Does Exposure to Burning and Heated Tobacco Affect the Abundance of Perio-Pathogenic Species in the Subgingival Biofilm?

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Article

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Abstract: This study investigated the impact of tobacco exposure, specifically through heating and burning, on periodontopathogens in the subgingival microbiome among clinically healthy individuals and those diagnosed with periodontitis. The sample comprised 66 subjects (26–56 years, median 38 yrs; 64% females) classified as non-smokers, classic cigarette smokers, and tobacco heating system (THS) smokers (each $N = 22$). Full-mouth periodontal examination was performed, and 330 paper-point samples from periodontal pockets were collected. Next-generation sequencing of 16S rRNA genes was conducted to identify the composition of subgingival microbiome. Periodontitis prevalence among the groups was ranked as THS (41%) < non-smokers (44%) < cigarette smokers (68%), without statistically significant differences between the groups. The number of perio-pathogenic species was higher in subjects with periodontitis compared to those without (median 7 vs. 6 species; $p = 0.005$) but without significant differences between exposure groups: non-smokers (6) = smokers (6) < THS (6.5). When combining exposure and periodontal status, each smoker group had more perio-pathogenic species than non-smokers: non-smokers without periodontitis (5) < smokers without periodontitis (5.5) < THS without periodontitis (6); non-smokers with periodontitis (6.5) < THS with periodontitis (7) = smokers with periodontitis (7). Multiple linear regression indicated periodontitis as the sole predictor of perio-pathogenic species quantity, irrespective of the type of tobacco consumption, sex, age, or oral hygiene ($R^2 = 0.163$; $p = 0.005$). In conclusion, the quantity of perio-pathogenic species in the subgingival microbiome was more influenced by periodontitis than by exposure to tobacco smoke, regardless of whether it was heated or burned.

Keywords: 16S rRNA; next-generation sequencing; cigarette smoking; dysbiosis; electronic nicotine delivery system; periodontal pathobionts; periodontitis; subgingival microbiome

1. Introduction

Periodontitis is a chronic inflammatory disease of the supporting tissues around the teeth which, if left untreated, leads to progressive and irreversible loss of certain components of the periodontium (e.g., collagen fibers and alveolar bone), eventually leading to tooth mobility and loss [1–3].

Dental biofilm (plaque) is the community of specific bacteria found on teeth surfaces, which is highly specific to the oral cavity and is responsible for two main oral diseases

in humans: dental caries and periodontal disease [4–6]. Periodontal pathogens are the pre-requisite for the illness to occur, but they also need to engage in interactive collaboration and unity with other dysbiotic microbes found in dental plaque [6–8]. In 1998, Socransky et al. presented the concept of bacterial complexes, coded by color according to their role in the formation of dental plaque. *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* are members of the red complex and are considered as the main periodontal pathogens. Pathogens of the orange complex, such as *Fusobacterium nucleatum*, *Prevotella intermedia*, *Campylobacter rectus* sp., and *Parvimonas micra*, are also frequently isolated and are predominantly associated with subgingival plaque samples in cases of periodontitis [9]. *Aggregatibacter actinomycetemcommitans* serotype b, *Selenomonas noxia*, and *Actinomyces viscosus* do not belong to any of the proposed complexes and are unrelated to each other or other complexes [10].

Smoking, as an important risk factor for periodontitis, influences the composition of the subgingival microbiome. Tobacco smoking is recognized as a modifying factor in the new classification of periodontal and peri-implant diseases and conditions, drafted in 2017, and represents a stage up-lifter, denoting rapid progression of the disease which may worsen the long-term prognosis and lead to poorer treatment results [11]. Cigarette smoke contains toxic and harmful substances including tar, nicotine, carbon monoxide, arsenic, hydrogen cyanide, benzene, and tobacco-derived nitrosamines, which are responsible for the abovementioned effects [12]. Tobacco and all kinds of different tobacco products available on the market have the same effect on human health. The World Health Organization and the European Respiratory Society have stated that all tobacco products are harmful, including heated ones, and that heated tobacco products are also addictive, carcinogenic, and damaging to the lungs and human health like classic cigarettes [13,14]. Polymicrobial oral communities are the first that come into contact with tobacco smoke or aerosols; therefore, it may be assumed that all potential negative (or positive) effects will be first manifested on them [15].

Tobacco heating system (THS) or Heat not Burn (HNB) products are new, modern, innovative, and tempting tobacco products advertised as having fewer negative consequences for human health. They produce only aerosol and no fire, ash, or smoke, as the tobacco is heated up to 350 °C and not burned like in classic cigarettes [12]. THS is a hybrid model combining electric and classic cigarettes. A chargeable battery provides power to the device, while a tobacco stick is inserted into a slot and then heated. Nevertheless, it still contains nicotine, tar, carbon monoxide, and ammonia, albeit in lower amounts [16–18]. IQOS (Phillip Morris International, Inc., Stamford, CT, US) is a representative type of this kind of novel hybrid product, which has been available on the Croatian market since 2017 [19].

The number of resident plaque microflora taken from cervical margins in THS smokers is reduced, while the number of opportunistic ones is increased [20]. *Bacteroides*, *Fusobacterium*, *Porphyromonas*, and *Campylobacter* species are more present in subgingival samples of smokers suffering from periodontal diseases than in non-smokers or former smokers [21,22]. Cigarette smoke affects the microbiological diversity of the surface of the buccal mucosa. Considering such an influence on the micro-organisms of the mucous membrane, it can be assumed that something similar may take place in the dental biofilm on the tooth surface [23].

It has been shown that cigarette smoke also affects the subgingival microbiological composition in healthy individuals, and it is responsible for the depletion of beneficial bacteria. Differences in bacterial communities between smokers and non-smokers with moderate periodontitis showed that bacteria of the *Bacteroides* genus are more common in non-smokers, while *Fusobacteria*, *Fretibacteria*, *Streptococci*, and *Veillonella* are more present in smokers [21]. A 2020 study which investigated the prevalence of periodontopathogens in healthy young adult smokers found that the levels of *Aggregatibacter actinomycetemcommitans*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum* were actually very similar between smokers and non-smokers [24].

In addition, periodontal disease and red complex bacteria are strongly related to various systemic diseases [25]. A link to rheumatoid arthritis has also been reported [26]. Recently published studies have stated that periodontal pathogens have a well-documented effect on synaptic impairment, neurodegenerative function, and neuroinflammation through the induction of chronic low-grade systemic inflammation, which can have an effect on the manifestation of Alzheimer's disease [27]. Patients infected with *P. gingivalis* have a higher risk of gastrointestinal cancer (odds ratio = 2.2), when compared to those who are uninfected [28]. Cardiovascular diseases can also be negatively affected by the disturbed oral microbiome. A significant number of very similar oral bacterial DNA were found in aortic valve specimens in patients suffering from periodontal disease [29]. When all these negative consequences from periodontal disease are added to those from smoking [30–32], the conclusion arises that cigarette smokers are a high-risk group that are susceptible to several serious and fatal diseases.

The aim of this study was to compare the prevalence and abundance of periodontopathogens in classic cigarette smokers, IQOS consumers, and non-smokers, in relation to clinically diagnosed periodontitis. The null hypothesis was that HNB tobacco modifies the subgingival microbiome profile in such a way that IQOS users without periodontal disease are expected to have more periodontopathogens than non-smokers but less than classic cigarette smokers.

2. Materials and Methods

The design of this study was an observational cross-sectional study with samples stratified according to tobacco exposure. The convenience sample consisted of 66 consecutive patients of the Dental Clinic, Clinical Hospital Center Rijeka, Croatia, who came for an examination at one of the six departments: Prosthodontics, Orthodontics, Oral medicine, Oral surgery, Periodontology, or Restorative dentistry with Endodontics. Three groups were formed (each N = 22): (I) subjects smoking classic cigarettes (S), (II) users of IQOS, and (III) subjects who have never smoked either classic cigarettes or used the IQOS system, that is, with non-smoking status (CTRL). According to periodontal examination, subjects were further classified into subgroups with periodontitis and without periodontitis. First, the IQOS group was formed, as they were least numerous, and those subjects were matched by age and gender with non-smokers and cigarette smokers. Samples were collected between 1 June 2022 and 1 June 2023.

The sample size was calculated based on previous research for differences in clinical periodontal status [33,34]. If the probing depth is expected to be 4.5 mm in smokers, 4.1 in IQOS, and 4.5 in non-smokers with a standard deviation of 0.4 in each group, considering the Bonferroni correction for multiple comparisons and drop-outs, 22 subjects are needed in each group to detect differences among groups. This calculation was made with a test power of 0.8 and a significance level of 0.05, using an online sample size calculator (v. 1.061; University of Vienna, Vienna, Austria) [35]. Power analysis also showed that, for the effect f = 0.4 with a test power of 0.8 and a significance level of 0.05, the minimum sample size for research was 66 respondents (22 per group). The calculation was conducted using the G*Power 3.1.9.4 software (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany).

The research question was formulated according to the PECO (Population, Exposure, Control or Comparison, Outcome) strategy [36]:

P (Population): Adult smokers;

E (Exposure): Aerosol from IQOS and tobacco cigarette smoke;

C (Comparison): Non-smokers;

O (Outcome): Microbial composition of subgingival plaque and periodontitis.

The clinical question in "PECO" format was the following: Is there a significant difference in microbial composition of subgingival dental plaque in IQOS users, when compared to classic cigarettes users and non-smoking subjects with or without periodontal disease?

2.1. Inclusion Criteria

Inclusion criteria were good general health, absence of any lesions below, at, or above the level of the oral mucosa, and a minimum of 20 healthy teeth. Smokers had to meet the criteria for smoking experience of at least 3 years (classic cigarette or THS) and daily consumption, which should not be less than 5 cigarettes or heat sticks per day. Selected subjects were only cigarette smokers for Group I, and the same rule of selection was applied for IQOS users as Group II.

2.2. Exclusion Criteria

Minors, pregnant women, subjects who use oral probiotics, subjects who have been under antibiotic therapy for the past six (6) months, subjects who use oral antiseptics based on chlorhexidine daily, subjects under immunosuppressive therapy, subjects under any medication therapy, and subjects who had previous periodontal therapy were excluded from the research.

The above listed exclusion criteria are modifiers of the supragingival and/or subgingival microbiological profile and, as such, can affect the results in the sampled microbiome. Gestational hormones during pregnancy act as growth factors for *Prevotella intermedia* [37]. Therapy with chlorhexidine (bisbiguanide antiseptic) preparations has a bacteriostatic effect at specific doses and may also be bactericidal, while therapy with oral probiotics can increase the population and number of probiotic bacteria and stop or hinder the reproduction of pathogenic species. Some systemic diseases, such as uncontrolled diabetes mellitus, lead to the dominance of certain perio-pathogenic species, including *Capnocytophaga*, *P. intermedia*, *C. rectus*, *P. gingivalis*, and *A. actinomycetemcommitans* [37].

2.3. Drop-Outs

Four subjects in the control group and three subjects in the smokers group were not fully processed (i.e., data were missing). Two subjects in the control group subsequently withdrew their study consent. Three microbiological samples were insufficient for DNA extraction and, for two more, the NGS sequencing results revealed insufficient data.

2.4. Anamnesis and Clinical Examination

The processing procedure of each subject consisted of taking anamnestic data, clinical examination of the oral cavity, analysis of panoramic dental radiographs, and taking paper-point sample swabs from gingival sulci or periodontal pockets. Clinical examination included the examination of all teeth except third molars. One experienced periodontist (I.M.) performed all periodontal diagnostic procedures.

The purpose of the clinical examination and analysis of panoramic dental radiographs was to establish the presence of periodontitis and to stage and grade the periodontal disease. For each group (CTRL, IQOS, and S), patients were additionally distributed into subgroups with periodontitis.

The clinical examination was performed using a millimeter graduated PCP-15 UNC manual periodontal probe (Hu-Friedy, Chicago, IL, US). The following periodontal indices were assessed: probing depth (PD), Full Mouth Plaque Score (FMPS), gingival recession (GR), and clinical attachment loss (CAL). PD and GR measurements were taken at six sites per tooth (mesio-buccal, middle of the buccal surface, disto-buccal, disto-oral, middle of the oral surface, and mesio-oral), as reported earlier [34].

The clinical procedures necessary for periodontal disease diagnosis were made according to the new 2017 classification. Presence of detectable bone loss in any area of the dentition through a visible cemento–enamelum junction or assessment of bone loss through panoramic dental radiographs, presence of an interdental CAL in more than one non-adjacent tooth, and buccal or oral recessions with PD higher than 3 mm are reliable signs of periodontal disease [38]. Panoramic dental radiographs (Planmeca ProMax, ProMax 3D Plus, manufactured by Planmeca OY, Helsinki, Finland) were taken and the

Planmeca Romexis software version 5.30.1015.28.03.19 was used for assessment by the same investigator (I.M.).

The participants were briefed thoroughly about the study and their voluntary participation, and they all signed written consents.

2.5. The Microbiological Diagnostic Procedure

2.5.1. Microbiological Sampling

The sampling procedure was carried out by taking paper-point sample swabs for microbiological analysis from five (5) selected gingival sulci or periodontal pockets of five (5) selected teeth. Selected periodontal pockets were the deepest ones found in subjects with periodontal disease. The teeth were isolated in a dry working field (through the placement of cotton rolls in the large salivary gland duct exits and the saliva ejector under the tongue), in order to prevent contamination of the paper points with saliva, and then additionally dried with compressed air. Supragingival biofilm was cleaned from the sampling area with a sterile cotton pellet, and one paper point size 45 (Roeko #45 Dental Paper Point, Coltene Whaladent, Cuyahoga Falls, OH, US) per tooth was inserted into the gingival sulcus or periodontal pocket for 30 s and then placed into an empty Eppendorf tube. The Eppendorf tube was then stored at a temperature of -20 °C. The samples were stored at this temperature until bacterial DNA isolation [33,39]. Isolation of bacterial DNA was performed within 48 h of collection.

2.5.2. Isolation of Bacterial DNA

Bacterial DNA samples were isolated from paper points containing subgingival biofilm using the commercially available Nucleospin Tissue kit (Macherey Nagel, Duren, Germany), according to a modified protocol for bacteria following the manufacturer's recommendations. Paper points with biofilm samples in 1.5 mL Eppendorf tubes were re-suspended in 200 µL of prepared G+ lysis buffer (according to the manufacturer's modified protocol for bacteria). The resulting suspension was vortexed, then 40 µL of freshly prepared lysozyme solution was added (also according to the manufacturer's instructions). This was followed by incubation at 37 °C for 1 h on a thermomixer with stirring at 850 rpm. Then, 40 µL of proteinase K was added and the suspension was incubated at 56 °C for 2 h (on a thermomixer with stirring at 850 rpm). Following this, 300 µL of B3 buffer was added and incubated at 70 °C for 10 min. After the Eppendorf tubes had cooled down, the precipitation of DNA followed with 300 µL of cooled 98% ethanol. Finally, DNA was eluted in 50 µL of elution buffer. The concentration and purity of genomic DNA was determined using a Qubit fluorometer [40–43].

2.5.3. Next-Generation Sequencing (NGS)

The amplification of hypervariable regions 2–9 of the 16s rRNA genes was performed with an Ion 16S Metagenomics kit (ThermoFisher, Waltham, MA, US), according to the manufacturer's instructions. A total of 3.5 ng of each isolated DNA sample was used. After amplification, purification, and joining of amplicons, 150 ng of each of the pooled amplicons were used to construct barcoded libraries using an Ion Plus Fragment Library kit (ThermoFisher, Waltham, MA, US), according to the manufacturer's instructions. The prepared libraries were then cleaned of unbound adapters and barcodes. Barcoded library concentrations were quantified using an Ion Universal Library Quantitation kit (ThermoFisher, Waltham, MA, US). Further steps involved PCR emulsion dilution with 10 pM of all samples pooled in 12 µL, followed by preparation of the basic template for sequencing with the Ion OneTouch™ 2 System device and an Ion PGM™ Hi-Q View Kit (ThermoFisher, Waltham, MA, US). Concentration of Ion Sphere beads-ISP (empty and polyclonal ISP washing) was performed with an Ion OneTouch ES kit and the Ion OneTouch 2 instrument (ThermoFisher, Waltham, MA, US), according to the manufacturer's instructions. Sequencing was carried out with an Ion PGM™ Hi-Q View Sequencing Kit on the Ion PGM™ System using the Ion 316™ chip. The analysis of the obtained results

was conducted using the IonReporter software 5.20 [44–47]. In the present study, our focus was on nine main periodontal pathobionts: *Aggregatibacter actinomycetemcommitans*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Treponema denticola*, *Tannerella forsythia*, *Prevotella intermedia*, *Parvimonas micra*, *Eubacterium nodatum*, and *Campylobacter rectus*.

2.5.4. Statistical Analysis

Frequencies between groups were compared through χ^2 and Fisher's exact tests. The Z-test for proportions with Bonferroni correction was used for multiple comparisons after the χ^2 test. Continuous variables were analyzed using the Spearman correlation, Kruskal–Wallis, and Mann–Whitney tests with Bonferroni correction of the *p*-value for multiple comparisons. The effect size for χ^2 and Fisher's tests was quantified using Cramer's V, for Kruskall–Wallis using the formula $\varepsilon^2 = H/[(n^2 - 1)/(n + 1)]$, and for the Mann–Whitney test using the formula $r = Z/\sqrt{N}$. The Cohen criteria was used for interpretation, as follows: $r = 0.25$–0.3, small effect size; 0.3–0.5, moderate; 0.5–0.7, large; and >0.7, very large. For interpretation of Cramer's V, the same criteria were used; while, for ε^2, squared values of r were used. Linear regression was conducted to analyze predictors of the number of perio-pathogenic species. For all statistical analyses, commercial software was used (SPSS IBM 22.0, IBM Corp., Armonk, NY, US).

3. Results

The subjects were 26–56 years old (median 38; interquartile range 34–54), and 42/66 (64%) were females. A total of 30 subjects (50.8%) had periodontal disease. All subjects had periodontitis stage III and grade B except smokers, who had stage III and grade C. There were seven dropouts and, so, 59 microbiological samples were analyzed. Periodontitis prevalence increased in the order of IQOS (41%) < non-smokers (44%) < cigarette smokers (68%), without statistically significant differences between groups (Table 1). FMPS was similar between the three groups (non-smokers 58.5, cigarette smokers 58.3, IQOS 57.1).

Table 1. Distribution of periodontal disease between control group (non-smokers, CTRL), IQOS users, and classic smokers (S).

	Presence of Periodontitis		
Exposure to Tobacco	**Non-Periodontitis**	**Periodontitis**	**Total**
CTRL (N (%))	10 (55.6%)	8 (44.4%)	18 (100%)
IQOS (N (%))	13 (59.1%)	9 (40.9%)	22 (100%)
S (N (%))	6 (31.6%)	13 (68.4%)	19 (100%)
Total (N (%))	29 (49.2%)	30 (50.8%)	59 (100%)

The number of perio-pathogenic species (scale 0–9 species) was similar in all three groups: non-smokers (median 6 species) = smokers (6) < IQOS (6.5); and it was similar between smokers and non-smokers (both median 6). It was significantly higher in those with periodontitis than without the disease, with moderate effect size (7 vs. 6; *p* = 0.005; r = −0.368; Figure 1). When combining exposure and periodontal status, each exposure group had more perio-pathogenic species than non-smokers: non-smokers without periodontitis (median 5 species) < smokers without periodontitis (5.5) < IQOS without periodontitis (6); non-smokers with periodontitis (6.5) < IQOS with periodontitis (7) = smokers with periodontitis (7); however, the differences did not reach the level of statistical significance. The number of perio-pathogenic species was not related to age, sex, or FMPS. In multiple linear regression, periodontitis was the only predictor of the number of perio-pathogenic species (dependent variable), when controlling for the effect of consumption of burning and heated tobacco, sex, age, and oral hygiene (adjusted $R^2 = 0.163$; *p* = 0.012). Periodontitis, burning and heated tobacco, age, sex, and FMPS were considered as independent variables.
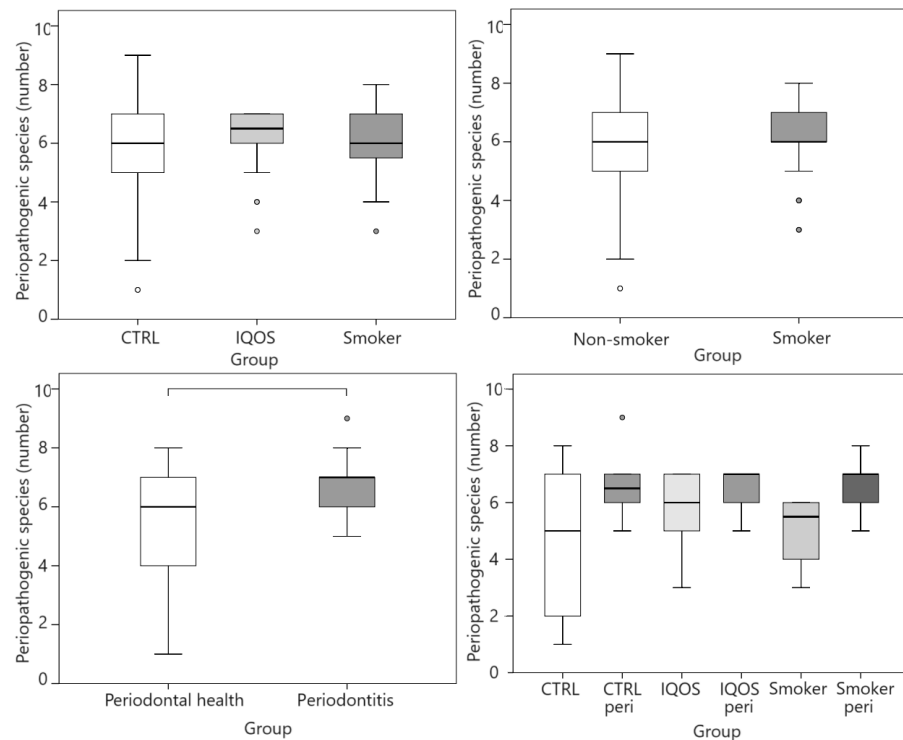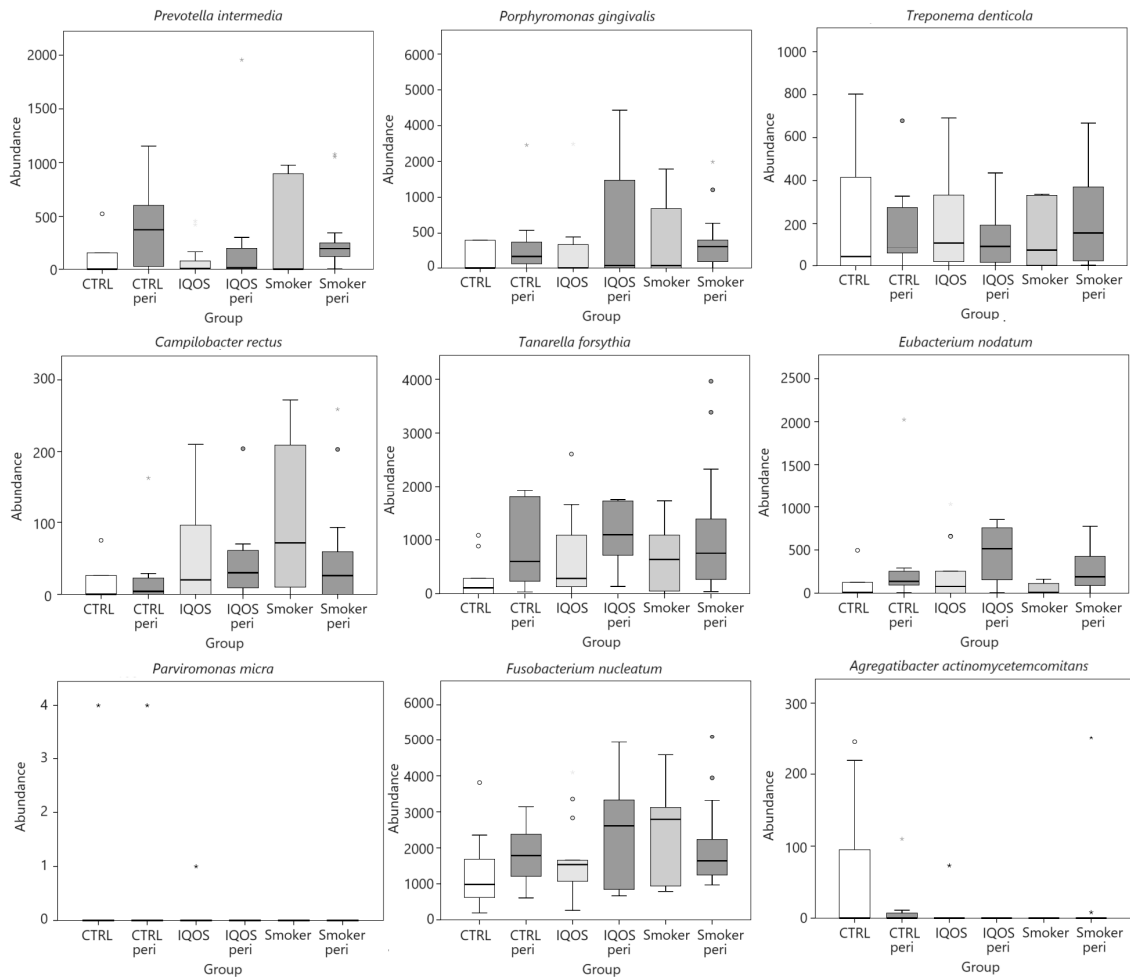
Figure 1. Number of perio-pathogenic species in groups formed according to tobacco exposure, presence of periodontal disease, and combination. Boxes represent interquartile ranges, horizontal lines represent medians, whiskers represent minimum and maximum values, and circles represent outliers. A horizontal line connects groups that differ significantly.

The median ratio of perio-pathogenic species in biofilm in the whole sample was <7%, with *F. nucleatum* being the highest (4%), followed by *T. forsythia* (3%), *P. gingivalis* (0.3%), *E. nodatum* (0.03%), and *T. denticola* (0.02%), while the ratio of other species was less than 0.01%. A significant difference between smokers, non-smokers, and IQOS in terms of the ratio of perio-pathogenic species was only present for *A. actinomycetemcomitans* ($p = 0.013$), where non-smokers had the highest and IQOS the lowest ratio. When comparing all types of smokers with non-smokers, they differed in terms of the ratios of *A. actinomycetemcomitans* and *C. rectus* ($p \leq 0.047$), with *A. actinomycetemcomitans* having a higher ratio in non-smokers and *C. rectus* in smokers. Subjects with periodontitis had higher ratios of *P. gingivalis*, *P. intermedia*, *T. forsythia*, and *E. nodatum* than those without periodontitis ($p \leq 0.039$), with the highest ratio in the case of *T. forsythia* (2 vs. 0.1%; $p = 0.009$).

According to tobacco exposure, the highest levels of *C. rectus* and *F. nucleatum* were observed in the smoker group, while *F. nucleatum* was the most dominant bacterium in the IQOS group. *A. actinomycetemcomitans* was the most abundant in the CTRL group.

When groups with tobacco exposure and the presence of periodontitis were compared, *P. intermedia* showed the highest abundance in the group of non-smokers with periodontitis (CTRL peri), while *P. gingivalis* and *T. denticola* were most dominant in cigarette smokers with periodontal disease (Smoker peri). *T. forsythia* and *E. nodatum* expressed the highest levels in the IQOS group with periodontitis (IQOS peri); see Figure 2.
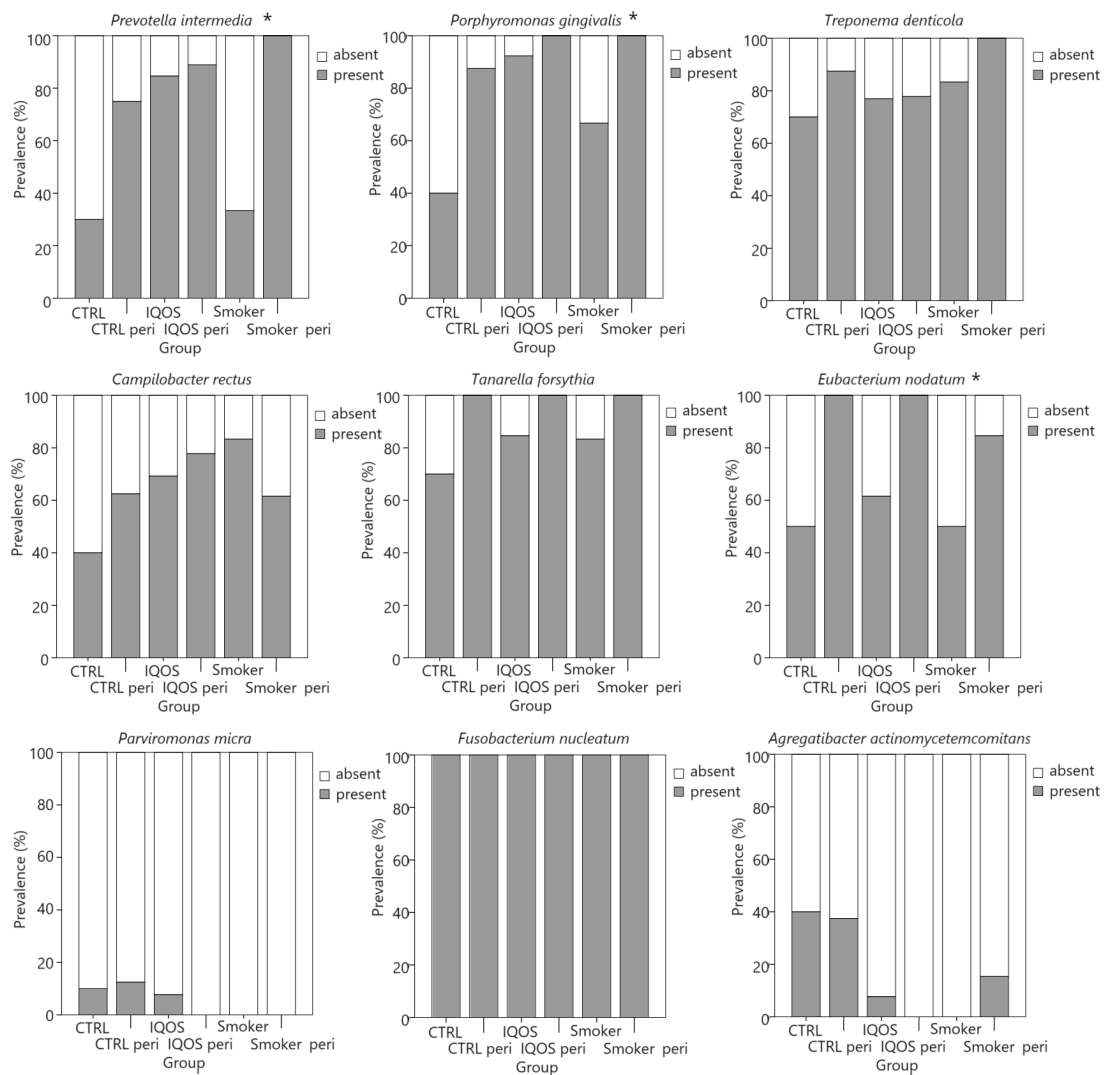
Figure 2. Abundance of perio-pathogenic species according to tobacco exposure and presence of periodontitis. Boxes represent interquartile ranges, horizontal lines represent medians, whiskers represent minimum and maximum values, and circles and asterisks represent outliers and extremes.

All classic cigarette smokers with periodontitis (Smokers peri) were positive for *P. intermedia*, *P. gingivalis*, *T. denticola*, *T. forsytha*, and *F. nucleatum* in subgingival dental plaque. In IQOS users with periodontitis (IQOS peri), *P. gingivalis*, *T. forsythia*, *E. nodatum*, and *F. nucleatum* were the most prevalent periodontal pathogens; while in non-smokers with periodontitis (CTRL peri), *T. forsythia*, *E. nodatum*, and *F. nucleatum* were the most frequent (Figure 3).

The composition of the bacterial flora was not influenced by age. *P. intermedia* was more abundant in males than females (median 305 vs. 6; $p = 0.003$; r = −0.385), but there were no differences in other bacteria by sex. Oral hygiene (assessed by FMPS) was related only to *P. gingivalis* (r = 0.280; $p = 0.032$), *T. forsythia* (r = 0.365; $p = 0.005$), and *E. nodatum* (r = 0.290; $p = 0.002$).

The prevalence of perio-pathogenic bacteria in groups formed by tobacco exposure is presented in Table 2, while the data for groups formed by the presence of periodontitis are shown in Table 3.

Figure 3. Prevalence of perio-pathogenic species according to tobacco exposure and presence of periodontitis. Asterisks denote bacteria whose prevalence significantly differed between groups.

Table 2. Prevalence of perio-pathogenic bacteria in control group (non-smokers, CTRL), IQOS users, and classic smokers (S).

Bacteria	S (N (%))	IQOS (N (%))	CTRL (N (%))	p *	V
Prevotella intermedia	15/19 (79%)	19/22 (86%)	9/18 (50%)	0.028	0.348
Porphyromonas gingvalis	17/19 (90%)	21/22 (96%)	11/18 (61%)	0.010	0.393
Treponema denticola	18/19 (95%)	7/22 (77%)	14/18 (78%)	0.257	0.215
Campylobacter rectus	13/19 (68%)	16/22 (73%)	9/18 (50%)	0.297	0.203
Tannerella forsythia	18/19 (95%)	20/22 (91%)	15/18 (83%)	0.507	0.152
Eubacterium nodatum	14/19 (74%)	17/22 (77%)	13/18 (72%)	0.930	0.050
Parvimonas micra	0	1/22 (5%)	2/18 (11%)	0.303	0.201
Fusobacterium nucleatum	19/19 (100%)	22/22 (100%)	18/18 (100%)	-	-
Aggregatibacter actinomycetemcommitans	2/19 (11%)	1/21 (5%)	7/18 (39%)	0.010	0.393

* Chi square test.

Table 3. Prevalence of perio-pathogenic bacteria in subjects with and without periodontitis.

Bacteria	Periodontitis	No Periodontitis	*p* *	V
Prevotella intermedia	27/30 (90%)	16/29 (55%)	0.003	0.392
Porphyromonas gingvalis	29/30 (97%)	20/29 (69%)	0.005	0.369
Treponema denticola	27/30 (90%)	22/29 (76%)	0.148	0.188
Campylobacter rectus	20/30 (67%)	18/29 (62%)	0.712	0.048
Tannerella forsythia	30/30 (100%)	23/29 (79%)	0.001	0.342
Eubacterium nodatum	28/30 (93%)	16/29 (55%)	0.011	0.438
Parvimonas micra	1/30 (3%)	2/29 (7%)	0.533	0.081
Fusobacterium nucleatum	30/30 (100%)	29/29 (100%)	-	-
Aggregatibacter actinomycetemcommitans	5/30 (17%)	5/29 (17%)	0.958	0.008

* Chi square test.

The results in Tables 2 and 3 demonstrate that the prevalence of *P. intermedia* was higher in smokers (S and IQOS) and in periodontitis-affected subjects. *P. gingivalis* showed a higher prevalence in the same groups as *P. intermedia*. *T. denticola* was more prevalent in cigarette smokers and in those with periodontitis. The prevalence of *C. rectus* was higher in the periodontitis group and somewhat higher in IQOS smokers. *T. forsythia* had the highest prevalence in the periodontitis group and in cigarette smokers. *E. nodatum* was most prevalent in the IQOS and periodontitis groups. *P. micra* was prevalent in groups without periodontitis and in the non-smoker group. *F. nucleatum* was equally prevalent in all tobacco-exposed groups and in those with or without periodontal disease. *A. actinomycetemcommitans* was equally prevalent in subjects with or without periodontitis, with a higher prevalence in non-smokers.

4. Discussion

The presented results do not support the hypothesis that heated and not burned tobacco increases the levels of subgingival periodontopathogens in IQOS users without periodontal disease, when compared to non-smokers. Furthermore, the second hypothesis was that classic cigarette smokers have a higher prevalence and higher abundance of perio-pathogens when compared to IQOS users. This hypothesis can be partially accepted. *P. intermedia* and *P. gingivalis* appear to be specific to smokers with periodontal disease, but the prevalence of these two pathogens was higher in smokers regardless of whether tobacco is burned or heated.

This research demonstrated that exposure to tobacco, regardless of whether it is burned (as in conventional cigarettes) or heated (as in THS products), has a modest effect on the composition of subgingival periodontopathogen biofilms. Periodontal disease has a more pronounced effect on the abundance and diversity of subgingival dental microbiome than tobacco exposure. Our research study focused on nine main periodontal pathogen bacteria, and the analyzed results are mostly in line with previously conducted studies, with some new and surprising findings [43].

The present research demonstrated that subgingival dental plaque in classic cigarette smokers had the highest prevalence of *T. forsythia* and *T. denticola*, while *P. micra* is the least frequent in this group. Plaque in IQOS users abounds with *P. intermedia* and *E. nodatum*, followed by *C. rectus* and *P. gingivalis,* while *A. actinomycetemcommitans* and *T. denticola* were the least-present periodontopathogens. The most frequent periodontopathogens in non-smokers were *P. micra* and *A. actinomycetemcommitans*, while *P. intermedia, T. forsythia*, and *E. nodatum* were rarely found in this group. *E. nodatum* is an orange complex bacterium that, according to the literature, is found in severe and advanced stages of periodontitis and is more frequent in smokers than in non-smokers [10]. Our investigation confirmed previous studies in this regard. We found that the numbers of *E. nodatum* were much higher in subjects with periodontitis and in IQOS consumers with periodontitis. According to the literature, *E. nodatum* is also more frequent in smokers [10]. Our study results indicated that *E. nodatum* was the most numerous in IQOS users, when compared to classic smokers.

Therefore, it appears that in the case of *E. nodatum,* IQOS aerosol affects its abundance more than cigarette smoke. This unexpected result suggests that IQOS users are at higher risk of developing severe forms of periodontal disease.

The keystone perio-pathogens *P. gingivalis* and *T. forsythia* were found in higher amounts in the subgingival plaque of patients suffering from periodontitis when compared to those without periodontitis. *T. denticola* was present in similar numbers in all groups (i.e., smokers and non-smokers, with or without periodontal disease) and, so, we cannot strictly connect it to any group. However, its prevalence was highest in smokers with periodontitis. The greatest prevalence of periodontal pathogens found in patients with periodontitis were *P. gingivalis*, *P. intermedia,* and *E. nodatum*. *P. gingivalis* and *P. intermedia* are more specific for smokers suffering from periodontal disease, regardless of whether the tobacco is heated or burned. Studies have confirmed the positive correlation between red complex bacteria and the severity of periodontitis. The presence of red complex bacteria in periodontal pockets clinically demonstrated more destruction of periodontal tissues in severe forms of periodontal disease, when compared to moderate or mild forms [25,48–52]. A surprising result observed in the present investigation was the highest abundance of *A. actinomycemcommitans* in the non-smoker group without periodontal disease, as this information is not supported by the vast majority of published studies focusing on its prevalence [37]. Additionally, *A. actinomycemcommitans* was the most deficient in IQOS users, regardless of whether they had or did not have periodontal disease. These data may be explained by the IQOS aerosol having some kind of inhibitory effect on the growth and reproduction of *A. actinomycemcommitans*; however, future larger-scale investigations with bigger samples can provide more exact insights regarding the accuracy of our findings.

Periodontitis, as a multifactorial and complex disease, cannot be manifested without its primary and basic component: micro-organisms. This is an irreplaceable ingredient without which the inflammatory disease cannot occur. The oral cavity microbiome is a dynamic and diverse orchestra which consists of small, interconnected fractions. These polymicrobial fractions need to interact and work together to resist the host immune system, determinate and create an environment favorable to their growth, and provoke the destruction of periodontal tissues via inflammation [3,49,53,54]. The fact that perio-pathogenic bacteria are present in gingival sulci or pockets does not mean that illness will occur [10,49]. Full-mouth extraction therapy can not eliminate all periodontal pathogenic bacteria which has already been proved [55]. Another interesting research study on this topic actually found significant quantities of red complex periodontal pathogenic bacteria on complete denture patients [56]. Microbial shifts, host inflammatory responses, and environmental factors such as smoking and genetics are all etiological factors that are involved in the development of periodontitis [57–59]. This information can explain our finding regarding *A. actinomycemcommitans,* which was abundant in non-smokers without periodontal disease. *A. actinomycemcommitans* may need some other bacteria, component, host or environmental factor, or some other piece of the puzzle to promote its pathogenicity, occurrence, and manifestation of full virulence potential. Furthermore, as *A. actinomycemcommitans* serotype B and JP2 clones have been recorded in past studies, it may be possible that only a small subset of clone types of *A. actinomycemcommitans*, and not all types, are responsible for the localized aggressive destructive appearance of *A. actinomycemcommitans*; however, this speculation is beyond the scope of our study [10].

We must note that, even though all regarding the quantities of subgingival microbiomes were not related to the study groups at a significant level, it appears that smoking favors the colonization of almost all perio-pathogenic bacteria in smokers, when compared to non-smokers. Tobacco products of all kinds are harmful—some more than others—and their detrimental effect on the subgingival microbiome was confirmed in this study. IQOS aerosol exhibited an increasing effect on the quantity and prevalence of *P. gingivalis*, *F. nucleatum*, and *P. intermedia*, three of the most important pathogens; thus, we cannot consider it to be safe and benign. It seems that, although IQOS is presented as a less-harmful tobacco product with less severe consequences for human health, it still has a detrimen-

tal effect on the periodontal tissues in terms of the prevalence and abundance of certain periodontal pathogens.

There were some limitations and concerns associated with our investigation. The study was observational and, so, all data and samples were collected before eventual periodontal therapy. It would be interesting if resampling was carried out after periodontal treatment in a longitudinal study. In this way, detailed insights, on a per-bacteria basis, into how initial periodontal therapy affects the numbers and prevalence of micro-organisms when different tobacco products are used would be enabled. Our study showed that increased bacterial load is related mainly to the presence of the disease, rather than the smoking habit. However, there was no evidence of a potential stratification according to the severity and the extent of the disease, which could clarify the results a bit more. The sample size was small, but metagenomic studies such as the present one are mostly small due to the high cost of analyses, complexity of the procedure itself, and interpretation of the large amount of received data. NGS technology has revolutionized genomics research with the speed and extent of the received results, but it still has some disadvantages beyond those already mentioned above. It is prone to errors and biases in sequencing data, which can affect the accuracy and completeness of the results [40–42]. However, its advantages in terms of enabling the sequencing of microbial communities provide invaluable contributions to science. NGS technology can enable and upgrade the possibility of personalized dental medicine through the identification of microbial communities found in different lesions caused by bacteria in the oral cavity [42,43]. Personalized treatment protocols targeting, for example, specific periodontopathogens, is one of possible applications.

This research is the first of its kind that used 16s rRNA NGS analysis to determine the exact composition of perio-pathogenic polymicrobial communities in subgingival dental plaque in IQOS users, classic smokers, and non-smokers with or without periodontal disease. The data obtained in this study have the potential to help in better understanding the effect of IQOS aerosols on the periodontal microbiome. No tobacco products can be completely safe. The consequences for human health can be minimized but, for now, not nullified. In the case of IQOS usage, there are concerns regarding its short- and long-term health effects due to its novelty on the world market. Studies have confirmed that IQOS users are exposed to lower levels of some toxicants, when compared to cigarette smoke; however, their exposure to higher levels of some (not known and not sufficiently researched) other toxicants and health consequences are yet to be revealed in the future [17]. Smoking does not have a stronger influence on the oral subgingival microbiome than periodontal disease, but it also cannot be claimed that smoking is harmless and without any consequences no matter which tobacco product we use, as our research demonstrated.

Guidelines for future research would include a longitudinal study concept, ideally before and after periodontal therapy was conducted. It would be interesting to carry out resampling every few months through one year, in order to monitor the reduction (stagnation or growth?) in the periodontal pathogens after periodontal therapy and recall visits. The additional inclusion of different tobacco products (e.g., e-cigarettes, cigars, hookah, and so on) and comparing their effects on the diversity and abundance of the periodontal microbiome using 16S rRNA sequencing would certainly clarify the effects of different kinds of tobacco products on the periodontal microbiome.

5. Conclusions

The quantity of subgingival perio-pathogenic species was more affected by periodontitis than by exposure to tobacco smoke, regardless of whether tobacco was heated or burned.

Institutional Review Board Statement: This study followed the Declaration of Helsinki guidelines regarding medical protocol and ethics, and the protocol was approved by the Ethics Committee of Faculty of Dental Medicine University of Rijeka (2170-137-006-01-23-60, 4 April 2022) and Clinical Hospital Center Rijeka, Croatia (2170-29-02/1-23-2, 6 March 2022).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper.

Data Availability Statement: The data sets used and/or analyzed during the current study are available from the Digital Academic Repository of the University of Rijeka, Faculty of Dental Medicine (Špalj S. Okolišni čimbenici i mikrobiološke interakcije u strukturi dentalnog biofilma: istraživački podaci. [Internet]. Fakultet dentalne medicine; 2023, Available from: https://urn.nsk.hr/urn:nbn:hr:271:843444, accessed on 4 January 2024).

Conflicts of Interest: The authors clearly declare no commercial interests with the manufacturer of the tobacco heating system IQOS®, PMI International Inc.

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