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Effect of Electronic Cigarettes on Oral Microbial Flora

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Abstract:

Background: Despite well-documented adverse effects of tobacco consumption, cigarettes use is still rising and part of this increase is related to the popularization of alternative electronic nicotine delivery devices, such as electronic cigarettes (ECs). The aim of the performed research was to assess the effect of electronic cigarettes aerosol on the oral microbiota, using culture methods.

Methodology: 30 ten-week-old WAG rats (female 76-81 g and male 86-94 g) were randomly distributed in two groups, as follows: Group 1 – control animals (n = 10); Group 2 – EC aerosol exposed (n = 20). EC aerosol exposures were carried out by using the Boyarchuck chamber. During the study, the rat oral microbiota were collected four times: at the beginning of the experiment, on the 30th, 60th and 90th days. Microorganisms were identified using standard microbiological techniques.

Results: EC exposure to Group 2 rats resulted in a depletion of colonies commensal microbes and a greater incidence of atypical species such as *Klebsiella pneumoniae*, *Acinetobacter lwoffii*, *Candida albicans* compared to Group 1 on day 90. The test of independence between frequency distribution of opportunistic microbes and duration of EC exposure showed a significance for *Klebsiella pneumoniae* – $\chi^2 = 8.017$, $p = 0.0456$, *Acinetobacter lwoffii* – $\chi^2 = 36.772$, $p = 0.0001$, and *Candida albicans* – $\chi^2 = 8.689$, $p = 0.0337$.

Conclusions: The impact of electronic cigarettes facilitated colonization of the oral cavity by opportunistic bacteria and yeast.

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INTRODUCTION

Studies have reported about the influence of oral health on an increased risk of gastrointestinal pathology due to changes in oral microflora.

There is a long list of health risk factors associated with cigarette smoking [1]. Tobacco smoke contains a highly complex mixture of compounds that cause serious cardiovascular, respiratory, gastrointestinal tract diseases, including oral, pharyngeal, laryngeal and lung carcinomas [2, 3]. Studies have reported about the influence of oral health on an increased risk of gastrointestinal pathology due to changes of oral microflora [4, 5]. The oral microbiome refers to an ecological community of diverse bacterial species associated with human oral mucosa. Oral commensal microflora forms a bacterial biofilm preventing the attachment of harmful microorganisms to the mucosal surfaces. This microbiota participates in a wide variety of metabolic and immunological functions including differentiation of mucosa, maturation of local immune system, degradation of toxins, maintenance of the pro-inflammatory and anti-inflammatory balance, provision of "colonization resistance" and prevention of systemic diseases [6, 7]. Studies have shown that cigarette smoking contributes to the link between oral microflora dysbiosis and gastrointestinal diseases of tobacco smokers [8-10]. Despite well-documented adverse effects of tobacco consumption, cigarettes use is still rising in Ukraine and part of the increase is related to the popularization of alternative electronic nicotine delivery devices, such as electronic cigarettes (ECs). Electronic cigarette (EC) was suggested as a device free from tobacco carcinogens that may assist people in quitting smoking [11].

To our knowledge, there are studies that address the tobacco cigarettes, dokha and shisha smoking effects on oral microbiota [9, 12, 13]. However, information about the effect of EC aerosol exposure to oral bacteria remains scarce and limited to a small number of species isolated in culture media [14]. More analyses using molecular methods are still required.

The aim of this study was to assess the effect of electronic cigarettes aerosol on the oral microbiota.

2. METHODOLOGY

Experimental Design

Subjects

Thirty ten-week-old Wistar Albino Glaxo (WAG) rats (female 76-81g and male 86-94g) were obtained from

the Vivarium of National Medical University of Kharkiv. They were kept at a controlled temperature ($21 \pm 2^{\circ}\text{C}$) and relative humidity (50-70%), at 12-12 hr light-dark cycle and free access to rodent chow and water ad libitum. All experimental procedures were carried out in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The animals were randomly subdivided into two groups. Group 1 (control group) consisted of 10 intact animals (females=5 and males=5). Group 2 consisted of 20 animals (females=10 and males=10) exposed to EC aerosol.

EC aerosol was generated by using the Boyarchuck chamber operating in a one-pass mode with the aerosol feed controlled externally by a metering pump. This avoided the EC aerosol recirculation that may lead to the physical damage of respiratory epithelial cells. The functionality of the Boyarchuck chamber is based on aerosol and fresh air mixture generated via fresh air forced through a small orifice meeting a flow of incoming EC aerosol.

At the beginning of the experiment, rats were allowed a period of 2 weeks to adapt to the Boyarchuck chamber. For time adaption, all rats were exposed to the fresh airflow.

EC liquid consists of 50/50 propylene glycol/glycerin (by volume fraction), 6 mg/ml nicotine, ethyl maltol and sucralose.

The Boyarchuck chamber was turned on, and rats of Group 2 were exposed to EC aerosol for 15 min. To exposure, dilution air was delivered at 6 l/min and aerosol EC was added at 6 l/min. The EC aerosol was delivered at a rate of 1 l/min per port, a value close to the puffing flow rate.

Sample Collection

To analyze the rat oral cavity microbiota, samples were collected four times: at the beginning of the experiment, on the 30th, 60th and 90th days. The samples were collected from the rat oral cavity using swabs soaked in 0.9% NaCl sterile solution. Then swabs were inserted in test tubes with 0.95 ml of sterilized physiological solution.

Culture and Identification of Isolates

Each sample was subsequently diluted up to 10^7 and with the aid of sterile calibrated wire loop 0.002 ml was inoculated onto Petri dishes containing microbiological

growth media. To detect the bacterial flora, the Petri dishes containing Blood agar, Mannitol Salt agar, Eosine Methylene Blue agar, MacConkey agar were incubated at 37°C in a bacteriological chamber for 48 hours. Sabouraud dextrose agar was used for elucidating fungal members and incubated at 37°C for 7 days. After incubation, the number of microbe colonies were registered as colony-forming units per ml (CFU/ml).

The bacterial isolates were examined microscopically by Gram staining technique and Lactophenol cotton blue mounts. Our Primary screening biochemical tests included: Coagulase test, Catalase test, Oxidase test, Urease test, IMVIC test (Indole, Methyl red, Voges-Proskauer and Citrate utilization tests) and germ tube test.

3. DATA ANALYSIS

The statistical analyses were performed using the STATISTICA software (StatSoftInc., USA, version 7.0). Friedman's test was used to compare multiple dependent variables. The differences of variables amongst different groups were analyzed using Mann-Whitney U-test for comparison of two groups, or Kruskal-Wallis test for more than two groups comparison. Wilcoxon matched-pairs test was used to compare two dependent variables. Discrete variables were measured and frequency distribution tables were constructed. Data were presented as Median values (Me), lower and upper quartile, i.e. Me [Lq; Uq]. Statistical significance was accepted if $p < 0.05$.

4. RESULTS AND DISCUSSION

At the beginning of the experiment, in order of frequency, the detected oral Gram-positive aerobic bacteria were of the following strains: *Bacillus spp.*, *Staphylococcus spp.*, *Streptococcus spp.*, *Enterococcus spp.*, *Corynebacterium spp.* and Gram-negative bacteria: *Enterobacter spp.*, *Escherichia spp.* Table 1 presents the distribution of bacterial species isolated from the oral cavity of WAG rats. Gram-positive and Gram-negative bacteria of all isolates comprised 89.2% and 10.8% in Group 1 and 88.5% and 11.5% in Group 2, respectively. A predominance of Gram-positive cocci and bacilli on the surfaces of the tongue and gums were detected in all WAG rats, at the beginning of the experiment. There was no statistically significant difference between Group 1 and Group 2.

During the entire observation period, no significant changes in the species composition of the microflora

were noted in Group 1 rats (Table 2). On the 30th day of EC exposure, there was inhibition in the following commensal microbes: *Bacillus spp.*, *Staphylococcus epidermidis*, *Streptococcus viridans* (Figure 1). At the same time, the number of colonies of the following opportunistic bacteria was increased: *Staphylococcus aureus*, *Enterococcus faecalis*, *Corynebacterium spp.* (Figure 2), *Enterobacter cloacae*, *Enterobacter aerogenes*, *Escherichia coli* (Figure 3) and *Acinetobacter lwoffii*, *Klebsiella pneumoniae* and *Candida albicans* appeared, which are not typical for the mouth in rats (Figure 4). On the 90th day, ECs exposure resulted in an increase in the colonies of *Acinetobacter lwoffii*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Staphylococcus aureus* and *Candida albicans*.

A test of dependence between an increasing count of isolated microorganisms and aerosol EC exposure time was conducted by way of the Friedman ANOVA Chi-Square, which showed Chi Sqr. =120.00, $p = 0.0000$ for *Staphylococcus aureus*, Chi Sqr. =119.90 $p = 0.0000$ – *Enterococcus faecalis*, Chi Sqr. =136.68, $p = 0.0000$ – *Enterobacter aerogenes*, Chi Sqr. = 139.64, $p = 0.0000$ – *Acinetobacter lwoffii*, Chi Sqr. = 139.54, $p = 0.0000$ – *Candida albicans*. There was a statistically significant relationship between the count of CFU/ml *Klebsiella pneumoniae* and duration of EC aerosol exposure $Z = 6.16$, $p = 0.0000$ (Wilcoxon test). This suggests an association between EC smoking and an increase in the number of opportunistic microbes. On the contrary, commensal flora exhibited a significant depletion of *Bacillus spp.*, *Staphylococcus epidermidis* and *Streptococcus viridians* during EC exposure to the *Bacillus spp.*, *Staphylococcus epidermidis*, *Streptococcus viridans* (Friedman ANOVA Chi-Square test $p = 0.0000$).

On the ninetieth day, the relative abundance of the oral microbiome differed between Group 1 and Group 2 (Table 2). The five most abundant genus persisted were *Bacillus spp.*, *Staphylococcus spp.*, *Streptococcus spp.*, *Enterococcus spp.*, *Corynebacterium spp.*, which accounted for 89.2% in Group 1. The Group 2 showed significantly elevated numbers opportunistic species: *Enterobacter cloacae*, *Enterobacter aerogenes*, *Escherichia coli* compared with Group 1 ($H = 54.59$, $p = 0.0000$, $H = 60.30$, $p = 0.0000$ and $H = 87.58$, $p = 0.0000$, respectively) (Table 2). Interestingly, *Acinetobacter lwoffii*, *Candida albicans* and *Klebsiella pneumoniae*, were detected firstly on the 30th and 60th day of EC exposure, respectively. On day

Table 1: The Distribution CFU of Microbial Isolates, (%)

Microbial isolates	Study period, day	Group 1 (n=10)								Group 2 (n=20)							
		0,0E+00	1,0E+01	1,0E+02	1,0E+03	1,0E+04	1,0E+05	1,0E+06	0,0E+00	1,0E+01	1,0E+02	1,0E+03	1,0E+04	1,0E+05	1,0E+06		
Bacillus spp.	0						50%	50%						60%	40%		
	30						60%	60%						80%	20%		
	60						60%	60%				15%	80%	5%			
	90						70%	70%				35%	65%				
Staphylococcus epidermidis	0				70%	30%						70%	30%				
	30				80%	20%					5%	70%	25%				
	60				80%	20%					45%	55%					
	90				70%	30%					45%	55%					
Staphylococcus aureus	0		60%	40%							65%						
	30		80%	20%						65%							
	60		80%	20%						30%	45%	25%					
	90		80%	20%							55%	25%	20%				
Streptococcus viridans	0				60%	40%								50%			
	30				60%	40%								45%			
	60				50%	50%					45%	50%	5%				
	90				50%	50%					70%	30%					
Enterococcus faecalis	0		50%	50%						30%	70%						
	30		50%	50%						30%	70%						
	60		40%	60%						10%	40%	50%					
	90		40%	60%							15%	85%					
Corynebacterium spp.	0		40%	60%						45%	55%						
	30		40%	60%						45%	55%						
	60		40%	60%							75	25%					
	90		20%	80							45	55%					

(Table 1). Continued.

Microbial isolates	Study period, day	Group 1 (n=10)								Group 2 (n=20)					
		0,0E+00	1,0E+01	1,0E+02	1,0E+03	1,0E+04	1,0E+05	1,0E+06	0,0E+00	1,0E+01	1,0E+02	1,0E+03	1,0E+04	1,0E+05	1,0E+06
Enterobacter cloacae	0		30%	70%											
	30		20%	80%											
	60		30%	70%							10%				
	90		40%	60%							65%	35%			
Enterobacter aerogenes	0		40%	60%											
	30		60%	40%							70%				
	60		60%	40%							80%				
	90		50%	50%							75%	25%			
Klebsiella pneumoniae	0	100%													
	30	100%													
	60	100%									100%				
	90	100%									40%	60%			
Escherichia coli	0			100%											
	30		5%	95%											
	60		10%	90%											
	90		10%	90%											
Acinetobacter Iwoffii	0	100%													
	30	100%													
	60	100%													
	90	100%													
Candida albicans	0	100%													
	30	100%													
	60	100%													
	90	100%													

Table 2: CFUs of Detected Microorganisms from Rat Oral Cavity, (Me [Lq; Uq])

Microbial species	Group 1 (n=10)					Group 2 (n=20)				
	Study period, day									
	0 day	30 day	60 day	90 day	0 day	30 day	60 day	90 day		
Bacillus spp.	2.5×10 ⁶ [5.2×10 ⁵ ;4.8×10 ⁶]	5.4×10 ⁵ [5.1×10 ⁵ ;5.2×10 ⁶]	5.3×10 ⁵ [5.0×10 ⁵ ;4.6×10 ⁶]	5.5×10 ⁵ [5.2×10 ⁵ ;4.3×10 ⁶]	5.8×10 ⁵ [5.1×10 ⁵ ;4.8×10 ⁶]	4.9×10 ⁵ [4.5×10 ⁴ ;5.4×10 ⁵]	4.2×10 ⁵ [4.0×10 ⁵ ;4.7×10 ⁵]	3.7×10 ⁵ [4.1×10 ⁴ ;4.2×10 ⁵]	Chi Sqr.= 139.68, p=0.0000	
Staphylococcus epidermidis	3.6×10 ³ [3.1×10 ³ ;2.9×10 ⁴]	3.1×10 ³ [2.7×10 ³ ;3.9×10 ³]	3.5×10 ³ [3.3×10 ³ ;4.2×10 ³]	3.8×10 ³ [3.6×10 ³ ;3.3×10 ⁴]	3.9×10 ³ [3.3×10 ³ ;3.1×10 ⁴]	3.2×10 ³ [2.5×10 ³ ;1.5×10 ⁴]	2.6×10 ³ [3.6×10 ² ;3.2×10 ³]	2.1×10 ³ [2.8×10 ² ;2.9×10 ³]	Kruskal-Wallis test: H=69.20, p=0.0000	
Staphylococcus aureus	3.2×10 ¹ [2.5×10 ¹ ;1.8×10 ²]	2.9×10 ¹ [2.5×10 ¹ ;3.1×10 ¹]	2.9×10 ¹ [2.7×10 ¹ ;3.4×10 ¹]	2.6×10 ¹ [2.3×10 ¹ ;3.3×10 ¹]	3.4×10 ¹ [2.3×10 ¹ ;2.0×10 ²]	3.9×10 ¹ [3.0×10 ¹ ;2.5×10 ²]	3.2×10 ² [4.2×10 ¹ ;1.6×10 ²]	4.5×10 ² [3.7×10 ² ;3.9×10 ³]	Kruskal-Wallis test: H=52.53, p=0.0000	
Streptococcus viridans	3.0×10 ³ [2.5×10 ³ ;2.8×10 ⁴]	2.8×10 ³ [2.3×10 ³ ;4.3×10 ⁴]	1.2×10 ⁴ [2.4×10 ³ ;4.1×10 ⁴]	1.4×10 ⁴ [2.7×10 ³ ;3.6×10 ⁴]	1.9×10 ⁴ [3.4×10 ³ ;3.8×10 ⁴]	4.0×10 ³ [3.0×10 ³ ;3.7×10 ⁴]	2.6×10 ³ [3.1×10 ² ;3.2×10 ³]	2.9×10 ² [2.7×10 ² ;2.4×10 ³]	Kruskal-Wallis test: H= 67.59, p=0.0000	
Enterococcus faecalis	1.4×10 ² [3.5×10 ¹ ;2.6×10 ²]	1.2×10 ² [3.3×10 ¹ ;2.6×10 ²]	2.4×10 ² [3.5×10 ¹ ;2.9×10 ²]	2.1×10 ² [3.7×10 ¹ ;2.8×10 ²]	1.7×10 ² [3.7×10 ¹ ;2.1×10 ²]	2.4×10 ² [4.3×10 ¹ ;2.8×10 ²]	1.3×10 ³ [2.9×10 ² ;2.6×10 ³]	2.7×10 ³ [2.2×10 ³ ;3.1×10 ³]	Kruskal-Wallis test: H=47.38, p=0.0000	
Corynebacterium spp.	1.5×10 ² [2.6×10 ¹ ;1.9×10 ²]	1.8×10 ² [2.4×10 ¹ ;2.2×10 ²]	2.0×10 ² [3.0×10 ¹ ;2.2×10 ²]	2.1×10 ² [1.7×10 ² ;2.2×10 ²]	1.8×10 ² [3.2×10 ¹ ;2.2×10 ²]	2.3×10 ² [4.0×10 ¹ ;2.7×10 ²]	3.9×10 ² [3.3×10 ² ;1.8×10 ³]	3.0×10 ³ [4.0×10 ² ;3.5×10 ³]	Kruskal-Wallis test: H=82.14, p=0.0000	

(Table 2). Continued.

Microbial species	Group 1 (n=10)					Group 2 (n=20)				
	Study period, day									
	0 day	30 day	60 day	90 day	0 day	30 day	60 day	90 day		
Enterobacter cloacae	2.4×10 ² [3.7×10 ¹ ;2.8×10 ²]	2.3×10 ² [1.9×10 ² ;3.0×10 ²]	2.3×10 ² [3.9×10 ¹ ;2.4×10 ²]	2.1×10 ² [4.0×10 ¹ ;2.5×10 ²]	2.2×10 ² [4.5×10 ¹ ;2.7×10 ²]	2.7×10 ² [5.5×10 ¹ ;3.0×10 ²]	3.3×10 ² [2.6×10 ² ;3.6×10 ²]	4.0×10 ² [3.6×10 ² ;2.9×10 ³]	Chi Sqr.= 138.93, p=0.0000	
Enterobacter aerogenes	2.2×10 ² [3.6×10 ¹ ;3.0×10 ²]	4.6×10 ¹ [3.5×10 ¹ ;2.7×10 ²]	4.5×10 ¹ [3.9×10 ¹ ;3.0×10 ²]	1.6×10 ² [4.1×10 ¹ ;3.1×10 ²]	2.7×10 ² [4.4×10 ¹ ;3.3×10 ²]	3.0×10 ³ [4.8×10 ¹ ;3.6×10 ²]	3.3×10 ² [3.1×10 ² ;4.0×10 ²]	4.2×10 ² [3.6×10 ² ;1.7×10 ³]	Kruskal-Wallis test: H=54.59, p=0.0000	
Klebsiella pneumoniae	0 [0:0]	0 [0:0]	0 [0:0]	0 [0:0]	0 [0:0]	0 [0:0]	3.9×10 ² [3.5×10 ² ;4.5×10 ²]	2.0×10 ² [4.3×10 ² ;2.8×10 ³]	Kruskal-Wallis test: H=60.30, p=0.0000	
Escherichia coli	2.9×10 ² [2.6×10 ² ;3.0×10 ²]	3.0×10 ² [2.5×10 ² ;3.3×10 ²]	3.3×10 ² [3.1×10 ² ;3.8×10 ²]	3.0×10 ² [2.6×10 ² ;3.6×10 ²]	4.3×10 ² [3.7×10 ² ;4.7×10 ²]	3.1×10 ² [2.5×10 ² ;3.6×10 ²]	2.9×10 ³ [4.7×10 ² ;3.7×10 ³]	3.7×10 ³ [3.0×10 ³ ;4.2×10 ³]	Kruskal-Wallis test: H=114.94, p=0.0000	
Acinetobacter lwoffii	0 [0:0]	0 [0:0]	0 [0:0]	0 [0:0]	0 [0:0]	3.6×10 ¹ [3.0×10 ¹ ;4.2×10 ¹]	4.4×10 ² [3.4×10 ² ;5.0×10 ²]	3.6×10 ³ [2.7×10 ³ ;4.3×10 ³]	Kruskal-Wallis test: H=87.58, p=0.0000	
Candida albicans	0 [0:0]	0 [0:0]	0 [0:0]	0 [0:0]	0 [0:0]	3.0×10 ² [2.8×10 ² ;3.4×10 ²]	5.8×10 ² [5.1×10 ² ;1.6×10 ³]	3.0×10 ³ [2.3×10 ³ ;3.5×10 ³]	Kruskal-Wallis test: H=116.40, p=0.0000	
									Kruskal-Wallis test: H=115.61, p=0.0000	

Data presented as Median, lower and upper quartile. Kruskal-Wallis test for independent samples. Friedman test (Chi-Sqr) for dependent samples.

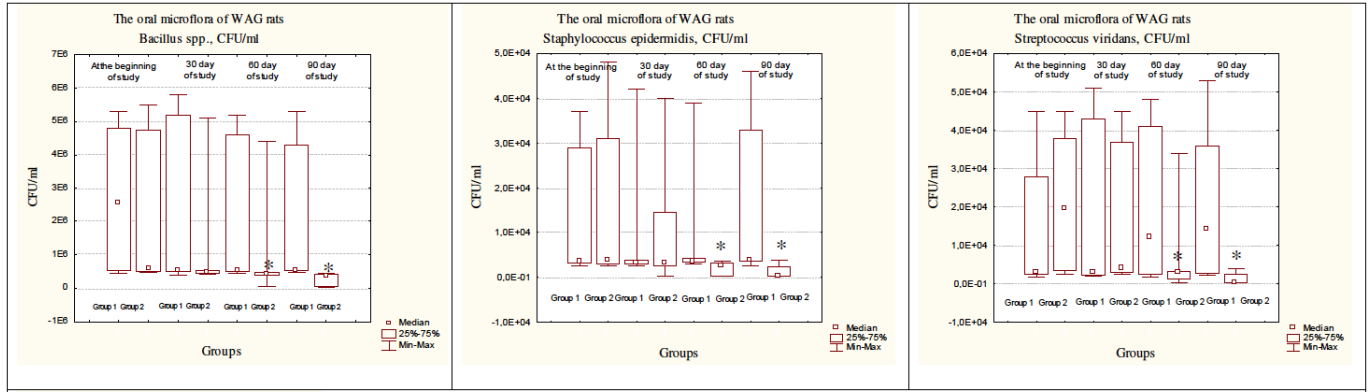


Figure 1: Characterization of the commensal microbes during experiment. Data are presented as box-plot. The median is denoted by the horizontal line within the box. The box indicates the interquartile range. *p < 0.05 Group 2 vs Group 1.

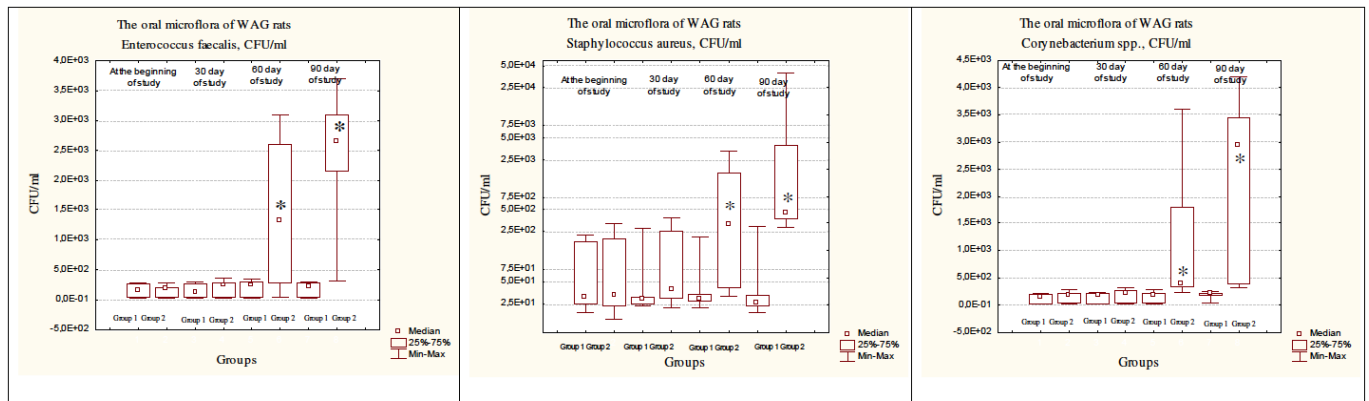


Figure 2: Characterization of the Gram-positive opportunistic microbes during experiment. Data are presented as box-plot. The median is denoted by the horizontal line within the box. The box indicates the interquartile range. *p < 0.05 Group 2 vs Group 1.

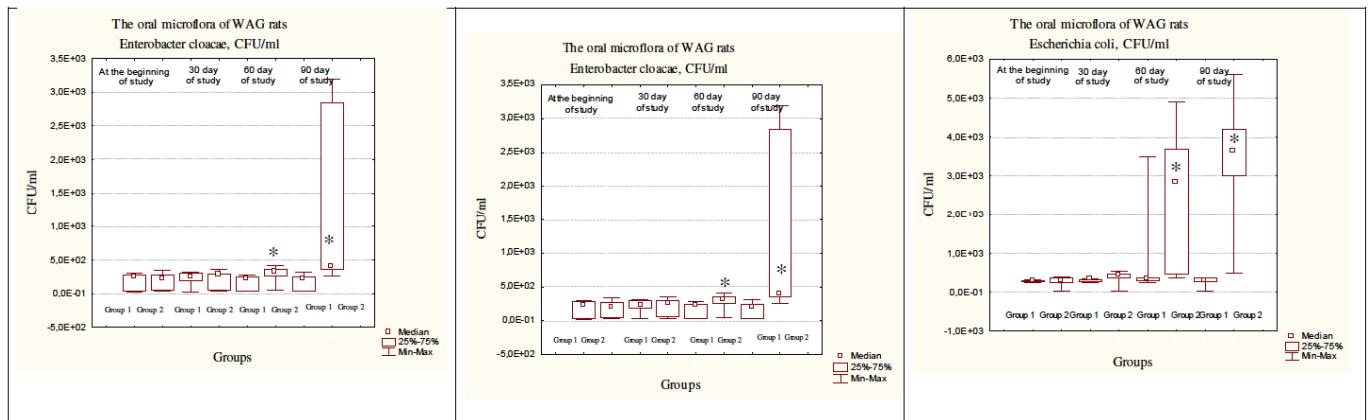


Figure 3: Characterization of the Gram-negative opportunistic microbes during experiment. Data are presented as box-plot. The median is denoted by the horizontal line within the box. The box indicates the interquartile range. *p < 0.05 Group 2 vs Group 1.

90, *Klebsiella pneumoniae*, *Acinetobacter lwoffii* and *Candida albicans* were present only in Group 2. The test of independence between frequency distribution of opportunistic microbes and duration of EC exposure was conducted by way of the Pearson Chi-Square test, which showed a significance for *Klebsiella pneumoniae* – $\chi^2= 8.017$, $p=0.0456$, *Acinetobacter lwoffii* – $\chi^2=$

36.772, $p=0.0001$, and *Candida albicans* – $\chi^2=8.689$, $p=0.0337$. It is expected that time of EC exposure will have a distinct effect on the growth of oral abnormal microbes [15].

The popularity of ECs smoking is growing throughout the world and in Ukraine, especially among the young

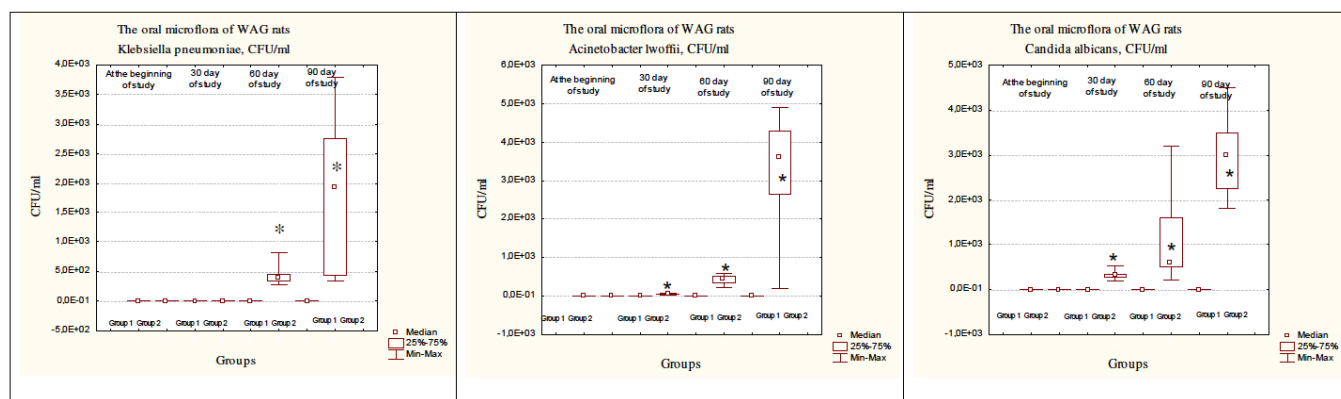


Figure 4: Characterization of the opportunistic microflora during experiment. Data are presented as box-plot. The median is denoted by the horizontal line within the box. The box indicates the interquartile range. * $p < 0.05$ Group 2 vs Group 1.

population [16]. This spread due to the misconceptions that EC aerosol is less harmful and less dependent than conventional cigarette smoke [17]. Although the prevalence of ECs is increasing, there are only a few research reports about their harmful effects on oral microbiota.

This study was performed to evaluate possible changes in the rat oral microbiota associated with the EC aerosol exposure. We assessed the rat oral microbiome before and its structural changes during EC aerosol exposure. At the beginning of the study, the similarity was detected in the microbial profile of Group 1 and Group 2 with the prevalence of health-compatible microflora. The following microorganisms were found: *Bacillus spp.*, *Staphylococcus spp.*, *Streptococcus spp.*, *Enterococcus spp.*, *Corynebacterium spp.*, *Enterobacter spp.* and *Escherichia spp.* The isolated oral microflora in this study is in agreement with a previous study indicating that human oral bacteria correspond to the bacterial species isolated from the rats' oral cavity [18].

Over the 90th day of the study, the statistical analysis revealed significant differences between Group 1 and Group 2 (Mann-Whitney U test). There was a reduction in the CFU/ml values of *Bacillus sp.*, *Staphylococcus epidermidis*, *Streptococcus viridans* was observed in a rat of Group 2 relative to Group 1 ($p_{1-2}=0.0000$, $p_{1-2}=0.0000$, $p_{1-2}=0.0000$, respectively). The Group 2 presented higher CFU counts of *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Escherichia coli* than did the Group 1 ($p_{1-2}=0.0000$, $p_{1-2}=0.0000$, $p_{1-2}=0.0000$, $p_{1-2}=0.0000$, $p_{1-2}=0.0000$, respectively). These data corroborate the information found in the literature, which shows increased counts of opportunistic microorganisms in the oral cavity of EC consumers

[19]. Meanwhile, the total microbial count in Group 2 had a tendency to reduce by 1.044 times ($p=0.7219$).

In our study, *Acinetobacter lwoffii*, *Candida albicans* and *Klebsiella pneumoniae* were not isolated from Group 1 rats; however, they were isolated from Group 2 on the 30th, 60th and 90th days of experiment, respectively, and their significantly higher colony forming units per milliliter (CFUs/ml) were found on the 90th day.

We observed that the frequency distribution of *Klebsiella pneumoniae*, *Acinetobacter lwoffii*, and *Candida albicans* were significantly higher in Group 2 rats on the 90th day compared to their data on the 60th day of the study: *Klebsiella pneumoniae* – $\chi^2= 8.017$, $p=0.0456$, *Acinetobacter lwoffii* – $\chi^2= 36.772$, $p=0.0001$, and *Candida albicans* – $\chi^2=8.689$, $p=0.0337$, respectively. Therefore, it was concluded that the increased frequency of distribution of these *microorganisms* is associated with a longer exposure time to EC aerosol.

Although viscous aerosols containing sweet flavours of ECs, as has been recently reported, provide attachment and food sources for pathogenic oral bacteria, *Streptococcus mutans*, we did not isolate this species in either group of our experiment [14].

The results of the study provide evidence about favorable conditions for some opportunistic species in rat oral cavities influenced by EC aerosol. The depletion of microorganisms belonging to the commensal species; higher prevalence and abundance of several opportunistic pathogens (*Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Candida albicans*); together with a reduction of total microbial count in Group 2 have also been elucidated in other studies [20, 21].

The symbiosis between oral microorganisms is based on mutual benefits. The commensals maintain homeostasis by not allowing opportunistic and pathogenic microorganisms to adhere to the mucosa. The opportunistic species become pathogenic only after they breach the barrier of the commensals, thus causing diseases [22].

The results of the study provide evidence about favorable conditions for some opportunistic species in rat oral cavities exposed to EC aerosol. While underlying triggers for dysbiosis in Group 2 rats are unknown, the current explanation of adverse effect EC is linked with increased production of acetaldehyde, acrolein, formaldehyde, metallic nanoparticles (copper, tin, lead, chromium, nickel, and cadmium), and reactive oxygen species in consumers. The activation of the heating element and the vaporization of EC liquids are two possible sources of release of these carbonyls, metals, and reactive oxygen species from electronic cigarettes [23-25]. Tendency to depletion of total microbial count in rats of Group 2 might be caused by ethyl maltol. In one study, researchers found that ethyl maltol destabilized the cell membrane by chelating Mg^{2+} or Ca^{2+} in a pH dependent manner [26]. Thus, it is plausible that ethyl maltol acted as a potent antimicrobial agent against microbes.

Moreover, acetaldehyde and sucralose (1,6-Dichloro-1,6-dideoxy- β -D-fructofuranosyl-4-chloro-4-deoxy- α -D-galactopyranoside) accumulation rates may alter the microbiota quantitatively. High concentrations of acetaldehyde and sucralose seem to cause an increase in aerobic Gram-positive bacteria, such as *Staphylococcus aureus* and *Corynebacterium spp.* [27].

Numerous factors influent on condition of the oral cavity such as social habits [10, 28]; condition of other human systems [29, 30] their pathological and physiological processes [31, 32] even in persons with active physical lifestyle [33, 34]; changes in different tissue [35, 36]. With an increase in ECs usage among youth, based on the literature on EC effects on oral microflora and our research findings, the issue of potential longer-term deleterious consequences of EC on the health of the young population must be raised.

The present study was limited by culture-based methods. We recognize that culture-based analytical technologies cannot reveal the diversity of the oral microbiome completely; however, the present study provides a rich insight into the adverse effects of EC on rat oral microbiome which may promote further health

risks. Despite limitations, our study provides insight into the adverse effect of EC on rat oral microbiome and the health risks.

5. CONCLUSIONS

The EC aerosol exposure had a critical influence on the oral microbiota. It resulted in a higher abundance of "misplaced" microbial species and progression toward dysbiosis.

CONTRIBUTION OF EACH AUTHOR ACCORDING TO THE KEY

A - research concept and design, B - data collection, C - data analysis and interpretation, D - article writing, E - critical review of the article, F - final approval of the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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