Comparison of the phenotypic profile of antimicrobial resistance in the oral microbiota of non-smokers, tobacco smokers, and electronic cigarette vapers - a pilot study

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ABSTRACT

Introduction: The microbiota is a community of microorganisms that live in a specific environment. Their type and number depend on multiple internal and external factors. Oral is the second most diverse and populated microbiota of the body. Smoking and vaping induce changes in its composition, and it has been demonstrated that they can lead to an increase in antimicrobial resistance. Objective: To compare the phenotypic profile of antimicrobial resistance in the oral microbiota of non-smokers, tobacco users, and electronic cigarette vapers. Methods: An observational, descriptive, cross-sectional, and comparative study was carried out. Three groups of non-smokers, smokers of conventional tobacco, and electronic cigarette (EC) vapers of tobacco flavored e-juice were formed. Oral cavity samples were obtained, incubated, and seeded in agar plates. Bacteria were isolated and identified performing Gram staining, oxidase, indole, and biochemical test panels. Susceptibility tests were performed using a MicroScan autoSCAN-4 system and the Kirby–Bauer test. Results: Variation was observed in the populations of bacteria that were isolated in each of the groups, but the non-smokers showed the most pathogens. In the non-smoking group, Staphylococcus sciuri was the most common bacteria, Staphylococcus sciuri and Enterobacter cloacae were the most abundant in the smoking group, and in the EC vapers group, the most common bacteria were Staphylococcus epidermidis and Staphylococcus sciuri. Conclusion: Multidrug resistance was observed in all the groups. However, EC vapers showed the highest proportions of antimicrobial resistance, raising a major concern.

Key words: cigarette; e-cigarettes; vaping; microbiota; drug resistance; antimicrobial drug resistance.

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Firmicutes, healthy individuals include Actinobacteria, Bacteroidetes, Microbiome Project, the most commonly found bacteria in not been identified. Still, since the beginning of the Human composition remains controversial as several species have colonization, reducing nitrate species, and facilitating homeostasis by protecting the mouth from pathogenic populated microbiota of the body. It plays a key role in The oral microbiota is the second most diverse and microorganisms, such as fungi, protozoa, and viruses that live in a specific environment. It comprises adherent microorganisms can also be found until that can stick to the surfaces of gums and teeth; however, non-adherent microorganisms have demonstrated that active or passive exposure to cigarette smoke eliminates indigenous bacteria, such as Peptostreptococcus and Prevotella, and allows the presence of pathogenic bacteria as Haemophilus influenzae, Moraxella catarrhalis, and Streptococcus pneumonia in the upper airways. Furthermore, Lacoma A. et al. have reported that it also increases the profile of antimicrobial resistance in Staphylococcus aureus. Smoking tobacco cigarettes is considered the leading cause of preventable diseases worldwide as it increases the risk of developing cardiovascular disease and certain types of cancer, among others. Brook I. and Gober A.E. have demonstrated that active or passive exposure to cigarette smoke eliminates indigenous bacteria, such as Peptostreptococcus and Prevotella, and allows the presence of pathogenic bacteria as Haemophilus influenzae, Moraxella catarrhalis, and Streptococcus pneumonia in the upper airways. Furthermore, Lacoma A. et al. have reported that it also increases the profile of antimicrobial resistance in Staphylococcus aureus. Electronic cigarettes (ECs), also known as vape pens, emerged as a possible solution to fight the tobacco epidemic and their use has rapidly increased, especially among adolescents. They are battery-powered devices that generally contain fewer toxic substances than conventional tobacco cigarettes; however, several health issues, including death, have been addressed. Recently, some researchers have analyzed the effects of ECs in the oral microbiota. For example, Campos M.A. et al. compared if there were any differences in the oral microbiota when tobacco smokers replaced his habit with EC vaping. Stewart C.J. et al. compared the oral and gut microbiota of tobacco smokers and EC users, but, to our knowledge, none have studied their antimicrobial resistance profile. The objective of our study was to compare the phenotypic profile of antimicrobial resistance in the oral microbiota of non-smokers, tobacco users, and EC vapers. Due to the size of the sample studied, this pilot study is part of a larger one,
which will be carried out when conditions allow it. Depending on the results obtained then, we will determine the statistical treatment to be carried out.

2. MATERIALS AND METHODS

An observational, descriptive, cross-sectional, and comparative study was carried out.

2.1 Study population

The study population consisted of three groups: non-smokers \((n = 11)\), conventional tobacco smokers \((n = 8)\), and EC vapers of tobacco flavored E-juice \((n = 11)\). Each group was made up of female individuals whose ages ranged from 18 to 21 years. It was sought that the volunteers had a similar consumption rate of tobacco or tobacco-flavored E-juice among them. In order to participate in this study, the volunteers had to answer a few questions to make sure they suffered no diabetes, hypertension or obesity; they were not under any pharmacological treatment and showed no clinical manifestations of respiratory infection, such as fever, headache, conjunctivitis, rhinorrhea, sore throat or cough at the time of the study. Then, they signed an informed consent.

2.2 Sample collection

Sterile plastic bottles (Delta-Lab, Barcelona, Spain) and a bottle with 250 ml of drinking water were given to each person. The samples were taken in the morning, before subjects ate and brushed their teeth. They rinsed their oral cavity and gargled with drinking water that was spat in the sterile plastic bottle. Samples were sent to campus immediately in less than 60 minutes.

2.3 Bacterial isolation

Blood and heart infusion (BHI) broth mediums (Becton Dickinson; Franklin Lakes, NJ, USA) were inoculated with 10 ml samples. Subsequently, they were incubated at 37 °C for 24 h. After incubation, 10 μL BHI broth medium was taken and seeded in blood agar (Becton Dickinson; Franklin Lakes, NJ, USA) using the cross-streak method and incubated again at 37 °C for 24 h. Pure colonies were obtained from the blood agars where the samples were seeded. The identification consisted of Gram staining, oxidase, indole, and biochemical test panels: The oxidase test was done using a BBL DrySlide Oxidase kit (Becton Dickinson; Franklin Lakes, NJ, USA), according to the manufacturer’s specifications.

2.4 Biochemical tests

The biochemical tests were performed using a MicroScan autoSCAN-4 (System Beckman Coulter®; Kraemer Blvd. Brea, CA, USA). The MicroScan Pos Combo Panel Type 33 (PC33) and MicroScan Neg Combo Panel Type 68 (System Beckman Coulter®; Kraemer Blvd. Brea, CA, USA) systems were used. In both cases, the manufacturer’s specifications were followed using Prompt® Inoculation System-D (inoculation system wands) and Pluronic® suspension (30 ml of stabilized aqueous surfactants), Inoculator-d grids, and the MicroScan RENOK system. The plates of the MicroScan Pos Combo Panel systems were incubated at 37 °C for 18 h, without CO₂, and with 40–60% humidity. The plates were revealed with peptidase reagents, potassium hydroxide, Kovac’s reagent, 0.5 N, N-Dimethyl-1-Naphtylamine, 10% ferric chloride, sulfanilic acid, and 1-Naphthol (System Beckman Coulter®; Kraemer Blvd. Brea, CA, USA). Finally, they were read using a MicroScan autoSCAN-4 reader (System Beckman Coulter®; Kraemer Blvd. Brea, CA, USA) and the data were analyzed with LabPro Command Center System Ink version 4.42 (System Beckman Coulter®; Kraemer Blvd. Brea, CA, USA).

2.5 Susceptibility tests

Susceptibility tests were performed using two methods: MicroScan autoSCAN-4 system (System Beckman Coulter®; Kraemer Blvd. Brea, CA, USA) and the traditional disk diffusion technique (Kirby–Bauer method) as shown below.

Positive Combo Panel Type 33 (µg/mL): amoxicillin/clavulanic acid 4 µg/2 µL; ampicillin 2−8 µg; ampicillin/sulbactam 8 µg/4 µg−16 µg/8 µg; ceftriaxone 8 µg, 32 µg; ciprofloxacin 1−2 µg; clindamycin 0.5−4 µg; erythromycin 0.5−4 µg; gentamicin 4−8 µg; levofloxacin 1−4 µg; maxifloxacin 0.5−4 µg; oxacillin 0.25−2 µg; penicillin 0.3 µg, 0.12 µg−0.25 µg, 2 µg, 8 µg; rifampicin 1−4 µg; Synercid 0.5−2 µg; tetracycline 4−8 µg; trimethoprim/sulfamethoxazole 0.5/9.5−2/38 µg; and vancomycin 0.25−16 µg.

Negative combo panel type 68 (µg/mL): amikacin 16−32 µg; amoxicillin/clavulanic acid 8 µg/4 µg−16/8 µg; ampicillin 8−16 µg; ampicillin/sulbactam 8 µg/4 µg−16/8 µg; cefazolin 2−4 µg; cefepime 4−16 µg; ceftriaxone 1−2 µg, 8 µg, 32 µg; cefturoxime 4−16 µg; ciprofloxacin 1−2 µg; ertapenem 0.5−2 µg; gentamicin 2−8 µg; imipenem 1−8 µg; levofloxacin 2−4 µg; meropenem 1−8 µg; piperacillin/tazobactam 16−64 µg; tetracycline 4−8 µg; tigecycline 2−4 µg; tobramycin 2−4 µg; and trimethoprim/sulfamethoxazole 2 µg/38 µg.

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The Kirby–Bauer method was based on the Clinical and Laboratory Standards Institute protocol. The pure colonies obtained from the blood agar were re-suspended in bacteria suspension, depositing two or three medium-sized colonies (2–3 mm) in a broth inoculum of BBL Crystal (Becton Dickinson; Franklin Lakes, NJ, USA). The inoculum obtained was adjusted using a Mac Farland 0.5 reader (expected CFU/mL $1.5 \times 10^9$) and seeded on Mueller Hinton 150 × 15 mm2 BD medium agar (Becton Dickinson; Franklin Lakes, NJ, USA). The antibiotic discs were placed using a Sensi-Disk dispensing system. The antibiotic panel was composed of amoxicillin/clavulanic acid (20 μg/10 μg), cefazolin (30 μg), cefepime (30 μg), cefoperazone (75 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), meropenem (10 μg), penicillin (10 μg), tetracycline (30 μg), and vancomycin (5 μg) (Franklin Lakes, NJ, USA).

3. RESULTS

3.1 Bacterial population isolated

A variation was observed in the populations of bacteria that were isolated in each of the groups. The largest number of bacterial species (15), each with a different frequency, was isolated from non-smokers. Within this group, the following results were found: *Staphylococcus sciuri* was the most frequent microorganism with a total of 5 (20.8%), followed by *Micrococcus* and related species with a frequency of 3 (12.5%) (Figure 1). In the smoking group, eight bacteria species were isolated, each with a different frequency. The *Staphylococcus sciuri* and *Enterobacter cloacae* bacteria had higher prevalence with 4 (20%) isolates (Figure 2). In the EC vapor group, a total of ten species of bacteria were isolated, each with a different frequency. It was found that the species with the highest prevalence were *Staphylococcus epidermidis* and *Staphylococcus sciuri*, with a frequency of 3 (21.4%) (Figure 3).

3.2 Phenotypic profile of antimicrobial resistance

To obtain a complete picture of the phenotypic profile of antimicrobial resistance of each isolate, the results obtained by the Kirby–Bauer method and those obtained from the MicroScan system should be complementary. In the non-smoking group, the antibiotics which presented 50% or more percentage of resistance were Penicillin (83.33%), Erythromycin (70.83%), Ceftriaxone (64.28%), Vancomycin (62.50%), Ampicillin (60%), Amoxicillin/Clavulanic Acid (54.54%), and Amoxicillin/Clavulanic Acid (50%). In the smoking group, the antibiotics which presented 50% resistance or higher were Penicillin (90%), Erythromycin (80%), Ampicillin (71%), Vancomycin (70%), and Amoxicillin/Clavulanic Acid (79%), Cefazolin (77%), Vancomycin (69%), Ceftriaxone (60%), and Cefepime (54%) (Figure 4).

![Bacteria isolated from the oral microbiota of non-smokers](https://doi.org/10.36105/psrua.2021v1n1.01)
Figure 2. Bacteria isolated from the oral microbiota of smokers: *Staphylococcus sciuri* and *Enterobacter cloacae* bacteria had a higher prevalence of 4 (20%). The remaining isolated microorganisms had a frequency of 1 (10%): *Micrococcus* and related species, *Staphylococcus aureus*, *Staphylococcus mitis*, *Staphylococcus auricularis*, *Staphylococcus hyicus*, and *Rhodococcus equi*.

Figure 3. Bacteria isolated from the oral microbiota of vape smokers: *Staphylococcus epidermidis* and *Staphylococcus sciuri* with a frequency of 3 (21.4%). The remaining eight species found presented the same frequency of 1 (7.1%): *Enterobacter cloacae*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Micrococcus* and related species, *Enterococcus faecalis*, *Streptococcus salivarius*, and *Kluyvera cryocrescens*.

4. DISCUSSION

The microbiota comprises structurally, and functionally organized communities of microorganisms attached to the skin and mucosal surfaces that can communicate and collaborate to maintain ecological stability. Its composition is entirely different between body sites and it depends on several internal and external factors, such as genetic background, gestational date (preterm or term birth), type of birth (vaginal delivery or cesarean), milk feeding methods (breast milk or artificial milk), age (neonate or elder), dietary habits (predominance of carbohydrates or fatty acid intake), personal hygiene (periodicity, methods, and products),

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smoking (active or passive), antibiotic administration (glycopeptides, lincosamides, macrolides, and quinolones), and environmental exposure. A person’s microbiota is shaped throughout their entire life. The most diverse and studied microbiota are those in the gastrointestinal tract and the oral cavity. A deviation from symbiosis leads to dysbiosis, and it has been demonstrated that the smoke of the cigarettes produces changes in the bacterial population of the oral cavity.

The most diverse and studied microbiota are those in the gastrointestinal tract and the oral cavity. The latter is a complex anatomic region containing several structures that can become microbial habitats, such as gingiva, gingival sulcus, teeth, tongue, lips, cheeks, and hard and soft palates. This complexity promotes the existence of very varied ecological niches that include aerobic and anaerobic environments. The taxa which are expected to be present in the oral cavity of healthy people include Gram-positive bacteria, such as Abiotrophia spp., Actinomyces spp., Bifidobacterium spp., Corynebacterium spp., Eubacterium spp., Lactobacillus spp., Peptostreptococcus spp., Propionibacterium spp., Pseudoramibacter spp., Rothia spp., Stomatococcus spp., and Streptococcus spp.; Gram-negative bacteria, such as Campylobacter spp., Capnocytophaga spp., Desulfovibrio spp., Desulfovibacter spp., Eikenella spp., Fusobacterium spp., Haemophilus spp., Leptotrichia spp., Moraxella spp., Neisseria spp., Prevotella spp., Selenomonas spp., Simonsiella spp., Treponema spp., Veillonella spp., and Wolinella spp.; fungi like Aspergillus spp., Aureobasidium spp., Candida spp., Cladosporium spp., Cryptococcus spp., Fusarium spp., and Saccharomyces spp.; and protozoa such as Entamoeba gingivalis and Trichomonas tenax. The presence of archaea and viruses has also been described.

Figure 4. Antimicrobial resistance profile of bacteria from the oral microbiota of non-smokers. The resistance obtained in non-smokers was Penicillin 83.33%, Erythromycin 70.83%, Ceftriaxone 64.28%, Vancomycin 62.50%, Ampicillin 60%, Amoxicillin/Clavulanic Acid 54.54%, Ampicillin/Sublactam 50%, Cefepime 42.85%, Cefazolin 38.09%, Tetracycline 29.16%, Levofloxacin 18.75%, Cefoperazone 11.76%, Ciprofloxacin 8.33%, and Meropenem 5.0%. The resistance in the smokers group was Penicillin 90%, Erythromycin 80%, Ampicillin 71%, Vancomycin 70%, Amoxicillin/Clavulanic Acid 50%, Cefepime 40%, Ampicillin/Sublactam 33%, Cefepoxacin 30%, Cefazolin 30%, Ceftriaxone 30%, Meropenem 22%, Tetracycline 20%, Cefoperazone 17%, and Levofloxacin 14%. The resistance obtained in the Vape Smokers group was Penicillin 92%, Ampicillin 80%, Erythromycin and Amoxicillin/Clavulanic Acid 79%, Cefazolin 77%, Vancomycin 69%, Ceftriaxone 60%, Cefepime 54%, Ampicillin/Sublactam 44%, Meropenem 33%, Tetracycline 29%, Ciprofloxacin 23%, Cefoperazone 22%, and Levofloxacin 8%.
smokers. Yu G. et al. showed that the alpha biodiversity (understood as the heterogeneity of a bacterial community based on the number of species present and their relative abundance) was higher in the oral cavity of non-smokers than in that of smokers, as the latter lose richness of the oral mucosa. Consequently, Shen P. et al. discussed the notion that cigarette smoke predisposes the nasal colonization by *Streptococcus pneumoniae*. In our study, variation was observed in the populations of bacteria that were isolated in each of the groups, but the non-smokers exhibited the most. This correlates with the evidence available in the literature, being the alpha biodiversity of the non-smokers higher than that of smokers and EC vapers.

In a comparative pilot study of the oral microbiota among non-smokers, tobacco smokers and EC vapers, significant alterations were seen in the tobacco smokers but no alterations were detected in EC vapers compared to the non-smokers. Furthermore, it has been reported that the EC vapor of flavorless E-juice, unlike cigarette smoke, does not cause inhibition of the oral microbiota bacterial growth, especially in commensal bacteria as *Streptococcus* spp. On the other hand, the sweetening or flavoring components of cigarettes and ECs can contribute to oral dysbiosis. This has been demonstrated in mentholated cigarettes and it has been suggested that this may also happen with flavored E-juices. In our study, we observed that all the groups presented pathogenic bacteria: The non-smoking group had *Enterobacter gergoviae*, *Klebsiella oxytoca*, and *Pseudomonas aeruginosa*; the smoking group had *Enterobacter cloacae* and *Rhodococcus equi*; and the EC vapor group had *Enterobacter cloacae*, *Enterococcus faecalis*, *Kluyvera cryocrescens*, and *Stenotrophomonas maltophilia*. However, EC vapers had the highest percentage in samples, followed by the smoking group and the non-smokers. Although the proportion of pathogens is low in the last group, the presence of bacteria should be studied, especially that of *Pseudomonas aeruginosa*, a commensal pathogen that causes necrotizing pneumonia.

Furthermore, Lacoma A. et al. reported that cigarette smoke increases the profile of antimicrobial resistance in *Staphylococcus aureus*, and as the use and acceptance of ECs is continuously rising, it is important to know whether it also increases the profile of antimicrobial resistance. In our study, multidrug resistance was seen in each of the groups, but the highest levels were present in smokers and EC vapers, especially in the latter. Penicillin was the only antibiotic with pharmacological resistance (over 80%) in all the groups. The antibiotics with the lowest pharmacological resistance level were Meropenem (5%) among non-smokers, Levofloxacin (14%) in smokers, and Levofloxacin (8%) in the vape smokers’ group. In contrast, Amoxicillin/Clavulanic Acid and Cefazolin showed an important increase in resistance due EC smoking habit. It is important to highlight that drugs with a high percentage of resistance are not an option in case of infection.

The presence of multidrug-resistant bacteria in all the groups evidences the need for urgent measures to fight a global health problem, as multidrug-resistant infections are expected to be the first cause of death by 2050. Specifically, smoking has not been considered a factor driving antimicrobial resistance; however, current evidence supports it, mainly due to the changes to the oral microbiota. To our knowledge, this report is the first phenotypic profile of antimicrobial resistance by EC vapers in our country. This pilot study has multiple limitations, such as the size of the sample and the homogeneity of the sex; still, it is a basis to conduct more comprehensive studies that clarify the proposed variables.

As originally stated, it should be noted that the microbiota within the different populations studied presented differences. In the case of EC vapers, there was a difference in part due to the microorganisms found in the microbiota of this population. The greatest resistance to antibiotics was found in the three populations studied within this group. Furthermore, EC vapers had the highest amount of pathogenic bacteria within the oral cavity. This is undoubtedly a relevant finding since ECs originally emerged as an alternative to replace conventional cigarettes. In their beginnings, ECs were considered an alternative with less harmful effects than those caused by the conventional cigarette. However, this and other studies have shown that ECs also have harmful effects in those who use them. This is relevant to our study given that we found pathogenic bacteria along with these effects, which affect the body. For example, in the study by Kassinen and Krogius-Kurikka cited in the National Center for Biotechnology Information Search database, the irritable bowel syndrome, one of the most common gastrointestinal disorders, was found to be largely bacteria-mediated. We found a large number of Gram-negative and Gram-positive bacteria from the phyla Proteobacteria and Firmicutes, respectively. Within the first phylum are *Enterobacter cloacae*, *Enterococcus faecalis*, *Kluyvera cryocrescens*, and *Stenotrophomonas maltophilia*. These pathogenic bacteria found in higher concentrations among EC vapers promote gastrointestinal disorders, as irritable bowel syndrome, eventually involved in amino acid synthesis, cell junctions, and inflammatory response. They are also related to a weakening of the epithelial barrier, which would finally explain diseases such as irritable bowel syndrome. This is where the importance of our work lies since these changes were found within the group of EC vapers.
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