (St. Louis, MO). The
101 samples of the smokeless tobacco products were stored at -20°C. The LIVE/DEAD BacLight bacterial
102 viability and counting kit was purchased from Life Technologies (Grand Island, NY).

103

104 ***2.2 Preparation of smokeless tobacco aqueous extracts (STAEs)***

105 The extraction of smokeless tobacco aqueous extracts (STAEs) was done according to previous
106 studies [20, 28, 29] with some modifications. Briefly, 5 g of STP was placed in a 50-ml centrifuge tube
107 containing 25 ml Milli-Q water and sonicated in a Branson 3200 ultrasonic cleaner for 1 h, and then the
108 mixtures were centrifuged at 12,000 x g for 1 h at 4 °C. The supernatants were adjusted to pH 7.0 and
109 filtered through 0.2 µm pore size filter. The filtered extracts were lyophilized, using a Labconco
110 FreeZone Console Freeze Dry Systems (Kansas City, MO). The dried extracts were weighted and re-
111 suspended to 10, 100 and 500 mg/ml stock solutions in Milli-Q water.

112

113 ***2.3 Detection of nicotine contents***

114 Nicotine content in STAEs was detected by high-performance liquid chromatography (HPLC).
115 The HPLC system (Agilent Technologies 1200 Series, Santa Clara, CA) consisted of G1379B degasser,

116 G1312A binary pump, G1329A autosampler, and G1315D Diode Array Detector. The analytical
117 column used as the stationary phase was a LiChroCART, Purospher STAR RP-18 endcapped (125 cm
118 $\times$ 4 mm i.d., 5 μ m particle size) (Merck Millipore, Billerica, MA). The mobile phase was a mixture of
119 0.272 g of KH_2PO_4 , 0.184 g of sodium *n*-heptane sulfonate, 820 ml of water (HPLC-grade), and 180 ml
120 of methanol (HPLC-grade). The pH of the mobile phase was adjusted by drop wise addition of ortho-
121 phosphoric acid (pH $\approx$ 3.2). The flow rate used was 1.0 ml/min and the injection volume of 1 μ l. The
122 wavelength was fixed at 254 nm [30].

123

124 ***2.4 Bacterial strains and growth conditions***

125 Twenty-nine oral bacterial strains were obtained from the American Type Culture Collection
126 (ATCC, Manassas, VA) and 9 oral bacterial strains were kindly given by BEI Resources (managed by
127 ATCC) (Table 1). One colony per bacterial strain was inoculated into 40 ml Brain Heart Infusion (BHI)
128 broth or modified BHI broth in a 50-ml Falcon centrifuge tube, grown at 37 °C overnight in different
129 atmospheres (Table 1), and used as a seed culture. The bacterial seed culture was inoculated into 2 ml
130 (final volume) of medium with 1, 5, or 10% (v/v) inoculum in a 15 ml Falcon tube, which contained
131 different concentrations of STAEs or TSNA in an anaerobic chamber (Coy Laboratory Products INC.,
132 Grass, MI) with an anaerobic gas mixture, 85% N_2 -10% H_2 -5% CO_2 . Then the cultures were incubated
133 at 37 °C without agitation (Table 1). After 24 (18 h for 6 strains which were grew rapidly) and 48 h (36
134 h for the strains which were grew rapidly) incubation, the cultures were collected for bacterial cell
135 growth and viability assays as described below. All experiments were done in triplicate.

136

137 ***2.5 Assessment of bacterial growth and cell viability***

138 Bacterial growth and the bacterial cell viability were analyzed by flow cytometry (Accuri C6

flow cytometer, San Jose, CA) [31, 32]. Samples collected from the bacterial cultures were stained according to the manufacturer's instructions using the LIVE/DEAD BacLight Bacterial Viability and Counting Kit (Life Technologies). Ten microliters of bacterial culture were added into a mixture of 987 μ l of 0.85% NaCl, 1.5 μ l of 3.34 mM SYTO 9 nucleic acid stain and 1.5 μ l of 30 mM propidium iodide in a flow cytometry analysis tube, and then incubated for 15 min at room temperature, protected from light. Green and red fluorescence were recorded in the FL1 channel (530 ± 15 nm) and the FL3 channel (> 670 nm), respectively. All parameters were collected as logarithmic. The flow rate was kept below 5,000 events per second and at least 20,000 cells for each sample were counted. Data were analyzed using CFlow Plus software (Accuri Cytometers). In the density plots of light-scattering properties, bacterial cells were gated from irrelevant counts. In the density plots of fluorescence, the distinct bacterial populations (P1: live cells and P2: dead cells) were gated based on the different viability stages. Cell viability (%) = percent of P1 (green cells)/(percent of P1 + percent of P2 (red cells)) X 100. Cell Viability Changes = Cell Viability_{in presence of STAE} – Cell Viability_{in absence of STAE}. Total cell numbers = live cell numbers + dead cell numbers. Accuri C6 flow cytometry was calibrated using 8-peak Spherotech Validation Beads.

2.6 Statistical analysis

Student's T-test was employed for analyzing cell growth and cell viability data from triplicated culture samples. For cell growth, cell number is the mean of $\log_{10}$ cell number measured by flow cytometry from triplicated incubations ($SD < 0.10$, P values < 0.05). Cell viability is the mean percentage measured by flow cytometry from triplicate incubations ($SD \leq 5\%$, P values < 0.05).

3. Results and discussion

162 3.1 Effects of STAEs on the cell growth of oral bacteria

163 Nicotine is the primary addictive and dependence producing substance in smokeless tobacco
164 products, and nicotine dependence drives the development and maintenance of addiction to tobacco
165 products (<https://profiles.nlm.nih.gov/ps/access/NNBBFC.pdf>). To determine nicotine level, seven
166 brands of STPs were extracted with aqueous phase, and then were examined with HPLC. Nicotine data
167 were summarized in Table 2. The 38 oral bacterial strains used in the current study are normal
168 inhabitants of the human oral cavity but have sometimes been implicated in cases of periodontitis or
169 other oral disease. Therefore these bacterial strains can be considered as opportunistic pathogens [8-
170 10]. In our preliminary experiment, we found that artificial saliva [33] did not significantly affect the
171 extraction rate of STAE comparing the extraction with water. The aqueous extraction condition is more
172 similar to chewing extraction conditions in the human mouth than organic solvent extraction condition,
173 thus STAEs were used to investigate effects of STPs on the growth and viability of oral bacteria. The
174 doses of STAE (1, 10, 50 mg/ml) used in this study are similar to physiological conditions in the mouth
175 of the smokeless tobacco users [34, 35]. Exposure to STAEs affected the growth of all tested strains in
176 a concentration-dependent manner, although there were strain-to-strain variations (Fig. 1, Fig.2). In the
177 presence of 1 mg/ml STAEs, the growth of 4 strains was inhibited 0.3-2.14 log₁₀ fold, while the growth
178 of 14 strains was enhanced 0.3-1.76 log₁₀ fold by some of the tested STPs, and the growth of 21 strains
179 was not significantly affected (Fig.1, Fig. 2 and Table S1). A concentration of 1 mg/ml STAEs from all
180 seven STPs inhibited the growth of *Propionibacterium acnes*, but promoted the growth of
181 *Streptococcus constellatus* and *S. sanguinis*.

182 In the presence of 10 mg/ml STAEs, the growth of 17 strains was inhibited 0.3-2.11 log₁₀ fold by
183 some of the tested STPs, the growth of 18 strains was enhanced 0.3-0.94 log₁₀ fold by some of the
184 tested STPs, and the growth of 4 strains were not affected (Fig.1, Fig. 2 and Table S1). At 10 mg/ml,
185 STAEs from all seven STPs inhibited the growth of 4 strains, including *Aggregatibacter*

186 *actinomycetemcomitans*, *Actinomyces naeslundii*, *Eikenella corrodens*, and *Fusobacterium nucleatum*;
187 and promoted the growth of 7 strains, including *Eubacterium nodatum*, *Gemella morbillorum*,
188 *Peptostreptococcus micros*, *Streptococcus anginosus*, *S. constellatus*, *S. sanguinis*, and *Veillonella*
189 *parvula*.

190 In the presence of 50 mg/ml STAEs, the growth of 32 strains was inhibited 0.3-2.96 log₁₀ fold by
191 some of the tested STPs, the growth of 8 strains was enhanced 0.3-1 log₁₀ fold by some of the tested
192 STPs, and 2 strains were not significantly affected after 18-48 h (Fig.1, Fig. 2 and Table S1). The 50
193 mg/ml concentration of STAEs from all seven STPs inhibited the growth of 21 strains and only
194 promoted the growth of *E. nodatum*.

195 At a low concentration of STAEs (1 mg/ml), the growth of more than half of the tested bacterial
196 strains was not significantly affected. With increasing concentrations of STAEs from 1 to 50 mg/ml, the
197 number of bacterial strains whose growth was enhanced increased at first, and then decreased, which
198 might be due to the conflicting effects of nutrients and toxic compounds in STPs. At a high
199 concentration of STAEs (50 mg/ml), the growth of most strains was inhibited. Similar to the results of
200 previous studies [18, 20], we also observed that smokeless tobacco extracts stimulated the growth of *S.*
201 *sanguinis* at 1 mg/ml and 10 mg/ml (Fig.1, Fig. 2 and Table S1). This species serves as a tether for the
202 attachment of other oral microorganisms that colonize the tooth surface, forms dental plaque, and
203 contributes to development of caries and periodontal disease [36]. In addition to *S. sanguinis*, we also
204 observed growth enhancement due to smokeless tobacco extracts of more bacterial strains, such as
205 *Bacteroides forsythus*, *E. nodatum*, *P. micros*, *S. anginosus*, and *S. constellatus*. *P. micros* is associated
206 with periodontal disease as well as several other polymicrobial infections and systemic diseases [13]. *E.*
207 *nodatum* has strong association with periodontitis, whether in the presence or absence of high levels of
208 the consensus pathogens [14]. *S. anginosus* was associated with oral infection [37], liver abscesses
209 [38], and esophageal cancer [39]. STAEs promoted the growth of these bacterial species associated
210 with oral diseases, whereas they inhibited the growth of other species tested, suggesting that smokeless

211 tobacco ingredients could disrupt the oral ecology and enhance the risk of oral disease.

212 When incubated for 18 or 24 h, there were 22 and 19 strains inhibited by 50 mg/ml STAE of Snus
213 1 (Camel Snus Large Robust) and 2 (Marlboro Snus Mint), respectively; on the other hand, there were
214 26 -30 strains inhibited by STAEs from five brands of snuffs for more than two fold, which implies that
215 the snuffs may be more harmful to the oral bacteria than snus.

216

217 **3.2 Effects of STAEs on the cell viability of oral bacteria**

218 To investigate whether STAEs could affect the cell viability of oral bacteria, cell viabilities of 38
219 oral bacterial strains grown with or without STAEs were determined by flow cytometry measurements.
220 STAEs decreased cell viability (20.3-93.3%) for 27 strains in a concentration-dependent manner (Fig. 3
221 and Table S2). In the presence of 1 mg/ml STAEs, the viability of 4 strains decreased 21.1-66.5%; in
222 the presence of 10 mg/ml STAEs, that of 9 strains decreased 20.3-85.7%; in the presence of 50 mg/ml
223 STAEs, viability of 27 strains decreased 22.0-93.3%. Cell viabilities of four bacterial strains were
224 slightly reduced (10.5-19.8%) by STAEs from some of the tested STPs. STAEs slightly enhanced cell
225 viability (10.2-15.7%) for four strains, including *F. nucleatum*, *Fusobacterium periodonticum*, and *G.*
226 *morbilorum*, and *Treponema socranskii* at 1 mg/ml or 10 mg/ml. STAEs from all seven STPs had no
227 significant effect on the cell viability of 7 strains, including *B. forsythus*, *Campylobacter rectus*,
228 *Capnocytophaga sputigena*, *E. nodatum*, *P. acnes*, *S. anginosus*, and *Streptococcus oralis*. Previous
229 studies indicate that *B. forsythus*, *E. nodatum*, *T. socranskii* are associated with periodontal disease [40];
230 *C. rectus* can cause oral abscess [14, 41]; and *S. anginosus* and *S. oralis* can cause oral infection and
231 other organ abscesses [38, 42].

232

233 3.3 Effects of TSNA_s on the cell growth and viability of oral bacteria

234 TSNA_s are considered the most important carcinogens in STPs [23, 24]. However, few studies
235 investigated effects of TSNA_s on the growth and viability of oral bacteria. Three important TSNA_s
236 (NNN, NNK, and NNAL) were added to bacterial cultures to investigate the effects on the cell growth
237 and viability of 38 oral bacteria species/subspecies by flow cytometry measurements. Cell growth of 24
238 strains was not significantly affected by tested TSNA_s (Fig. 4 and Table S3), *P. micros* was enhanced
239 0.10-0.54 log₁₀ fold by all three TSNA_s, and cell growth of 12 other strains was enhanced 0.1-0.26
240 log₁₀ fold, including *C. sputigena* and *Porphyromonas gingivalis* (Fig. 4 and Table S3). The three
241 strains, *C. sputigena*, *P. micros* and *P. gingivalis*, are all potentially associated with periodontal disease
242 [11, 13, 43]. Cell growth of *V. parvula* was inhibited with a decrease of 0.33-0.36 log₁₀ fold, and 2
243 other strains slightly decreased (0.10-0.13 log₁₀ fold) (Fig. 4). In general, cell growth of the oral
244 bacteria was not significantly affected by TSNA_s at the tested concentrations. For cell viability, 32 of
245 38 strains were not significantly affected by TSNA_s (Fig. 5 and Table S4). Cell viabilities of *C. rectus*
246 and *F. periodonticum* decreased 56.6-69.9%, and 4 other strains were slightly decreased (11.0-19.8%).
247 As TSNA_s are well known to be important carcinogens in STPs [23, 24], use of STPs may have
248 negative effects on oral health. Similar as above, cell viability for the oral bacteria was not significantly
249 affected by TSNA_s at the tested concentrations, indicating that other ingredients in the smokeless
250 tobacco products may be the responsible for the growth inhibition and decreasing cell viability of the
251 bacteria.

252

253 4. Conclusions

254 STAEs affected the growth and viability of some oral bacterial species in a concentration-
255 dependent manner. Among the bacterial species in which the growth rate was enhanced by STAEs,
256 some were associated with opportunistic pathogens which potentially cause oral diseases (e.g., gingival

and periodontal inflammation and dental caries). STAEs from snuffs inhibited more bacterial strains than those from snus indicating that the snuffs may be more toxic to the oral bacteria than snus. Our results indicate that STPs might have adverse effects on maintaining a healthy ecological balance of oral bacteria in humans. Since the human oral microbiome is a diverse and complex ecosystem, future research to evaluate the toxicological effects of smokeless tobacco on the oral microbiota *in vivo* and the genotoxicity of smokeless tobacco products and their metabolites produced by oral microbiota, which may provide new insight into how smokeless tobacco causes diseases, is warranted. The new insights from this study might contribute to the understanding of the interactions of oral microbiome and smokeless tobacco. The methodology described in this study could potentially be used as an effective assay to compare the relative toxicity of various smokeless tobacco products on oral bacteria.

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Figure legends

Figure 1 The cell growth of oral bacteria incubated with STAEs from different STP brands for 18 or 24 h. The cell number change = $\text{Cell Number}_{\text{in presence of STAE}} / \text{Cell Number}_{\text{in absence of STAE}}$. When cell

number change $\geq 0.3 \log_{10}$, the change is considered as significant. A: incubated with STAE from Snus 1 (Camel Snus Large Robust); B: incubated with STAE from Snus 2 (Marlboro Snus Mint); C: incubated with STAE from Snuff 3 (Copenhagen Snuff Original Fine Cut); D: incubated with STAE from Snuff 4 (Copenhagen Long Cut Wintergreen); E: incubated with STAE from Snuff 5 (Grizzly Long Cut Premium Wintergreen); F: incubated with STAE from Snuff 6 (Skoal Long Cut Classic Wintergreen); G: incubated with STAE from Snuff 7 (Skoal Banditis Wintergreen). Statistically significant values were presented here, the rest data were shown in Table S1. * Strains were incubated for 18 h, while other strains were incubated for 24 h.

290

Figure 2 The cell growth changes of oral bacteria incubated with STAEs from different STP brands for 36 or 48 h. The cell number change = $\text{Cell Number}_{\text{in presence of STAE}} / \text{Cell Number}_{\text{in absence of STAE}}$. When cell number change $\geq 0.3 \log_{10}$, the change is considered as significant. A: incubated with STAE from Snus 1 (Camel Snus Large Robust); B: incubated with STAE from Snus 2 (Marlboro Snus Mint); C: incubated with STAE from Snuff 3 (Copenhagen Snuff Original Fine Cut); D: incubated with STAE from Snuff 4 (Copenhagen Long Cut Wintergreen); E: incubated with STAE from Snuff 5 (Grizzly Long Cut Premium Wintergreen); F: incubated with STAE from Snuff 6 (Skoal Long Cut Classic Wintergreen); G: incubated with STAE from Snuff 7 (Skoal Banditis Wintergreen). Statistically significant values were presented here, the rest data were shown in Table S1. * Strains were incubated for 36 h, while other strains were incubated for 48 h.

301

Figure 3 The cell viability changes of oral bacteria incubated with STAE from different STP brands. Cell Viability Changes = $\text{Cell Viability}_{\text{in presence of STAE}} - \text{Cell Viability}_{\text{in absence of STAE}}$. When cell viability change $\geq 20\%$, the change is considered as significant. A: incubated with STAE from Snus 1 (Camel Snus Large Robust); B: incubated with STAE from Snus 2 (Marlboro Snus Mint); C: incubated with STAE from Snuff 3 (Copenhagen Snuff Original Fine Cut); D: incubated with STAE from Snuff 4

307 (Copenhagen Long Cut Wintergreen); E: incubated with STAE from Snuff 5 (Grizzly Long Cut
308 Premium Wintergreen); F: incubated with STAE from Snuff 6 (Skoal Long Cut Classic Wintergreen);
309 G: incubated with STAE from Snuff 7 (Skoal Banditis Wintergreen). * Strains were incubated for 18 h,
310 while other strains were incubated for 24 h.

311

312 **Figure 4 The cell growth changes of oral bacteria incubated with TSNA.** The cell number change
313 = $\text{Cell Number}_{\text{in presence of STAE}} / \text{Cell Number}_{\text{in absence of STAE}}$. When cell number change $\geq 0.3 \log_{10}$, the
314 change is considered as significant. A: incubated with NNN for 24 h; B: incubated with NNAL for 24
315 h; C: incubated with NNK for 24 h; D: incubated with NNN for 48 h; E: incubated with NNAL for 48
316 h; F: incubated with NNK for 48 h. * Strains were incubated for 18 h; † strains were incubated for 36 h.

317

318 **Figure 5 The cell viability changes of oral bacteria incubated with TSNA.** Cell Viability Changes
319 = $\text{Cell Viability}_{\text{in presence of STAE}} - \text{Cell Viability}_{\text{in absence of STAE}}$. A: incubated with NNN for 24 h; B:
320 incubated with NNAL for 24 h; C: incubated with NNK for 24 h. * Strains were incubated for 18 h.

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430

Fig. 3

Fig. 4

Fig. 5

Table 1. The oral bacterial strains and culture conditions used in the study

Strain name	Culture medium ^a	Inoculum (%)	Atmosphere	Source
<i>Aggregatibacter actinomycetemcomitans</i> ATCC 29524	BHI	5	CO ₂ (5%)	ATCC
<i>Actinomyces gerencseriae</i> F0344	BYKH	5	Anaerobic	BEI
<i>Actinomyces israelii</i> F0345	BHI	10	Anaerobic	BEI
<i>Actinomyces naeslundii</i> ATCC 12104	BHI	5	Anaerobic	ATCC
<i>Actinomyces odontolyticus</i> F0309	BYKH	5	Anaerobic	BEI
<i>Bacteroides forsythus</i> ATCC 43037	BHI	10	Anaerobic	ATCC
<i>Capnocytophaga gingivalis</i> ATCC 33624	BHI	1	Anaerobic	ATCC
<i>Campylobacter gracilis</i> ATCC 33236	BHIF	5	Anaerobic	ATCC
<i>Campylobacter rectus</i> ATCC 33238	BHIF	5	Anaerobic	ATCC
<i>Campylobacter showae</i> ATCC 51146	BHIF	5	Anaerobic	ATCC
<i>Capnocytophaga sputigena</i> ATCC 33612	BHI	5	Anaerobic	ATCC
<i>Eikenella corrodens</i> ATCC 23834	BHI	5	CO ₂ (5%)	ATCC
<i>Eubacterium nodatum</i> ATCC 33099	BYKH	1	Anaerobic	ATCC
<i>Eubacterium saburreum</i> ATCC 43850	BHI	1	Anaerobic	ATCC
<i>Fusobacterium nucleatum nucleatum</i> ATCC 23726	BHI	1	Anaerobic	ATCC
<i>Fusobacterium nucleatum polymorphum</i> F0401	BHI	1	Anaerobic	BEI
<i>Fusobacterium nucleatum vincentii</i> ATCC 49256	BHI	1	Anaerobic	ATCC
<i>Fusobacterium periodonticum</i> ATCC 33693	BHI	1	Anaerobic	ATCC
<i>Gemella morbillorum</i> M424	BYKH	10	Anaerobic	BEI
<i>Leptotrichia buccalis</i> ATCC 14201	BHI	1	Anaerobic	ATCC
<i>Neisseria mucosa</i> C102	BYKHL	5	Anaerobic	BEI
<i>Propionibacterium acnes</i> ATCC 6919	BYKHL	1	Anaerobic	ATCC
<i>Porphyromonas gingivalis</i> ATCC 33277	TSB-YKHL	1	Anaerobic	ATCC
<i>Prevotella intermedia</i> ATCC 25611	TSB-YKHL	5	Anaerobic	ATCC
<i>Prevotella melaninogenica</i> ATCC 25845	BHI	10	Anaerobic	ATCC
<i>Peptostreptococcus micros</i> ATCC 33270	BHI	5	Anaerobic	ATCC
<i>Prevotella nigrescens</i> ATCC 33563	BHI	10	Anaerobic	ATCC
<i>Streptococcus anginosus</i> F0211	BHI	5	Anaerobic	BEI
<i>Streptococcus constellatus</i> ATCC 27513	BHI	5	Anaerobic	ATCC
<i>Selenomonas noxia</i> 43527	BYKHL	1	Anaerobic	BEI
<i>Staphylococcus intermedius</i> ATCC 51874	BHI	5	Anaerobic	ATCC
<i>Streptococcus gordonii</i> ATCC 33399	BHI	5	Anaerobic	ATCC
<i>Streptococcus mitis</i> ATCC 903	BHI	5	Anaerobic	ATCC
<i>Streptococcus oralis</i> ATCC 35037	BHI	5	Anaerobic	ATCC
<i>Streptococcus sanguinis</i> ATCC 29667	BHI	5	Anaerobic	ATCC
<i>Treponema denticola</i> 33520	BHI	5	Anaerobic	BEI
<i>Treponema socranskii</i> ATCC 35535	BHI	10	Anaerobic	ATCC
<i>Veillonella parvula</i> ATCC 17745	BHI	10	Anaerobic	ATCC

^aBHI, brain-heart infusion medium; BYKH, brain-heart infusion medium with yeast extract, vitamin K1 solution and hemin solution; BYKHL, brain-heart infusion medium with yeast extract, vitamin K1 solution, hemin solution and L-cysteine HCl; TSB-YKHL, Tryptic Soy Broth with yeast extract, vitamin K1 solution, hemin solution and L-cysteine HCl; BHIF, brain-heart infusion medium with formate and fumarate.

Table 2. Nicotine contents in STPs^a

STP ^b	Nicotine/STP (mg/g)
Snus 1	4.77
Snus 2	4.57
Snuff 3	6.72
Snuff 4	6.00
Snuff 5	5.12
Snuff 6	5.11
Snuff 7	5.03

^a The data were shown in the mean of triplicate ($SD \leq 0.05$); ^b Snus 1 (Camel Snus Large Robust); Snus 2 (Marlboro Snus Mint); Snuff 3 (Copenhagen Snuff Original Fine Cut); Snuff 4 (Copenhagen Long Cut Wintergreen); Snuff 5 (Grizzly Long Cut Premium Wintergreen); Snuff 6 (Skoal Long Cut Classic Wintergreen); Snuff 7 (Skoal Banditis Wintergreen).