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IMPACT OF SMOKELESS AND SMOKING TOBACCO ON SUBGINGIVAL MICROBIAL COMPOSITION: A COMPARATIVE STUDY

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ABSTRACT

BACKGROUND. Environmental perturbations such as tobacco use causes increased bacterial diversity in the subgingival microbiome. Despite the recognized impact of tobacco on oral health, there is a notable gap in the literature regarding the specific characteristics of the subgingival microbiome among Indian tobacco users.

OBJECTIVE. This study seeks to provide a comparative analysis of subgingival microbial profile of smokeless tobacco users and smokers with an otherwise healthy periodontal environment.

METHOD. This cross-sectional study at a Tertiary Dental Hospital in India recruited 118 participants: 52 non-tobacco users (Group 1), 36 smokeless tobacco users (Group 2), and 30 smokers (Group 3). Subgingival samples were collected from mesial surfaces of teeth (16, 46) using sterile paper points and analysed via the streak plate method for bacterial profiling. Clinical examinations assessed oral hygiene, gingival, and periodontal health using indices: Bleeding on Probing (BoP), Pocket Depth (PD), and Approximate Plaque Index (API). Categorical variables were analysed using the Chi-square test, and odds ratios were calculated.

RESULT. Gender distribution was 76.3% male and 23.7% female ($p < 0.05$). Group 2 had a significantly higher prevalence of Gram-positive cocci (100%) and Gram-negative coccobacilli (94.4%) compared to Group 1, with a 12.4 times increased risk for Gram-negative coccobacilli ($p < 0.05$). Group 2 also showed a higher occurrence of *Aggregatibacter* (88.9%) and a 3.5 times increased risk ($p < 0.05$). Group 3 exhibited significantly more Gram-positive cocci and Gram-negative coccobacilli than Group 1, with 3.8 times and 4.7 times increased risks, respectively ($p < 0.05$). *Rothia* species were significantly more common in smokers (13.3%) than non-tobacco users (0%) ($p < 0.05$).

CONCLUSION. Despite the absence of periodontal disease, the elevated presence of *Aggregatibacter*, *Enterococcus*, *Klebsiella*, and *Rothia* species indicates a shift towards increased bacterial diversity and a higher risk of future periodontitis.

Keywords: *smokeless tobacco, smoking, microbiota, gingiva, comparative study*

INTRODUCTION

Tobacco consumption is a major public health menace around the world. Globally, 80% of the 1.25 billion tobacco users reside in low-and-middle-income countries contributing greatly to tobacco associated illness, disability and death (1). Tobacco use is attributed as a risk factor for noncommunicable diseases including chronic respiratory diseases, cancer, diabetes, and cardiovascular disease, as well as for certain communicable diseases such as tuberculosis (2). In addition, all major forms of tobacco has significant detrimental impact on oral health. It can lead to specific oral health problems such as halitosis, dental caries, periodontal disease, peri-implantitis, alveolar osteitis, oral potentially malignant disorders and oral cancer among others (3). A causal relationship between smoking and periodontal disease has long been established. There is sufficient evidence that tobacco aggravates the progression and development of periodontal disease. It has been found to cause direct inhibition of normal fibroblastic function, increased collagen degradation, delaying of healing process and accelerating invasion of pathogenic bacteria (4). Tobacco smoke has also been linked to long-term microvascular dysfunction resulting from decreased perfusion caused by multiple vasoconstrictive insults, contributing to the pathophysiology of periodontal disease (5). Similarly, use of smokeless tobacco (SLT) is associated with increased plaque and calculus deposition and gingival recession, ultimately leading to periodontitis (6).

In addition to the macro-level changes caused by tobacco use, it also has an effect on the oral microbiome homeostasis. Oral microbiome is the second largest microbial community in humans after the gut, and harbours over 500–700 different bacterial species (7). The variety of microbiota in the oral cavity is due to the presence of two different types of surfaces that support microbial colonization: the shedding mucosal surfaces and the solid surfaces such as teeth or dentures (8). A healthy core microbiome consists of genera *Streptococcus*, *Veillonella, Neisseria,* and *Actinomyces* (8). While studies have reported dominance of *Streptococcus* in the microbial landscape, the top coverage genera also includes *Haemophilus, Rothia, Neisseria*, and *Veillonella* (9). However, the microbial characterization of subgingival plaque community exhibits a shift with changes in periodontal tissue. For instance, in chronic periodontitis patients, the microbiota is dominated by *Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria,* and *Proteobacteria* and in addition, the proportion of *Spirochaetes* and *Synergistetes* is increased compared with healthy samples (10,11). It has also been reported that ecological subgingival microbiome community in periodontitis is marked by the rise of newly dominant taxa with higher proportions of *Spirochetes, Synergistetes,*

Firmicutes and *Chloroflexi* without displacing the primary species associated with healthy periodontium (12).

Environmental perturbations such as tobacco use causes increased bacterial diversity in the subgingival microbiome (13). Smoking, in particular, has an impact on the pathogenicity of periodontopathogens with augmented biofilm formation of microbes such as *P. gingivalis* (14). Smokers exhibit highly diverse and relatively unstable initial colonization of subgingival biofilms, with early acquisition and colonization of pathogens in oral biofilms (15). Research has consistently reported reduction in beneficial bacteria and increased colonization of pathogenic bacteria among tobacco users (16). In case of smokeless tobacco users, oral bacterial composition shows Gram-negative anaerobes as majority of altered bacterial genera involved in biofilm formation (17). Moreover, evidence points out its role in creating a pro-inflammatory environment in the oral cavity (18). India, as the second-largest tobacco consumer in the world, faces a dual challenge from both smoking and smokeless tobacco use (19). Despite the recognized impact of tobacco on oral health, there is a notable gap in the literature regarding the specific characteristics of the subgingival microbiome among Indian tobacco users. While some studies have explored oral dysbiosis in tobacco users, the subgingival microbiome among Indian users remains under-researched (17,20). This gap underscores the need for further investigation into how tobacco use affects the subgingival microbiome within this population, specifically in the absence of any periodontal condition since such conditions can alter the subgingival microbiome. Therefore, this study aimed to examine the subgingival microbiome profile among smoking and smokeless tobacco users, with otherwise healthy periodontal environment, visiting a Dental Hospital in India.

METHODOLOGY

Study setting. The present study was conducted among patients visiting the Tobacco Cessation Centre of Government College of Dentistry, Indore, India. A series of subgingival samples were collected over a period of 4 months.

Ethical approval and informed consent. The study protocol received approval from the Institutional Ethics Committee, ensuring adherence to ethical standards and the protection of human research subjects. All potential participants were thoroughly informed about the study's objectives, procedures, as well as any probable risks. Informed consent was obtained from each participant only after confirming their full understanding of the study details and their rights as participants.

Sample size determination. A priori sample size calculation was conducted using G*Power 3.1.9.4 (Heinrich Heine University, Düsseldorf, Germany) to determine the minimum required sample size for 3 groups. To achieve a statistical power of 0.80 (80%) with an alpha error probability of 0.05, an anticipated medium effect size (w) of 0.3, and 2 degrees of freedom (df = 2), the minimum required sample size was calculated to be 108 participants. To account for data loss due to procedural issues or unforeseen circumstances, we recruited 118 participants in our study.

Study subjects & selection. The study targeted young adults aged 20 to 35 years, including both males and females. The patients visiting the department were examined for their general oral and periodontal health. Patients who showed no clinical signs of periodontal disease and presented with good systemic and oral health were included in the study. Further, individuals who did not consume tobacco in any form were assigned to Group 1 (Non-Tobacco Users), individuals who consumed SLT in any form at least 1 packet per day were assigned to Group 2 (Smokeless Tobacco Users) and individuals who smoked at least one pack of cigarettes per day were assigned to Group 3 (Smoking Tobacco Users). Participants were excluded from the study if they had: diagnosed periodontal disease, dental anomalies or malocclusion, tobacco use history outside of the study criteria (occasional or former tobacco users), presence of systemic disease, active oral infections (e.g.: candidiasis), pregnancy, fewer than 20 teeth in the oral cavity, antibiotic use (six months prior to enrollment), and history of recent dental treatments.

Patients were systematically selected for examination and sample collection. During the recruitment process, 200 individuals were assessed, with 52 assigned to Group 1, 36 to Group 2, and 30 to Group 3. The remaining individuals were excluded in accordance with the exclusion criteria. All participants were instructed to avoid eating, or drinking anything other than water for at least two hours before the clinical examination and bacterial sample collection. Variability in oral hygiene practices was minimized by instructing all participants to refrain from brushing, flossing, or using mouth rinses for at least 12 hours prior to sample collection.

Bacteriological assessment. To obtain samples for the microbiological analysis, sterile endodontic paper points (DiaDent ISO Color-Coded 2% Paper Points, South Korea) were used. These paper points were inserted cautiously into the sulcus of the mesial surfaces of the following index teeth: right first maxillary and mandibular molars (16, 46). After 30 seconds, the paper points were withdrawn, and each paper point was immediately submerged in saline (transport medium). The collected samples were transported to a laboratory for microbiological analysis, aiming to evaluate the presence and composition of bacteria. The streak plate method

is a microbiological technique employed for the isolation and identification of bacterial colonies within a mixed culture. Using a sterile inoculating loop, the bacterial sample was streaked onto the surface of a solid agar medium in a petri dish. The process involved multiple streaks, each aimed at diluting the bacterial concentration and promoting the growth of isolated colonies. After incubation, the resulting colonies were observed and, if necessary, subculture for further analysis. This method was performed for obtaining pure cultures and studying the distinct characteristics of individual bacterial colonies.

Clinical examination. All the study subjects were clinically examined using indicators for oral hygiene, gingival and periodontal health. Oral examination was carried out using a standard Williams Probe (#6, GDC Fine Crafted Dental Pvt Ltd., India) and a mouth mirror. The probe tip, made of stainless steel, has a diameter of 0.5 mm and was calibrated before each session to ensure accuracy, with a precision of ±0.2 mm. The following parameters were evaluated: Bleeding on Probing (BoP) to assess the gingival health, Pocket Depth (PD) to assess the periodontal health and Approximate Plaque Index (API) to evaluate the oral hygiene of study participants.

***Statistical data analysis*.** Data was entered into the Microsoft Excel Data and analysed using SPSS (Statistical Package for Social Sciences) 25.0 version, IBM, Chicago. Categorical variables were analysed using the Chi-square test. Continuous variables were expressed as Mean± SD and assessed using One-Way ANOVA. Additionally, odds ratios were calculated to evaluate the associations between tobacco use and the presence of specific bacteria, p value <0.05 was considered statistically significant.

RESULTS

The study involved 200 subjects including 52 subjects with no history of tobacco use (Group 1), 36 subjects with a history of tobacco chewing (Group 2), and 30 subjects with history of smoking tobacco (Group 3). Table 1 shows the distribution of study participants according to their demographic variable (Age, Gender) and clinical indicators for their oral health. There was significant difference in the mean age of the subjects in 3 groups. Post hoc analysis revealed that the mean age of Group 1 subjects was significantly lesser compared to that of Group 2 and Group 3 subjects ($p <0.05$), whereas the age of subjects belonging to group 2 and group 3 did not differ significantly ($p >0.05$). Gender distribution showed 23.7% female and 76.3% male participants in the study ($p <0.05$).

The number of subjects showing presence of Gram-positive cocci, Gram positive bacilli, Gram-negative cocci, and Gram-negative coccobacilli were found to differ significantly

between Group 1 and Group 2 (p-value <.05). A greater occurrence of Gram-positive cocci and Gram-negative coccobacilli were found to be significantly associated with Group 2 (100% & 94.4% respectively) (p-value<.05). Assessment of odd's ratio revealed a 12.467 times increased risk of infection with Gram-negative coccobacilli among tobacco chewers (p-value<.05).

The number of subjects showing presence of *Aggregatibacter* was found to differ significantly between Group 1 and Group 2 (p-value <.05). A greater occurrence of *Aggregatibacter* was found to be significantly associated with Group 2 (88.9%) (p-value<.05). Assessment of odd's ratio revealed a 3.556 times increased risk of infection with *Aggregatibacter* among tobacco chewers (p-value<.05).

The number of subjects showing presence of Gram-positive cocci, Gram-negative cocci, and Gram-negative coccobacilli were found to differ significantly between Group 1 and Group 3 (p-value <.05). A greater occurrence of Gram-positive cocci and Gram-negative coccobacilli were found to be significantly associated with Group 2 (100% & 94.4% respectively) (p-value<.05). Assessment of odd's ratio revealed a 3.833 times increased risk of infection with Gram-negative cocci and 4.767 times increased risk of infection with Gram negative coccobacilli among tobacco smokers (p-value<.05).

The number of subjects showing presence of *Rothia* species was found to differ significantly between Group 1 and Group 3 (p-value <.05). A greater occurrence of Rothia species found to be significantly associated with Group 3 (13.3%) compared to Group 1 (0.0%) (p-value<.05).

DISCUSSION

The subgingival microflora is a complex and diverse community, significantly impacted by environmental factors, including tobacco use. This study aims to examine the subgingival microbiome of Indian tobacco users, both in smokeless and smoking forms, with otherwise healthy periodontal conditions. Our study revealed a notable increase in Gram-positive cocci, Gram-positive bacilli, Gram-negative cocci, and coccobacilli in the subgingival environment of smokeless tobacco users compared to the control group. Similar results were reported by S. Sawant *et al.* (2023) with findings such as abundance of phyla consisting of Gram-negative organisms (Bacteroidetes and Proteobacteria) in tobacco chewers (21). The higher levels of Gram-negative cocci and bacilli in SLT users may reflect tobacco-induced inflammation and tissue damage. Gram-negative bacteria are often associated with periodontal disease and thrive in more inflamed or anaerobic conditions, which could be promoted by SLT use (22). Although

in our study the subjects did not show signs of periodontal disease, the presence of such organisms could be an early indicator of microbial shifts that may predispose individuals to future periodontal issues or reflect subclinical inflammatory changes in the oral environment.

When the control group was compared with those who smoked tobacco, significant difference was found in presence of Gram-positive cocci, Gram-negative cocci and Gram-negative coccobacilli ($p<0.05$). Cigarette smoke contains numerous toxicants which significantly disrupts the oral microbial environment. Some of the mechanism by which smoking affects the microbial ecology includes influencing bacterial adherence to oral mucosal surfaces, decreasing salivary pH, depleting oxygen and weakening host immunity (23-25). Microbial analysis from a study conducted by Bastiaan RJ et al. (1978) found similar results of increase in Gram-positive bacteria to Gram-negative bacteria in the smokers as compared to the nonsmokers (26). In another study, the bacterial diversity was also found to be significantly lower among tobacco smokers with altered Gram-positive bacterial microbiota with potential implications in tobacco related diseases (27).

Oral streptococci, a facultative anaerobic Gram-positive bacteria, plays a key role in preserving the equilibrium of the host-microbiome relationship in health, while also influencing immune responses during disease (28). The findings of this study indicate that a large percentage of participants in both Group I and Group II (78.8% vs 77.8%) exhibited the presence of *Streptococcus* species. *Streptococcus* is a potential marker for a healthy oral microbiome however, some *Streptococcus* species (e.g., *Streptococcus mutans* and *Streptococcus sobrinus*) are well-known contributors to dental caries and may indicate a shift from symbiotic to pathogenic role in the presence of tobacco use (29). Moreover, nicotine in the saliva of SLT users in concentration of 10^{-3} M is associated with increased growth of *S. mutans*, potentially increasing their risk of developing dental caries (30). Our findings also indicated that the likelihood of encountering Gram-negative, facultatively anaerobic bacterium *Aggregatibacter* in SLT users is 3.5 times higher compared to non-tobacco users (OR = 3.556, 95% CI = [1.077 – 5.742], p = <0.05). *Aggregatibacter* is a periodontopathogen and its increased presence could signify an environment conducive to the proliferation of pathogenic bacteria.

Available literature has demonstrated that regardless of the periodontal conditions of smokers, substantial changes in the subgingival microflora has been observed. This is particularly evident in the fact that smoking promotes the early acquisition and colonization of periodontal pathogens, leading to a subgingival microbial community in the healthy periodontium that is "at risk for harm" (31). While our results showed that the odds of presence

of *Aggregatibacte*r among smokers is 2.9 times that of non-smokers, the association was not found to be statistically significant (OR = 2.889, 95% CI = [0.865 –9.651], p=<0.05). A higher presence of species *Aggregatibacter actinomycetemcomitans* among smokers have been reported by previous studies as well (32,33). The presence of *Aggregatibacter* can induce inflammatory responses, contributing to tissue destruction in the periodontium. It is also recognized for its interaction with other bacteria, contributing to the development of a dysbiotic microbial community (34). However, in a modified subgingival microbiome model, 16S rRNA sequencing revealed that while *A. actinomycetemcomitans* altered the microbiome structure and composition, it had a mild effect on compromised epithelium function and reduced expression of tight junction proteins (35). Gram-negative bacteria like *Klebsiella* are opportunistic pathogens that are known to resist the effects of smoke and do not exhibit growth inhibition on exposure to tobacco smoke (36). In our study, while smokers had a higher number of individuals with Klebsiella presence, the difference was not statistically significant when compared to the control group. Al-Marzooq F et al. (2021) also reported that these bacteria were more abundant among users of different forms of smoking tobacco (37). This could potentially be attributed to subgingival dysbiosis, variations in oral hygiene practices or differences in the sample collection sites among participants. Smokers also often have a compromised immune response, which could allow for the proliferation, and thereby creating an abundance of Gram-negative bacteria (38). This study also notes an increase in the presence of *Rothia* species among smokers, which has been positively linked to pro-inflammatory cytokines (39).

Tobacco use, in both smokeless and smoked form is an important modifiable environmental risk factor for subgingival dysbiosis. It is important to understand subgingival dysbiosis as it can be a predisposing factor to certain conditions such as increased prevalence and severity of periodontal disease, can act as inflammatory stimulus to subsequent insulin resistance and increased vulnerability to systemic infections (40,41). Tobacco use is recognized for disrupting the oral microenvironment and increasing susceptibility to periodontitis by promoting a pathogen-rich, diverse, and commensal-depleted subgingival microbiota (42). Although participants in this study were periodontally healthy, the elevated presence of both Gram-positive and Gram-negative facultative anaerobic bacteria suggests increased risk of periodontal deterioration in the future.

The impact of tobacco use on the subgingival microbiome also varies with populations and geographic locations. Western studies report increased *Tannerella forsythia* and *Porphyromonas gingivalis* in smokers, while research from Asian cohorts has highlighted

enrichment of *Treponema* species (15,43). Additionally, differences in tobacco formulations and cultural practices may influence microbial composition. For instance, smokeless tobacco products commonly used in South Asian populations have been associated with increased colonization by *Streptococcus mutans* and *Lactobacillus* species, which are less frequently reported in Western studies (44). These variations highlight need for region-specific prevention and treatment strategies for tobacco-induced oral diseases.

A limitation of our study is its cross-sectional design, which restricts the ability to establish a clear causal relationship between tobacco form and subgingival microbial characteristics. Additionally, the study has focused on Genus-level bacterial classification which may not capture species-specific differences that could play a critical role in oral diseases. A more detailed species-level (e.g., metagenomics or transcriptomics) could offer deeper insights into the microbial community dynamics. Another limitation of our study is the sample size and gender distribution. Future research incorporating a larger sample size and a more balanced gender distribution will enhance the generalizability of the findings. Lastly, the study has focused on periodontally healthy tobacco users, which limits the ability to fully understand the microbiome changes in those with periodontal disease. Hence, the results of this study may not be generalizable to individuals with existing periodontal conditions.

CONCLUSION

The characterization of subgingival bacteria in smokeless tobacco users and smokers in this study revealed a higher proportion of Gram-positive cocci, Gram-negative cocci, Gram-negative bacilli, and coccobacilli compared to non-tobacco users. A closer examination of the bacterial genera indicated a 3.5-fold increase in the prevalence of *Aggregatibacter* among smokeless tobacco users and a 2.9-fold increase among smokers, although the latter association was not statistically significant. Furthermore, although the number of SLT users with *Streptococcus* species was comparable, bacteria such as *Enterococcus*, *Klebsiella*, and *Rothia* species were present in greater numbers among SLT users compared to non-users. When comparing smokers to the control group, the study observed a reduction in the number of participants with presence of *Streptococcus* species and *Enterococcus*. Conversely, there was a higher occurrence of *Klebsiella* and *Rothia* species among smokers. Although the study participants were free of periodontal diseases, the elevated presence of *Aggregatibacter*, *Enterococcus*, *Klebsiella*, and *Rothia* species suggests a trend toward increased inflammatory conditions, which may lead to greater bacterial diversity and a heightened risk of developing periodontitis.

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Table 1. Distribution of study subjects with respect to their age and gender along with their mean pocket depth and plaque index and mean number of teeth with bleeding on probing present

	Group 1	Group 2	Group 3
Age* (Mean± SD)	23.3846 ± 0.97325	30.0556 ± 5.89888	31.6667 ± 3.85364
Gender** (n/%)			
Male	40 (76.9%)	24 (66.7%)	26 (86.7%)
Female	12 (23.1%)	12 (33.3%)	4 (13.3%)
Bleeding on Probing (Mean± SD)	0.3163 ± 0.00149	0.1293 ± 0.10884	0.1087 ± 0.08127
Pocket Depth (Mean± SD)	1.1962 ± 0.17393	1.1806 ± 0.17864	1.2427 ± 0.21644
Plaque Index* (Mean± SD)	1.3427 ± 0.17542	1.7236 ± 0.46239	1.6320 ± 0.41712

*Statistically significant at p<0.05 (Statistical Test Used: One Way ANOVA)
**Statistically significant at p<0.05 (Statistical Test Used: Chi square Test)

Table 2. Association between tobacco chewing and type of microorganisms present.

Microorganisms	GROUP		Total	Chi-square value	Df	Odds ratio (CI: 95%)	p-value
	Group 1 (n=52)	Group 2 (n=36)					
Gram positive cocci (n/%)	44 (84.6)	36 (100)	80 (90.9)	6.092[a]	1	-	.014*
Gram positive bacilli (n/%)	10 (19.2)	0 (0.0)	10 (11.4)	7.811[a]	1	-	.005*
Gram positive coccobacilli (n/%)	0 (0.0)	0 (0.0)	0 (0.0)	-	-	-	-
Gram negative cocci (n/%)	6 (11.5)	15 (41.6)	21 (23.8)	4.458[a]	1	0.183 (0.062 – 0.538)	.035*
Gram negative bacilli (n/%)	24 (46.1)	26 (72.2)	50 (56.8)	0.263 [a]	1	0.800 (0.341-1.877)	.608
Gram negative coccobacilli (n/%)	30 (57.7)	34 (94.4)	64 (72.7)	14.486	1	12.467 (8.703-14.488)	<.001*

Chi-square test. *p-value<.05 was considered statistically significant.
[a]No statistics (odds ratio) were computed because Gram-positive cocci, Gram-positive bacilli, Gram-positive coccobacilli, Gram-negative cocci and Gram-negative bacilli were constant.

Table 3. Association between tobacco chewing and presence of different microorganisms.

Microorganisms	GROUP		Total	Chi-square value	Df	Odds ratio (CI: 95%)	p-value
	Group 1 (n=52)	Group 2 (n=36)					
Streptococcus spp. (n/%)	41 (78.8)	28 (77.8)	69 (78.4)	.014	1	0.939 (0.335-2.629)	.905
Aggregatibacter (n/%)	36 (69.2)	32 (88.9)	68 (77.3)	4.681	1	3.556 (1.077-5.742)	.030*
Enterococcus (n/%)	16 (30.8)	12 (33.3)	28 (31.8)	.064	1	1.125 (0.453-2.793)	.800
Klebsiella (n/%)	6 (11.5)	16 (44.4)	22 (25.0)	2.256	1	0.163 (0.055-0.478)	.133
Rothia spp. (n/%)	0 (0.0)	2 (5.6)	2 (2.3)	2.956[a]	1	-	.086

Chi-square test. *p-value<.05 was considered statistically significant.
[a]No statistics (risk ratio) were computed because *Rothia* species were constant

Table 4. Association between tobacco smoking and type of microorganisms present.

Microorganisms	GROUP		Total	Chi-square value	Df	Odds ratio (CI: 95%)	p-value
	Group 1 (n=52)	Group 3 (n=30)					
Gram-positive cocci (n/%)	44 (84.6)	30 (100.0)	74 (90.2)	5.114[a]	1	-	.024*
Gram-positive bacilli (n/%)	10 (19.2)	2 (6.7)	12 (14.6)	2.404[a]	1	0.300 (0.061-1.474)	.121
Gram-positive coccobacilli (n/%)	0 (0.0)	0 (0.0)	0 (0.0)	-	-	-	-
Gram-negative cocci (n/%)	6 (11.5)	10 (33.3)	16 (19.5)	5.754	1	3.833 (1.226-11.988)	.016*
Gram-negative bacilli (n/%)	24 (46.1)	18 (60.0)	42 (51.2)	2.146	1	0.667 (0.268-1.657)	.143
Gram-negative coccobacilli (n/%)	30 (57.7)	26 (86.7)	56 (68.3)	7.376	1	4.767 (1.453-15.633)	.007*

Chi-square test. *p-value<.05 was considered statistically significant.
[a]No statistics (risk ratio) were computed because Gram-positive cocci and Gram-positive coccobacilli were constant.

Table 5. Association between smoking tobacco and presence of different microorganisms.

Microorganisms	GROUP		Total	Chi-square value	Df	Odds ratio (CI: 95%)	p-value
	Group 1 (n=52)	Group 3 (n=30)					
Streptococcus spp. (n/%)	41 (78.8)	19 (63.3)	60 (73.2)	2.332	1	0.463 (0.171-1.256)	.127
Aggregatibacter (n/%)	36 (69.2)	26 (86.7)	62 (75.6)	3.136	1	2.889 (0.865-9.651)	.077
Enterococcus (n/%)	16 (30.8)	7 (23.3)	23 (28.0)	.521	1	0.685 (0.244-1.920)	.470
Klebsiella (n/%)	6 (11.5)	8 (26.7)	14 (17.1)	.155	1	0.359 (0.111-1.163)	.694
Rothia spp. (n/%)	0 (0.0)	4 (13.3)	4 (4.9)	7.289[a]	1	-	.007*

Chi-square test. *p-value<.05 was considered statistically significant.
[a]No statistics (risk ratio) were computed because *Rothia* species was a constant.