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Effect of different stress factors induced alteration in oral commensally microbiota

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Abstract

Microbiome is the term that is used to represent the total ecological diversity of microbes However, different factors that disrupt homeostasis can also significantly alteration oral microbial community structure due to exposure to psychological or pathological stressors like hormonal disturbance associated with pregnancy, cancer diseases with chemo and radiotherapy and smoking as one of the stress factors that change the acidity and PH and affect the oral microbiota. The aim of the study is to investigate the effect of different stress factors on selected bacteria of the oral microbiota. 30 samples were collected from different participant with different stress factors. Culture of microorganisms, phenotypic identification and VITEK machine were used for microbial profiles. Streptococcus mutans is higher in case of cancers, pregnancy and smokers compared to non smokers. Veillonella specises increased in cancer patients and smokers compared to normal non smoking cases. Hormonal disturbance associated in pregnancy cause alteration in normal oral microbiome and increase veillonella species. Conclusions: cancer which is treated with chemotherapy and chemo-radiotherapy makes stress on normal oral flora and increase Streptococcus mutans and veillonella species which cause dental caries. Pregnancy cause alteration in normal ecosystem of oral microbiome and increase veillonella species which cause tooth decay. Smoking alters normal ecosystem of oral

microbiome and increase oral Streptococcus mutans and increase danger of dental caries and tooth decay.

Keywords: Oral cavity, Microbiota, Smokers, Nonsmokers, Culturing and VITEK

INTRODUCTION

The microbiome lives on and inside the human body and can be defined as the genetic material of all microbes which may be bacteria, fungi, protozoa and viruses **(Bordenstein., 2015).** There are dynamic interactions between human microbiota and the environment as human microbes flowing freely into the surfaces we interact with every second; There are several factors which affects oral microbiome. These factors are Physiochemical factors such as Temperature, Nutrient, PH, Endogenous nutrients, exogenous nutrients and Host related factors such as: Host immunity and hormones. The second most diverse microbial community in the body is the mouth after the gut and colon. There are over 700 species of bacteria. These bacteria colonize the hard surfaces of teeth and the soft tissues of the oral mucosa **(Relman., 2015).** In dysbiosis or dysbacteriosis (microbial imbalance inside the body), the equilibrium of the oral ecosystem is disrupted allowing disease-promoting bacteria to manifest and cause conditions such as dental caries, gingivitis and periodontitis. The oral microbial habitat is altered when the primary teeth is replaced with an adult dentition. Smoking, consuming acidic drinks and refined sugar cause defects in oral ecosystem leading to dental caries and periodontal disease **(Busscher, 2010).** Maintaining a healthy oral microbiome: After the acquisition of the oral microbiome, it is maintained by factors related to the host and the microbe and this involves processes which are not fully understood. Regular use of tooth pastes, stop smoking and alcohol. Taking care of oral hygiene is very important step to have healthy oral microbiome.The oral ecology is the science that assesses the interaction between the oral microbiome and its habitat Factors affecting oral microbiome are Physiochemical factors, temperature, PH, host immunity and hormones.

Different techniques in analysis of oral microbiome: The culture - based method. Traditionally identification of the species in any given sample was achieved by growing it in vitro on a suitable media. Culturing can be done on selective and also nonselective media. There is common non selective media which is the blood agar ,which allow the growth of a broad spectrum of organisms. Laboratory culturing under special conditions and using a range of media has allowed the isolation of a diverse the range of bacteria. However, there are some side effects for this method: Which are Narrow spectrum, It has been estimated that 50% to 60% of the distinct extend bacterial phyla in oral cavity still have no cultivable representative and low sensitivity **(Kolenbrander, 2000).** Over that the culture depended method is sensitive and need a highly skilled individual. There is growing need for the developing improved methods to cultivate and characterize the as yet uncultivated portion of oral microbiome so as to unpick portion of the oral micro biome so as to unpick its role in health and disease. Theoretically all bacteria can grow under a proper nutritional and physiochemical conditions **(clarridge et al, 2014).** A lot of microbiological studies have been conducted specific in patients undergoing cancer treatment. Radiotherapy-caused hyposalivation may change the oral microbiomes. alteration in the amount, quality and complexity of the oral microbiota also happen with chemotherapy, leading to a major imbalance of the ecosystem **(Sixou et al, 1998)**

It is dangerous when pathogenic and opportunistic micro-organisms colonizing the mouth in immune suppressed patients treated with cytostatic drugs. In leukemia patients it was found that Enterobacteria and genera such as Pseudomonas, Neisseria, and Veillonella in oral samples from granulocytopenic **(Peterson et al 1990).** Yeasts can also be predominant but not always (**Pompei et al 1993) .**However, researches are lacking to illustrate how different cancer treatments have an effect on oral microbiota as a whole. It can be estimated that all the oral cancer treatment modes, include surgery, radiotherapy, and cytostatic drugs, change oral micro-organisms in a different way with possible characteristic shifts in biofilm composition. For this reason more researches are needed in this area which is essential in order to get evidence-based guidelines to the maintaining acceptable dental health of cancer patients **(Meurman. 2010).**

Materials: During this study we have used various materials and equipment these include: Plastic, disposable Petri dishes, Falcon tubes and anaerobic jars.

Medias and Agar: 1. TYCSB media (Tryptone Yeast Extract Cystine w/ Sucrose & w/o Bacitracin Agar Base) was used to detect Streptococcus mutans. 2. Lactobacillus selective agar is a selective media for lactobacillus. 3. Veillonella Agar Base with added vancomycin is used for selective isolation of Veillonella species. Brain heart media also was used. Carbon Dioxide gas packs: were used to ensure an anaerobic environment. VITEK machine: in the Egyptian cancer institute was used to detect lactobacillus selective agar colonies. Saline was used to collect different samples as the participant rinsed with it. Sucrose, inulin, mannitol and sorbitol sugars were used for phenotypic identification of microorganism as they were used for sugar fermentation test.

Data collection: In a form of questionnaire attached to the consent form, we collect data about the participant in two sections, section one include data about: gender, age, smoker or non-smoke, if none is there is any history of smoking, Do they drink any kind of soft drinks, Do they have any problems with their teeth or any empty teeth sites and How many times do they brush their teeth. For the last 15 days, did they undergo any surgeries Yes (mention it) or take any antibiotics. Have they ever drink alcohol or suffering from any diseases for the female participant if she is pregnant or have a menstruation during taking the sample. Section II (Smokers only) and include questions like On average, how many of the following products do you currently smoke each (day/ week)? Also, let us know if you smoke the product, but not every (day/ week).kind of smoke eg. Manufactured cigarettes? Hand- rolled cigarettes?) Kreteks? Tobacco pipes? Cigars, cheroots, or cigarillos and Number of water pipe sessions, Have they visited a doctor or other health care provider in the past 12 months and if yes During any visit were they advised to quit smoking tobacco, Do they have any health problems due to smoking, Do they notice a change in teeth color or oral bad smell due to smoking. And finally additional notes that should mention participant's diseases, habits and other stress causes that are not included in the questionnaire. The whole work including the design, questionnaire and the consents are

submitted and approved by the ethics committee in faculty of pharmacy MSA University.

Methods: Sampling: 30 samples (15 smokers and 15 non-smokers) were collected. In a sterile falcon tubes filled with 10 ml saline, samples were collected from the participant (oral rinse sample). In sterile eppendorfs, 10µl from each sample was diluted in 90µl saline from 10^{-1} to 10^{-6}. Among smoker cases there were three females only. Among nonsmoker cases there were 8 females. Microorganisms on different types of media samples were cultured. The samples were put in an anaerobic jar with a gas pack and then incubated at 37 ^{0}c and left for two days. After two days different media were separated according to its type. Colonies on TYCSB were transferred to brain heart media at left in the incubator for two days. Turbidity indicates bacterial growth. Petri dishes were divided according to the dilution and microorganisms were cultured on different types of media (*Veillonella* agar base, *lactobacillus* selective agar and TYCSB media). 5µl from each dilution is transferred to the plate by pipette. Petri dishes are put in anaerobic jars with gas packs and incubated at 37 ^{0}C. Viable count: After incubation, number of colonies in different media and plates were counted. Phenotypic identification of microorganism: Gram staining was made for different colonies on different plates and examined under microscopes. TYCSB (Tryptone Yeast Extract Cystine w/ Sucrose & w/o Bacitracin Agar Base) was used to detect *Streptococcus mutans*. One colony is transferred by a sterile loop to brain heart broth and incubated at 37 0 C for 2 days. Sugar fermentation test is applied after incubation on inulin, sucrose, sorbitol and mannitol sugars. It gives positive, if changes from red to yellow.*Lactobacillus* selective agar is a selective media for lactobacillus. Sub-culture was applied to LBS colonies and they were transferred to Egyptian cancer institute to be detected by VITEK machine. *Veillonella* Agar Base with added vancomycin is used for selective isolation of *Veillonella* species. Identification was done by gram staining and examination under microscope.

RESULTS

Viable count: Colonies of different types of media were counted.
Note: (s) means smoker and (n) means nonsmoker

Sample number	**TYCSB colonies**	***Veillonella* agar base colonies**	***Lactobacillus* selective agar colonies**
Sample1 (n)	10^{-1} : 1	No yield	10^{-1} : 4 10^{-2} : 2
Sample 2 (s)	10^{-1} : to many to be counted	10^{-1} : 20 10^{-2} : 12	10^{-1} : 12
Sample 3 (s)	10^{-1} : to many to be counted 10^{-2} : 2 colonies	10^{-1} : 16 10^{-2} : 12	10^{-1} :2 10^{-2} : 2
Sample 4 (n)	10^{-1}: 1 10^{-2} : 1	No yield	10^{-1} : 17 10^{-2} : 16
Sample 5 (n)	10^{-1} : too many to be counted	0^{-1} : 1	10^{-1}: 16
Sample 6 (s)	10^{-1} : 23 10^{-2} : 20	10^{-1} : 12 10^{-2} : 12	10^{-1}: 6 10^{-2}: 2
Sample 7 (s)	10^{-1} : 24 10^{-2}: 1	10^{-1} : 13 10^{-2} : 8	10^{-1}: 10 10^{-2} : 2
Sample 8 (s)	10^{-1} : 30 10^{-2} : 28	10^{-1} : 16	10^{-1} : 12
Sample 9 (s)	10^{-1} : 31 10^{-2} : 28 10^{-3} : 12	10^{-1} : 13	10^{-1}: 8 10^{-2} : 6 10^{-3} : 1
Sample 10 (s)	10^{-1}: 28 10^{-2} : 17 10^{-3} : 6 10^{-4} : 1	10^{-1} : 11 10^{-2} : 9	10^{-1} : 16
Sample 11 (n)	Too many to be counted	10^{-1} : 1	10^{-1} : 18
Sample 12 (s)	10^{-1} : 28	10^{-1} :8 10^{-2} : 6	10^{-1} : 10
Sample 13 (s)	10^{-1} : 22 10^{-2} : 20	10^{-1}: 12 10^{-2} : 5	10^{-1}: 12
Sample 14 (n)	10^{-1} : 35 10^{-2}: 12 10^{-3} : 1	10^{-1} : 8 10^{-2} : 6	10^{-1} : 12 10^{-2} : 1
Sample 15 (s)	10^{-1} : too many to be counted 10^{-2} : 12	10^{-1} :5	10^{-1} : 2 10^{-2} : 2
Sample 16 (n)	10^{-1} : 1	No yield	10^{-1} : 6 10^{-2} : 1
Sample 17 (s)	10^{-1} : 40 10^{-2} : 39	10^{-1} : 5 10^{-2} : 2	10^{-1} : 5
Sample 18 (s)	10^{-1} : too many to be counted	10^{-1} : 7	10^{-1} : 17 10^{-2}: 2
Sample 19 (s)	10^{-1} : 36 10^{-2}: 29 10^{-3} : 12	10^{-1} : 22	10^{-1} : 3

Sample 20 (s)	10^{-1} : too many to be counted	10^{-1} : 15	10^{-1} : 8 10^{-2} : 2
Sample 21 (n)	10^{-1} : too many to be counted 10^{-2} : 16	10^{-1} : 35 10^{-2} : 12	10^{-1} : 22 10^{-2} : 2 10^{-3} : 1
Sample 22 (s)	10^{-1} : 31 10^{-2} : 19	10^{-1} : 1	10^{-1} : 7 10^{-2} : 1
Sample 23 (n)	10^{-1} : 4 10^{-2} : 1	10^{-1} : 15	10^{-1} : too mant to be counted 10^{-2} : 4
Sample 24 (n)	10^{-1} : 40 10^{-2} : 22	10^{-1} : 33 10^{-2} : 10	10^{-1} :20 10^{-2} : 2
Sample 25 (n)	10^{-1} : 2 10^{-2} :2	No yield	10^{-1} :6
Sample 26 (n)	10^{-1} : 1	10^{-1} : 1	10^{-1} : 8
Sample 27 (n)	10^{-1} : 4 10^{-2} : 2	No yield	10^{-1} : 4 10^{-2} : 1
Sample 28 (n)	10^{-1} : 1	10^{-1} : 1	10^{-1} : 3
Sample 29 (n)	10^{-1} : 3 10^{-2} : 1	No yield	10^{-1} : 2
Sample 30 (n)	10^{-1} : 1 10^{-2} :1	No yield	10^{-1} : 4

Table1 show the viable count on different types of media

On TYCSB, viable count of colonies in smoker participants was higher if compared with nonsmoker. The non-smoker yield was few or depleted in some participants. Five non-smoker participants (samples: 5, 11, 14,21and24), their viable count was too many to be counted. On *veillonella* agar base, viable count was higher in smoker if compared with nonsmokers which may be depleted in some participants. Three non-smoker participants (21, 23 and 24), their viable count was observed so high if compared with other nonsmokers. On *lactobacillus* selective agar, viable count in non-smoker and smokers was almost corresponding to each other. Microscopically examination: TYCSB colonies appear as gram positive cocci. *Veillonella* agar base colonies were gram negative cocci. *Lactobacillus* selective agar colonies appear as gram positive bacilli. Sugar fermentation test: It was applied after incubation on inulin, sucrose, sorbitol and mannitol sugars. It gives positive as it changed from red to yellow with all smokers' samples. It was negative with all nonsmokers' samples except samples 5,11,14, 21 and 24.

The VITEK machine: *Lactobacillus* species, the VITEK machine indicates that in all nonsmoker cases *lactobacillus acidophilus* was found except samples 5, 11, 14, 21 and 24. In samples 21 and 24, *lactobacillus casei*, *Lactobacillus fermentum* and

lactobacillus paracasei were found. In samples (5, 11 and 14), *lactobacillus fermentum* was found. In smokers, *Lactobacillus fermentum* and *lactobacillus acidophilus* were found. We found that there were no differences between male and female cases in all the experiment.

Case	Case description	Sugar fermentation test	VITEK results
1	Nonsmoker	-ve	*Lactobacillus acidophilus*
2	smoker	+ve	*Lactobacillus casei*
3	smoker	+ve	*Lactobacillus paracasei*
4	Nonsmoker	-ve	*Lactobacillus acidophilus*
5	Nonsmoker, regularly drink soft drinks and poor oral hygiene	+ve	*Lactobacillus fermentum*
6	smoker	+ve	*Lactobacillus casei*
7	smoker	+ve	*Lactobacillus paracasei*
8	smoker	+ve	*Lactobacillus fermentum*
9	smoker	+ve	*Lactobacillus casei*
10	smoker	+ve	*Lactobacillus paracasei*
11	Nonsmoker, regularly drink soft drinks and poor oral hygiene	+ve	*Lactobacillus fermentum*
12	smoker	+ve	*Lactobacillus casei*
13	smoker	+ve	*Lactobacillus paracasei*
14	Nonsmoker, regularly drink soft drinks and poor oral hygiene	+ve	*Lactobacillus fermentum*
15	smoker	+ve	*Lactobacillus casei*
16	Nonsmoker	-ve	*Lactobacillus acidophilus*
17	smoker	+ve	*Lactobacillus paracasei*
18	smoker	+ve	*Lactobacillus paracasei*
19	smoker	+ve	*Lactobacillus paracasei*
20	smoker	+ve	*Lactobacillus casei*
21	Nonsmoker, gingival cancer patient and is subjected to chemotherapy	+ve	*Lactobacillus fermentum*
22	smoker	+ve	*Lactobacillus fermentum*
23	Pregnant and nonsmoker	-ve	*Lactobacillus acidophilus*
24	Nonsmoker and colon cancer	+ve	*Lactobacillus casei*
25	Nonsmoker	-ve	*Lactobacillus acidophilus*
26	Nonsmoker	-ve	*Lactobacillus acidophilus*
27	Nonsmoker	-ve	*Lactobacillus acidophilus*
28	Nonsmoker	-ve	*Lactobacillus acidophilus*
29	Nonsmoker	-ve	*Lactobacillus acidophilus*
30	Nonsmoker	-ve	*Lactobacillus acidophilus*

Table2 show Case description and phenotypic results

DISCUSSION

Taking into account the many toxic materials which found in cigarette smoking, it is not unusual or strange thing that smoking strongly

changes the microbial flora of the mouth. *Streptococcus mutans* is higher in smokers and this increase tooth decay danger. It is observed that five non-smoker participants viable count was too many to be counted. In the questionnaire, three of them told us that they complain of dental caries and tooth decay and they regularly have soft drinks (samples 5, 11 and 14). Two of them were cancer patients (colon cancer and gingival cancer) and are subjected to radiotherapy and chemotherapy (samples 21 and 24) and one is a pregnant woman (sample 23). Smoking increases *veillonella* species in smokers if compared with non-smokers due to oxygen depletion. *Lactobacillus* species, the VITEK machine indicates that in all nonsmoker cases *lactobacillus acidophilus* was found except samples 5, 11, 14, 21 and 24. In samples 21 and 24, *lactobacillus casei*, *Lactobacillus fermentum* and *lactobacillus paracasei* were found *Lactobacillus casei*, *Lactobacillus fermentum*. In samples (5, 11 and 14), *lactobacillus fermentum* was found. In smokers, *Lactobacillus fermentum* and *lactobacillus acidophilus* were found and *lactobacillus paracasei* are associated with active dental caries lesions. There is a significant depletion of Proteobacteria and enrichment of Firmicutes and Actinobacteriain current compared with never smokers.

Each person's micriobiota varies in the certain bacterial lineages found, with a comparable level of co-variation. oral microbioms on mucosa vary between healthy and malignant sites and particular oral micriobiota species have been associated with malignancies but the evidence is still weak in this point However, in the oral cavity the role of microbiota in carcinogenesis is not known in many studies. Another problem is the cancer treatment-caused change in oral microbial population which may be a harmful and lead to potential pathogens and following other systemic health problems to the patients **(Meurman. 2010).**For that reason clinical guidelines and recommendations have been offered to control oral microbiota in patients with malignant disease, but also in this area the scientific evidence is still weak. More controlled studies are needed for further conclusion.

In case of cancer patients, this is in agreement with the fact that the microenvironment of solid tumors is typically hypoxic with low pH, thus favoring the survival of only acid tolerant bacteria **(Nagy et al., 1998).** Also **Rautemaa et al 2006** observed that the sampling site may affect the results and In oral cancer patients

optimal sampling may be complicated, accodingly, suitable sampling technique for both the conventional cultivation and the new molecular procedures needs to be emphasized **(Rusanen et al., 2009)** There is a necessity for standardized guidelines in this area.

A lot of microbioms studies have been conducted in particular in cancer treated patients. hyposalivation caused by Radiotherapy may influence the oral microbiota also change in quantity, complexity, and quality of the oral microbial populations can be found in chemotherapy **(Meurman. 2010). Almståhl et al 2008** state that *Lactobacillus* species were found in 92% of the subjects and the relative amount of the species was very high compared with the controls. Mutants streptococci were also found in high numbers; 31% in the patients vs. 23% in controls.

An important question is How permanent are the changes in oral microbiota after the cancer treatment. Radiotherapy or cytostatic therapy lead to variation in bacterial composition need not be permanent. In immunocomprmized patients with cytostatic drugs pathogenic and opportunistic bacteria colonizing the mouth may be harmful. Oral microbial population generally exisiting in biofilms cause a risk to immunocomprmized cancer patients if microbes get into blood circulation or reach to adjacent tissues. Giving that bacteremia complications are life-threatening to immune compermized patients so it is important to prevent such complications.

Smoking has a strong inhibitory effect on the growth of *Neisseria* species and less inhibitory effect on *streptococcus* species as observed through in vitro studies using culture-based methods **(Bardell, 1981).** Also **Belstrom et al., 2014** state that in the saliva of smokers, there is increase in *streptococcus sobrinus* and *Eubacterium* [11][G-3] *brachy* and they concloudes that the bacterial profile of saliva seems independent of food intake, but influenced by smoking and maybe socioeconomic status. Strict or facultative anaerobes over strict aerobes are the environment caused by smoking in smokers. Tobacco smoking has significant effects on oral bacterial flora **(Brook and Gober, 2007).** Tobacco smoking increases susceptibility to periodontitis **(Bagaitkaret al.,2008).** Tobacco smoking increases infection risk as it compromises the antibacterial function of leukocytes including neutrophils, monocytes, T-cells and B-cells **(Palmer et al., 2005).** In smokers with periodontitis, scaling

and root planning have been shown to be less effective .There are gingivalis, Treponema denticola and Tannerella forsythia which are anaerobic bacteria and associated with periodontal infections. lately, studies with overall oral bacterial ecology in humans have observed increased Streptococcus sobrinus and Eubacterium brachy in the saliva of smokers **(Belstrom et al., 2014),** decreased Neisseria, Porphyromonas and Gemella in oral wash samples from smokers **(Morris et al., 2013),** increase of Megasphaera, Streptococcus and Veillonella, and decrease of Capnocytophaga, Fusobacterium and Neisseria, in the oropharynx of smokers **(Charlson et al., 2010).**

In pregnant case, this increase in veillonella is due to increase saliva acidity especially in early stages of pregnancy which cause vomiting and nausea **(Kamma et al., 1999).** A strong correlation has been established between the saliva Lactobacillus count and dental caries, the higher the DMF index, the higher the number of persons harbouring a high Lactobacillus count. Qualitative studies confirmed the predominance of group casei species in dental plaque sampled on carious lesions. In cancer patient, L. casei is predominant and Lactobacillus fermentum is the most frequently found species. The latter is also more frequently found in dental plaque samples in smokers. **(Badet and Thebaud 2008)**

CONCLUSION:

Poor oral hygiene has been linked with high risk of oral carcinoma where the common risk factors are excessive consumption of alcohol and tobacco. A lot of studies needed to emphazise the role of oral microbiome in carcinogenesis. In our study oral cancer which is treated with chemotherapy and colon cancer which treated with chemo-radiotherapy makes stress on normal oral flora and increases *Streptococcus mutans* and *veillonella* species and both cause dental caries. Smoking alters normal ecosystem of oral microbiome. Smoking increase oral *Streptococcus mutans* and increase danger of dental caries and tooth decay. Smoking increases *veillonella* species which cause dental caries. Pregnancy cause alteration in normal ecosystem of oral microbiome and increase *veillonella* species which cause tooth decay. There was no difference between male and female cases.

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