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The Alterations in Some Salivary Biomarkers and Oral Microbiome in COVID-19 Patients and Healthy Individuals

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ABSTRACT

A saliva samples have been collected from 42 COVID-19 patient and a 42 healthy individuals and tested for salivary biomarkers of, α-amylase, lysozyme in addition to and mutans streptococci. Results showed non-significant difference between mean concentration of α-amylase in COVID-19 patients (5.48 µ/ml) and healthy individuals (6.05 µg/ml), high significant difference in mean concentration of lysozyme in COVID-19 patient (170.42 µg/ml) and healthy individuals (8.47 µg/ml), and non-significant difference in mean concentration of mutans streptococci in COVID-19 patients (6.88) × 10^6 CFU/ml and healthy individuals (6.58 × 10^6 CFU/ml).

Key words: COVID-19, SARS-CoV-2, Salivary lysozyme, Salivary α-amylase, *Mutans streptococci*

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INTRODUCTION

Saliva is a bio-fluid constituted of various fluids of secretions of salivary glands, respiratory secretions, crevicular and exfoliated epithelial cells. Presence of SARS-CoV-2 in saliva is attributed to the viral replication and RNA secretion in any cells and tissues related to the production of salivary components like salivary glands, periodontal tissues and the upper respiratory tract cells [1].

The microbial constituents of a eubiotic Humam Oral Microbiome (HOM) can inhibit colonization of pathogens through competitive exclusion and/or *via* facilitation of immune response to exclude the pathogen [2,3]. It is reported that a vital collaborative interactions happen between viruses and the micro biome and that the micro biome could be regulate and it, in turn, could be regulated by viruses through various mechanisms [4]. The oral micro biota can produce anti-viral substances (defensins) against various viruses, including respiratory tract viruses like coronaviruses, herpes viruses, adenoviruses, orthomyxoviruses and papillomaviruses [5]. Otherwise, invading viruses could results in dysbiosis and progression of disease [6].

The pandemic of coronavirus SARS-CoV-2, the causative of COVID-19 is respiratory virus that invades the oropharynx as the primary site of replication but the possible impact on oral micro biome through development of infection remains un-clarified. In particular, there are no date available concerning the non-bacterial constituents of HOM (fungi and viruses), that have been shown vital for other diseases. Regarding COVID-19, it is reported that the presence of gingival inflammation/periodontitis was associated with a 3.5-fold increase in the risk for admission to Intensive Care Units (ICU), a 4.5-fold increase in the probability for assisted ventilation and a risk of 8.81-fold increase in the probability for death as a consequences of COVID-19, separately from any other concomitant risk factors [7].

The functions of salivary biomarker of α-amyalse extensively studied for their biological activities but their correlation to each other in COVID-19 patient and healthy individuals to COVID-19 are still unrevealed. The current study is designed to assess whether there is any association between salivary α-amylase, lysozyme, and total a count of *mutans Streptococci*. The study aimed to measure the salivary α-amylase level in both groups (COVID-19 patients and heathy individuals), to measure the salivary lysozyme level in both groups (COVID-19 patients and control group), to calculate the total viable count of *mutans Streptococci* and *C. albicans* among COVID-19 patients and to evaluate the relationship between salivary α-amylase, lysozyme, and total viable a count of *mutans Streptococci* among COVID-19 patients.

MATERIALS AND METHODS

Saliva collection: After taking patients agreement for collecting saliva samples, checking that the patients didn't take antibiotics or any medications for the latest two weeks, then giving the patients plain tubes that numbered before sample collection. Saliva sample collection was made in early morning at time between 8 am to 10 am.

The amount of the collected saliva was between 1-3 ml of un-stimulated saliva by allowing the saliva to accumulate in the mouth and then spitting into a tube. After collecting the salivary sample from each patient, the tubes were placed in a cool box with ice to transfer them to the laboratory to be cultured within less than an hour, then 0.1 ml would be taken from the salivary sample by micro pipette for the serial dilution tubes using PBS. The resting saliva was centrifuged for 3000 rpm for 10 min, and the clear supernatant was e and stored in freezer at -20°C until the determinations of salivary α-Amylase, lysozyme and melatonin were done by ELISA test

Determination of lysozyme level

This kit was based on Competitive-ELISA detection method (Cell Biolabs, USA). The microtiter plate provided in this kit has been pre-coated with antibody. During the reaction, target in the sample or standard competes with a fixed amount of Biotin-Antigen. Excess conjugate and unbound sample or standard are washed from the plate. HRP-Streptavidin was added and unbound conjugates were washed away with wash buffer. Then TMB substrate solution is added to each well. The enzyme substrate reaction is terminated by the addition of a sulfuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of target in the samples is then determined by comparing the OD of the samples to the standard curve.

Determination of salivary α-Amylase

The ELISA test kit (LDN, Germany) provides a quantitative *in vitro* assay for free α-amylase in human saliva. The test kit contains microtiter strips each with 8 break off reagent wells coated with anti-rabbit antibodies. In the first reaction step, diluted patient samples are pipetted into the reagent wells together with peroxidase labelled α-amylase and a specific rabbit anti-α-amylase antibody. α-amylase from the patient sample and the labelled α-amylase in the conjugate compete for the free binding sites of the specific antibody. In the third incubation step, the bound peroxidase catalyses a colour reaction with the peroxidase substrate Tetra Methyl Benzidine (TMB). The intensity of the colour formed is inversely proportional to the concentration of α-amylase in the sample. The results for the samples are determined using the standard curve.

Cultivation of *mutans streptococci*

Saliva samples were centrifuged at 3000 rpm for 10 min. Precipitate has been discarded and the supernatant forwarded for culture and identification of *mutans Streptococci* and The supernatant has been serially 10-folds diluted in PBS enumeration of *mutans streptococci*, the supernatant of saliva was serially 10-folds diluted in PBS and streaked on MSBA agar for calculation of CFU/ml of *Mutans Streptococci*.

Statistical analysis: Statistical analysis and processing of the data were carried out using SPSS version 21 (Statistical Package for Social Sciences) under Windows 10. Data were subjected to the following:

Descriptive statistics

- Arithmetic Means (M), Standard Deviation (SD) and Standard Error (SE).
- Minimum (mini) and maximum (maxi).

Inferential statistics

The statistical tests that were used in this study:

- Anova test.
- L.S.D. test.
- Student's t-test and.
- Pearson correlation coefficients.

The level of significance was accepted at P< 0.05, and highly significance when P<0.01.

RESULTS

α-Amylase: Salivary α-amylase level in COVID-19 patients group and healthy individuals group is shown in Table 1 and Figure 1, which revealed the presence of mean value of salivary α-amylase among the COVID-19 patients group (5.48) u/ml less than healthy individuals group (6.05) u/ml, with a statistical non-significant difference between the two groups, the t-value was (1.602) and the p value was (0.117).

Table 1: Descriptive of α-amylase.

Variable	No. of cases	Minimum	Maximum	Mean	SE	SD
Healthy individuals	42	3.6	9.09	6.05	0.25	1.66
COVID-19 patients	42	1.79	11.2	5.48	0.33	2.18

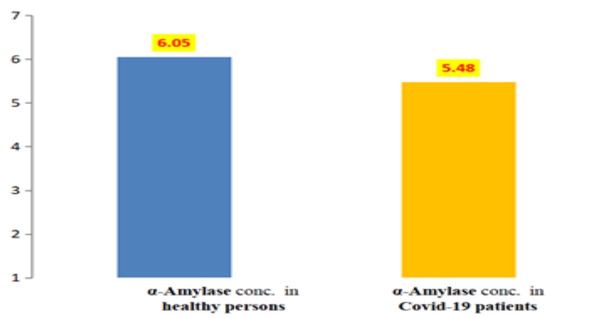

Figure 1: Means of a-amylase among COVID-19 patients and healthy individuals groups.

Lysozyme: Salivary lysozyme level in COVID-19 patients group and healthy individuals group is shown in Table 2 and Figure 2, which revealed the presence of higher mean value of salivary lysozyme among the COVID-19 patients group (170.42) g/ml than healthy individuals group (8.47) g/ml, with a statistical high significant difference between the two groups, the t-value was (7.668) and the p value was (0.001).

Table 2: Descriptive of lysozyme.

Variables	No. of cases	Minimum	Maximum	Mean	SE	SD
COVID-19 patients	42	24.38	513.3	170.42	21.11	136.86
Healthy individuals	42	4.76	9.29	8.47	0.16	1.07

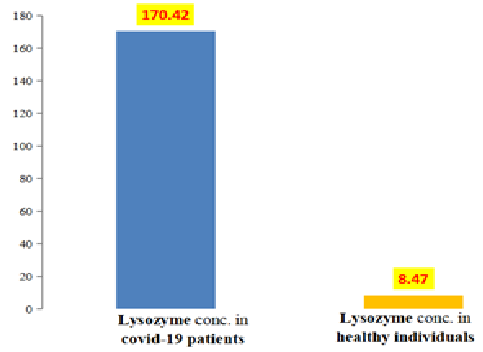

Figure 2: Means of lysozyme among COVID-19 patients and healthy individuals group.

Total Viable Count of *Mutans Streptococci* (CFU/ml)

Result in Table 3 and Figure 3 shows the statistical analysis of viable count (CFU/ml) × 10^6 of Total Viable Count of Salivary *Mutans streptococcus*, there was Non-significant difference between two groups, the t-value was (0.43), the p value (0.66), the mean value in healthy individuals group (6.58) × 10^6 CFU/ml was less than COVID-19 patients group mean value (6.88) × 10^6 CFU/ml

Table 3: Descriptive of *mutans streptococcus.*

Variables	No. of cases	Minimum	Maximum	Mean	SE	SD
Healthy individuals	42	2.8	11.28	6.58	0.36	2.39
COVID-19 patients	42	3	17.44	6.88	0.55	3.61

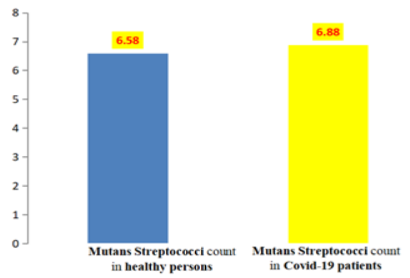

Figure 3: Means of Total viable count of salivary *mutans streptococcus* among COVID-19 patients and healthy individuals group.

DISCUSSION

Salivary lysozyme level in COVID-19 patients group and healthy individuals group

The presence of lysozyme in body fluids, including saliva, is an important factor in non-specific mechanisms towards microbial infections [19-21]. The high significant difference between the COVID-19 patients and healthy individuals groups is a strong marker that SARS-CoV-2 provokes the salivary glands to secrete a 20 fold of lysozyme concentration of healthy individuals to counteract the viral infection.

Lysozyme is bactericidal for gram-positive bacteria *via* hydrolyzing the β-1,4 glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid of the bacterial cell wall [22]. Furthermore, the lysozyme exerts antimicrobial activity through binding to negatively charged surfaces of microbes owing to its cationic nature [23,24]. The immunomodulatory action of lysozyme has only been appreciated in last few years.

Within neutrophils and marcophages, lysozyme works to increase their pro-inflammatory response, but when it secreted outside the above mentioned cells as well as epithelial cells, it limits inflammation through decreasing the chemotaxis and oxidative burst in neutrophils [25], it suppresses the production of IL-6 and TNF-α in macrophages [26], it binds and decreases the circulating levels of Advanced Glycation End products (AGEs) (that are pro-oxidative) in addition to increasing their renal excretion [27] and it disrupts the capacity of peptidoglycan to bind the complement factors that works as anaphylotoxins [22]. Moreover, when subjected to artificial conditions of gastro-intestinal conditions, thehydolyzate of lysozyme of hen egg white showed remarkable Angiotensin Converting Enzyme (ACE) and anti-oxidant activity [28,29]. As mentioned above, the oxidative stress (including participation of AGEs), cytokines of IL-6 and TNF-α, inflammation caused by macrophages and neutrophils and the Renin–Angiotensin System (RAS) are characteristic in the Acute Respiratory Distress Syndrome (ARDS) and/or severe COVID-19. It is most interested that the activity of lysozyme beside lactoferrin in neuroprotection of Alzheimer's patients through inhibition of amyloid-beta aggregation [30] could be an active mechanism in treatment of potential neurological manifestations in severe cases of COVD-19.
The increase in Salivary Antimicrobial Proteins (sAMPs) like lysozyme and lactoferrin in Lower Respiratory Tract Infection (LRTI) was reported in several previous studies [31,32]. The advantage of increasing level of lysozyme is to raise the immunomodulatory effects of lysozyme to counteract SARS-CoV-2 infection [25-28]

The viable total count of salivary *mutans streptococci* in COVID-19 patients group and healthy individuals group

The count concentration of *mutans streptococci* in COVID-19 patients and healthy individuals was attributed to the fact that the natural habitat of *mutans streptococci* is the oral cavity. The *mutans streptococci* have the ability to exert effective persistence in oral cavity due to formation of diverse human associated-biofilms [33-65] reported that the bacterial profile of oral microbiota in COVID-19 patients is characterized by dominance of Prevotella salivae and Veillonella infantium, whereas Neisseria perflava and Rothia mucilaginosa were dominant in healthy individuals along with *N. perflava*, *K. gabonensis*, *G. elegans*, *Porphyromonas pasteri*, *Gemella taiwanensis*, *R. mucilaginosa*, and *Streptococcus oralis*. *Mutans streptococci* concentration in oral cavity is unaffected by COVID-19 [66-95].

CONCLUSION

It is concluded that salivary lysozyme is highly responsive through COVID-19 to immunomodulate the infection. There is an inverse relationship between salivary melatonin level and COVID-19 that is resulted, probably, from fast utilization of melatonin as free radical scavenger to counteract the inflammation-generated high level of ROS. The secretory function of salivary glands for secretion of α-amylase is unaffected during COVID-19 due to the homeostatis of the enzyme in both COVID-19 patients and healthy individuals. Oral candidiasis is a sequeleae of COVID-19 as result of saliva low flow rate (Xerostomia) occurred during SARS-CoV-2 infection to salivary glands. The total count of *Mutans Streptococci* is stable during COVID-19.

RECOMMENDATIONS

It is recommended to study the effect of COVID-19 on oral microbiota of gram-positive and gram-negative bacteria and monitor the potential presence of bacteremia and/or septicemia in COVID-19 patients due to overgrowth of any member of oral bacteria, study level of other salivary biomarkers through course of COVID-19 pathology like C-Reactive Protein (CRP), myoglobin, creatine kinase isoform MB, α-2-macroglobulin, glycosylated hemoglobin (HbA1c) and various Interleukins (ILs), study the involvement of periodontal diseases in the COVID-19 due to depletion of salivary melatonin level in COVID-19 patients that is correlated to periodontal diseases, study the correlation between severity of COVID-19 and levels of salivary biomarkers and oral micro biome to uncover the contribution of salivary biomarkers and oral micro biome in determination of susceptibility of an individual to COVID-19.

CONFLICT OF INTEREST

The author declare that there are no conflicts of interest

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