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Salivary *Streptococcus mutans* and Lactobacilli Levels as Indicators of Dental Caries Development in Iranian Patients with Systemic Sclerosis

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ABSTRACT

Background and Aim: Systemic sclerosis is an autoimmune disorder with orofacial manifestations, including tooth decay. Lactobacilli can inhibit biofilm formation and growth of cariogenic pathogens, such as *Streptococcus mutans*. We aimed to assess the salivary levels of *S. mutans* and Lactobacilli as indicators of dental caries development in patients with systemic sclerosis.

Materials and Methods: In this cross-sectional study, 80 patients with systemic sclerosis were assigned into 2 groups, anti-centromere antibody (ACA) positive (n=42) and ACA-negative (n=38). Besides, 80 age- and gender-matched healthy individuals were enrolled as control. Unstimulated saliva was collected in sterile tubes. Blood agar and tomato juice agar were used to cultivate *S. mutans* and Lactobacilli. The number of colony-forming units per milliliter (CFU/mL) was calculated and compared between the groups.

Results: *S. mutans* in patients (median=1.6×107 CFU/mL; interquartile range (IQR): 1.1–3.1 ×107 CFU/mL) was significantly higher than control group (median=5.1×106 CFU/mL; IQR: 5.1–7.9 ×106 CFU/mL) (P<0.0001, Mann-Whitney U-test); however, the median Lactobacilli levels was similar between these groups (3.4×106 vs. 2.2×106 CFU/mL; P=0.095). The median concentrations of *S. mutans* (1.3×107 vs. 2.4×107 CFU/mL; P=0.342) and Lactobacilli (4.1×106 CFU/mL vs. 3.1×106 CFU/mL; P=0.515) in the ACA-positive and ACA-negative patients had no significant differences. There were no significant correlation coefficients between *S. mutans* and Lactobacilli levels in the study groups (P>0.05).

Conclusion: Our findings suggest the higher levels of salivary *S. mutans* in patients with systemic sclerosis might increase the likelihood of dental caries over time; however, it was not affected by the ACA status.

Keywords: Lactobacilli, Saliva, *Streptococcus mutans*, Systemic sclerosis

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1 Introduction

Systemic sclerosis, also known as scleroderma, is a rare chronic autoimmune disorder of connective tissue characterized by the progressive fibrosis of the skin and several internal organs **(1, 2)**. It is divided into diffuse cutaneous and limited cutaneous forms based on the extent of skin involvement. The peak incidence of systemic sclerosis is in the fifth decade of life and tends to affect women more than men. The exact etiology of the disease remains uncertain; however, a wide range of genetic, infectious, and environmental

factors are thought to contribute to the development of systemic sclerosis **(2-5)**.

Antinuclear antibodies (ANA), such as anti-topoisomerase I antibody (ATA), anti-centromere antibody (ACA), and anti-RNA polymerase III antibody (ARA), are the hallmarks of immune dysregulation and are frequently detected in patients with systemic sclerosis. They are helpful for the early diagnosis of the disease and are established as strong predictors of disease outcome **(6-8)**. Among autoantibodies, ACA is most commonly associated with systemic sclerosis, is useful in classifying systemic sclerosis variants, and often remains stable during disease. Positivity for ACA has been associated with a higher survival rate **(9-11)**. Currently, there is no proven curative therapy for systemic sclerosis and it still poses a great challenge to clinicians **(12, 13)**.

Patients with systemic sclerosis are at a higher risk for dental caries than the general population due to reduced mouth opening, salivary hypofunction, xerostomia, impaired oral hygiene, immunosuppression, bone resorption, and the pronounced dysfunction of hand joints **(14-18)**. Chu *et al.* **(19)** showed that Chinese people with systemic sclerosis had caries experience (DMFT=10.5), as well as lower resting salivary flow and pH values. *Streptococcus mutans* (*S. mutans*) is a gram-positive facultative anaerobic pathogen, the natural habitat of which is the oral cavity of humans. It is an acidogenic bacterium with the ability to form biofilm on tooth surfaces, known as dental plaque. *S. mutans* can secrete water-insoluble glucans in a sucrose-containing environment that enhances bacterial adhesion to the tooth surface. Consequently, bacterial co-aggregation leads to the development of highly virulent mixed-species biofilms in the oral cavity. Scientific evidence revealed that *S. mutans* is a primary pathogen and central facilitator of dental caries and decay lesions in the early stages mainly due to the irreversible dissolution of enamel minerals **(20-22)**. On the other hand, probiotic bacteria have shown several health benefits to the host because of modifying the microbiome. Some probiotic strains can interfere with the biofilm formation of cariogenic bacteria, such as *S. mutans* **(23-25)**. *Lactobacilli* strains are the most common probiotics found in commercial dental products, which potently reduce the growth of *S. mutans* and inhibit the biofilm formation of this cariogenic bacterium. However, little is known about the effects of *Lactobacilli* species on dental caries **(26-28)**.

The salivary *S. mutans* and *Lactobacilli* enumeration may be considered a reflection of the oral health of an individual. Moreover, they are closely associated with the development of dental caries. Therefore, the current study was designed to assess the salivary levels of these bacteria to find whether naturally occurring *S. mutans* and *Lactobacilli* are related to systemic sclerosis.

2.Materials and Methods

Ethical Considerations

Prior to any experiment, written informed consent was obtained from all participants in accordance with the Declaration of Helsinki and its later amendment. The study protocol was approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (IR.SUMS.DENTAL.REC.1399.096).

Study Design

This cross-sectional study was performed on 80 patients with a confirmed diagnosis of systemic sclerosis who were routinely visited in the Rheumatology Clinic of Hafez Hospital affiliated with Shiraz University of Medical Sciences, Iran, during August 2020-September 2020. Systemic sclerosis was diagnosed by a rheumatologist according to the criteria of the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR). Based on the medical records, patients were assigned into two groups ACA-positive (N=42) and ACA-negative (N=38).

The inclusion criteria were adult age, good general health, and not having used any antibiotics, chlorhexidine, sodium fluoride, or probiotic mouthwashes for 4 weeks before the study. Patients with systemic disorders, diabetes, oral breathing, chronic periodontitis, gingivitis, other autoimmune diseases rather than systemic sclerosis, and any medical conditions that could interfere with the study were excluded. All patients were referred to the dental specialized clinic affiliated with Shiraz University of Medical Sciences, Iran. Clinical oral health status was assessed for each patient by a single dentist examiner. The DMFT index for decayed, missing, and filled teeth was determined using the standard methods suggested by World Health Organization. In addition, the control group of this study consisted of 80 healthy individuals without any systemic and/or autoimmune diseases who were matched with the patients group in terms of age, gender, and DMFT index.

Two hours prior to sampling, all participants were requested to perform a thorough tooth-brushing and refrain from food and drink consumption. Unstimulated whole saliva sample (5 mL) was self-collected by each participant. During salivation, the study subjects were requested to keep their eyes open, avoid movements and speaking, and mentally stimulate salivary flow as far as possible. Collected

saliva samples were sent immediately to the laboratory for microbiological analyses.

Microbiological Assessments

Before plating, 100 µL of saliva sample was diluted with 1.9 mL of sterile water. Next, the aliquots of the suspension (50 µL) were directly spread on blood agar (Merck, Germany) and tomato juice agar (Merck, Germany) plates to isolate salivary *S. mutans* or *Lactobacilli*, respectively. Afterwards, plates were sealed, placed in an anaerobic jar, and incubated at 37°C for 48 h. Morphological characteristics of colonies were assessed. Colonies on solid plates were characterized by gram staining, catalase, biochemical tests, and resistance to bacitracin and optochin. Colony counting for each specimen was performed by 100-folds dilution with saline and then, 10 µL of each sample was transferred to the colony plates. The plates were set in microaerophilic conditions and were incubated for 1 day at 37°C. Finally, the colonies were counted using the Quebec colony counter (Reichert, USA), multiplied by the dilution factor, and expressed as the number of colony-forming units per milliliter (CFU/mL) of saliva. All bacteriological analyses were commenced within 2 h of sampling and were performed by a single-blinded expert microbiologist in three replicates.

Statistical Analysis

Statistical analyses were carried out using the IBM SPSS Statistics for Windows version 22 (IBM, Armonk, NY, USA). The normality of data distribution was checked using the Shapiro-Wilk test. Data with non-normal distribution were presented as the median and interquartile range (IQR). The Mann-Whitney U test was used to examine the differences between the study groups. Furthermore, the correlation between salivary *S. mutans* and *Lactobacilli* was evaluated utilizing the Pearson correlation coefficient test. P-value<0.05 was considered statistically significant.

3.Results

The patients group consisted of 74 women and 6 men with an average age of 50.7±11.2 years. Out of the 80 subjects in the control group, 92.5% were female (N=74) and 7.5% were male (N=6) with a mean age of 52.2±9.1 years. There were no significant differences between the case and control groups in terms of gender (*P*=1) and age (*P*=0.465). Moreover, two subgroups of systemic sclerosis, namely ACA-positive (N=42) and ACA-negative (N=38) patients were not significantly different in gender (*P*=0.527) and age (*P*=0.356). There were no statistically significant differences (*P*>0.05) in the mean DMFT values of patients and control groups, as well as the ACA-positive and ACA-negative subjects.

S. mutans and *Lactobacilli* were detected in 100% of unstimulated saliva samples collected from the case and control groups, and there were no missing data. However, the Mann-Whitney U-test demonstrated statistically significant differences between the study groups. The median salivary concentration of *S. mutans* in patients with systemic sclerosis (1.6×10^7 CFU/mL; IQR: $1.1\text{-}3.1\times10^7$ CFU/mL) was significantly higher than in the control group (5.1×10^6 CFU/mL; IQR: $5.1\text{-}7.9\times10^6$ CFU/mL) (*P*<0.0001). However, *Lactobacilli* levels were similar between patients (3.4×10^6 CFU/mL; IQR: $1.6\text{-}11.6\times10^6$ CFU/mL) and control subjects (2.2×10^6 CFU/mL; IQR: $1.2\text{-}8.1\times10^6$ CFU/mL) (*P*=0.095).

As shown in Figure 1, the median salivary concentration of *S. mutans* in the ACA-positive (1.3×10^7 CFU/mL; IQR: $1.1\text{-}3.1\times10^7$ CFU/mL) and ACA-negative (2.4×10^7 CFU/mL; IQR: $1.1\text{-}3.1\times10^7$ CFU/mL) subjects was not significantly different (P=0.342). In addition, no significant difference was observed between the ACA-positive (4.1×10^6 CFU/mL; IQR: $1.5\text{-}13.1\times10^6$ CFU/mL) and ACA-negative (3.1×10^6 CFU/mL; IQR: $1.3\text{-}11.4\times10^6$ CFU/mL) groups in terms of *Lactobacilli* count (P=0.515). No significant correlation was detected between the salivary concentration of *S. mutans* and *Lactobacilli* in either patients (R=0.099, P=0.38) or control (R=0.186, P=0.251) groups. There were no significant differences between males and females regarding salivary *S. mutans* and *Lactobacilli* concentrations (*P*>0.05).

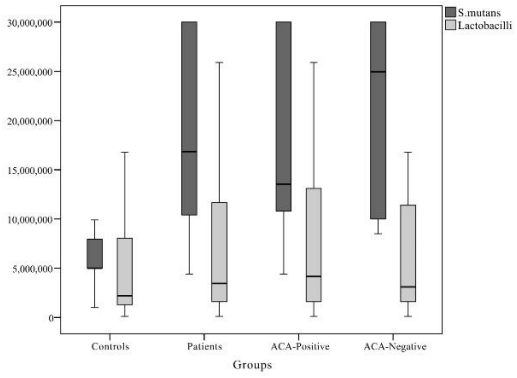

Figure 1. The levels of *S. mutans* and *Lactobacilli* in unstimulated saliva samples

4.Discussion

Orofacial manifestations, including dental caries, are common in systemic sclerosis **(5, 18, 19)**. Dental caries generally occurs in the presence of cariogenic bacteria and is a simple process in concept, but complicated in detail. The oral cavity contains a wide range of bacterial species, some of which have cariogenic properties **(29)**. Previous studies reported that the oral microbiome can change in autoimmune disorders. Therefore, the detection and quantitation of bacterial species in the oral cavity are necessary to understand the disease process **(30, 31)**. In this regard, the current study aimed to compare the total salivary levels of *S. mutans* and *Lactobacilli* between patients with systemic sclerosis and healthy individuals. Patients with systemic sclerosis were matched with the control group for DMFT index, gender, and age. Based on the findings of the present study, *S. mutans* and *Lactobacilli* were isolated from the unstimulated saliva samples of all participants. Higher levels of *S. mutans* and *Lactobacilli* were detected in the patients group.

Saliva is a non-invasive and easy-to-take sample, which is assumed to control the reproduction of microorganisms in the oral cavity. Stimulated saliva has been found to contain an estimated number of species over three times greater than unstimulated saliva **(32)**. Therefore, we used unstimulated saliva to measure *S. mutans* and *Lactobacilli* levels. Systemic sclerosis is generally associated with the impairment of saliva production. Disease severity and duration play a crucial role in saliva secretion and pH values. Furthermore, patients with systemic sclerosis have decreased ability for brushing teeth as a result of sclerotic changes in the fingers and hands **(5, 18, 19, 33-36)**. The immunosuppressive therapy commonly used in many autoimmune disorders, including systemic sclerosis, also creates a more permissive environment for oral pathogenic bacteria **(14-17)**.

The current preliminary research was not specifically designed to evaluate these relationships in systemic sclerosis. We investigated the occurrence of hyposalivation/xerostomia, alterations in the pH of saliva, sclerotic changes, and immunosuppression, which can contribute to the growth of cariogenic bacteria and might in part explain the greater *S. mutans* levels observed in patients. Although the salivary levels of *S. mutans* and *Lactobacilli*, as well as their possible roles in caries development, have been assessed in different studies **(33, 37-41)**, this issue has received no attention in systemic sclerosis.

Our data revealed that the salivary concentrations of *S. mutans* and *Lactobacilli* in ACA-positive and ACA-negative patients had no significant differences. The ACAs are most commonly associated with systemic sclerosis **(42)**. Moreover, they have also been reported in systemic lupus erythematosus, primary Raynaud's phenomenon, Rheumatoid arthritis, primary biliary cirrhosis, and Sjögren's syndrome. Baer *et al.* **(9)** found that ACA had an independent association with more severe exocrine glandular dysfunction and more pronounced labial salivary glandular inflammation. Consequently, dental practitioners should be aware of the potential systemic health problems related to tooth status in patients with systemic sclerosis and should periodically monitor such patients during the course of the disease.

The current study had a small sample size and potential unmeasured confounding. Ideally, patients in both genders with similar disease duration, activity, and severity would have been included for counting salivary *S. mutans* and *Lactobacilli* and investigating carries, but the enrollment of a sufficient number of such patients is difficult. On the other hand, the values obtained from a single saliva sample at one particular point of time could not well represent oral health status as dental caries develop over a considerable period, during which bacterial counts could fluctuate in response to the changing oral environment. Therefore, further comprehensive studies with a larger sample size that assess the dynamics of changes in salivary *S. mutans* and *Lactobacilli* during the progression of systemic sclerosis are warranted.

5. Conclusion

To the best of our knowledge, this paper is the first to assess the salivary levels of *S. mutans* and *Lactobacilli* in patients with systemic sclerosis. Our results support the hypothesis that total counts of salivary *S. mutans* in patients with systemic sclerosis is higher than in healthy individuals; however, the presence of anti-centromer antibodies could not have changed the amount of this cariogenic pathogen. Patients with systemic sclerosis should undergo regular dental check-ups to receive the proper preventive and restorative care and to detect potential issues before they worsen.

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Ethics Approval

Prior to any experiment, written informed consent was obtained from each participant, in accordance with the Declaration of Helsinki and its later amendment. The study was conducted according to the protocol approved by the local Ethics Committee of the Shiraz University of Medical Science, Shiraz, Iran (IR.SUMS.DENTAL.REC.1399.096).

Authors' Contribution

Study Design: Mardani M., Motamedifar M., Nazarinia M.A.

Collection of Data: Najafi S., Hadadi M., Motamedifar M., Nazarinia M.A.

Statistical Analysis: Najafi S., Hadadi M.

Draft of Manuscript: Mardani M.

Revisions: Motamedifar M., Nazarinia M.A.

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Conflict of Interest

The authors declare no conflict of interest.

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