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ORIGINAL ARTICLE

Comparison of Salivary Bacterial Flora in Iranian Patients with Hiatal Hernia and Healthy Subjects

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Abstract

Objective: To evaluate salivary microbial flora of patients with hiatal hernia and compare it with that of healthy subjects. **Material and Methods:** In this cross-sectional study, 50 patients with hiatal hernia measuring >1 cm and 50 healthy subjects (as the controls) were selected using simple random technique. One mL of salivary sample was taken from each patient, transferred into 50-mL Falcon tubes and immediately carried to the microbiology Laboratory of Tabriz Faculty of Medicine. The salivary samples were cultured on specific *Streptococcus viridans* (*S. mitis*, *S. mutans*, *S. salivarius* and *S. sanguis*), *Enterococcus* spp. and *Lactobacillus* culture media. Then the samples were incubated at 37°C for 7 days, followed by evaluation of the bacterial colonies. Statistical significance was defined at $p<0.05$. **Results:** A total of 34% of subjects with hiatal hernia and 26% healthy subjects exhibited *Lactobacillus gasseri* in their salivary samples; 16% of subjects with hernia and 6% of healthy subjects exhibited *Enterococci* spp. in their salivary samples. In addition, 82% of subjects with hernia and 72% of healthy subjects exhibited *S. mutans* in their salivary samples; 74% and 4% of subjects with hernia and 76% and 4% of healthy subjects exhibited gram-positive and gram-negative bacilli in their salivary samples, respectively. Furthermore, 98% of subjects with hernia and 86% of healthy subjects exhibited gram-positive cocci in their salivary samples, however without significant difference between the groups ($p>0.05$). **Conclusion:** No significant differences in the counts of *Lactobacillus* spp., *Enterococcus* spp., *Streptococcus viridans* and gram-positive and gram-negative bacterial species between healthy controls and subjects with hiatal hernia.

Keywords: Hernia, Hiatal; Gastrointestinal Microbiome, Saliva.

Introduction

The esophagus descends through the hiatus of the diaphragm and joins the stomach immediately below the diaphragm, at which the hiatus creates an anatomic constriction, preventing reflux of gastric contents into the esophagus. With loosening or widening of the hiatus, a part of the stomach protrudes into the thoracic cavity, giving rise to hiatal hernia. In recent years, the incidence of hiatal hernia has increased significantly and its prevalence has been reported to be 20-60% [1,2].

One of the problems with hiatal hernia is its close association with gastro-esophageal reflux disease (GERD) [1,3,4]; in this context, reflux symptoms are more common in subjects with hiatal hernia compared to healthy subjects [3] and the majority of subjects with pathologic GERD have hiatal hernia [5]. Regurgitation of gastric contents into the esophagus is a normal physiologic event, referred to as GERD if it is associated with symptoms and signs and the related problems [6]. Microaspiration of reflux contents into the airways might result in respiratory problems such as laryngitis, chronic coughing, hoarseness of the voice and asthma. Acid injuries of the oropharynx lead to pain in the throat, otalgia, gingivitis, tooth sensitivity to cold and heat, tooth erosion and pulpitis [6-8].

Several studies have reported that the most common oral manifestation of GERD is tooth erosion [9-11]. In addition, these studies have well shown that salivary factors such as the salivary flow rate, pH, buffering capacity, pellicle protective capacity and individual susceptibility affect demineralization of tooth hard structures [9]. However, there are ambiguities and controversies in relation to the mechanism of the direct effect of gastric acid on the oral cavity and its relationship with the development of caries, salivary flow and buffering capacity and colonization of microorganisms. Some studies have shown higher levels of dental caries in patients with GERD [10,11]; however, some have reported no significant differences in dental caries rate between healthy individuals and patients with GERD [12]. In addition, there are some reports indicating a lower rate of dental caries in patients with GERD compared to healthy subjects [13].

Furthermore, there are conflicting reports about bacterial colonization in the oral cavity. One study showed lower colonization of *Lactobacillus* spp. and *Streptococcus* spp. in patients with GERD compared to the control group [13]. However, in another study, colonization of *Streptococci* spp. in children with GERD was higher than that in the control group [11]. The oral microbiota, which is a complex ecosystem of oral cavity microbes and consists of several thousand bacterial species [14], has a great role in oral diseases, including dental caries and periodontics, and some systemic conditions, including bacterial endocarditis, aspiration pneumonia, premature birth, immature infants and cardiovascular diseases [15].

Controlling pathogens in oral biofilms might prevent microorganisms from reaching the cardiovascular and pulmonary tissues and exacerbating an existing condition [16]. In addition, the presence of the resident microflora prevents disease by decreasing the odds of colonization by exogenous species; in this context, it might be useful to make attempts to preserve and control these organisms [17]. Therefore, understanding changes occurring in the oral cavity over time or due to

disease or other host-related disorders might help researchers discover new interventions and better treatment modalities for diseases and their complications [18]. Given what was discussed above and since only a limited number of studies are available on changes in the resident bacterial flora in the saliva of patients with hiatal hernia, the present study was undertaken to evaluate the salivary microbial flora of patients with hiatal hernia and compare it with that in healthy subjects.

Material and Methods

Study Design and Sampling

The present descriptive/cross-sectional study was carried out from March 2014 to March 2015 in Imam Reza Educational/Treatment Center in Tabriz, Iran.

Fifty patients who had undergone endoscopic examinations for different reasons by an alimentary tract specialist and had been diagnosed with hiatal hernia measuring >1cm [19] were included in the study; in addition, 50 subjects who had undergone endoscopy for different medical reasons and did not have hiatal hernia were included in the study as a control group. The sample size was estimated at 48 subjects in each group with the use of ratio estimation by considering α=0.05, a study power of 80% and a difference of 15% in the frequency distribution of bacterial flora; however, 50 subjects were included in each group.

Subjects with hiatal hernia were selected by convenient sampling technique, and the control subjects were selected randomly using simple random sampling technique using Research Randomizer website (https://www.randomizer.org).

Inclusion criteria consisted of an age range of 20-75 years, no use of any medication for the treatment of hiatal hernia and esophageal reflux (H2 blockers, protein pump inhibitors, histamine receptor antagonists such as famotidine, ranitidine, etc.), antifungal agents, antibiotics, medications that change the pH and microbial flora of the alimentary tract during the previous month, no smoking during the previous 2-hour period, absence of any dental treatment during the previous 24-hour period, no tooth brushing during the 45-minute period before sampling, and the subjects interest in taking part in the research project [19].

Exclusion criteria consisted of any advanced systemic condition such as diabetes, hormonal disturbances, thyroid disorders, tuberculosis, inflammatory bowel disease and cancer, any severe oral disease such as generalized periodontitis, rampart caries, fistula with active drainage of pus, any advanced mucosal disease such as rubor and inflammation, etc, use of antibiotics in the recent month or use of a mouthwash in recent 3 hours, and any other alimentary tract conditions such as esophagitis [15,19,20].

Data Collection

After endoscopy procedures, the subjects were placed in the lists of healthy or afflicted subjects in term of the presence or absence of hernia; in addition, some subjects were excluded due to the presence of other systemic conditions. One mL of salivary sample was collected from each subject

and transferred to a sterile 50-mL Falcon tube [21]. The samples were immediately transferred to the Microbiology Laboratory of Faculty of Medicine, Tabriz University of Medical Sciences. Then, 0.05 mL of each salivary sample was transferred onto the culture media of *Streptococcus viridians* bacterial species (*S. mitis*, *S. mutans*, *S. salivarius*, *S. sanguis*), *Enterococcus* spp. and *Lactobacillus* spp. with the use of a sterile swab. The samples were incubated at 37°C for 7 days, followed by evaluation of the bacterial colonies [15,22].

To evaluate *S. mutans* colonies, which is a facultative anaerobic gram-positive coccus, *S. mitis-salivarius* culture medium was used at 37°C in association with 10% CO_2. Normally, incubation in an environment with 10% CO_2 results in acceleration of hemolysis. Differentiation of isolated *Streptococci* was carried out based on biochemical characteristics in microbiology protocols [23,24]. Sucrose agar was used to evaluate proliferation of *S. sanguis*. Identification of other *viridians streptococci* was carried out based on biochemical tests and hemolysis characteristics on blood agar. RSL (Rogosa selective lactobacilli agar) with 10% CO_2 concentration was used to culture *Lactobacilli*. Oral Lactobacilli such as *Lactobacillus fermentum* and *L. gasseri* grow on Rogosa agar culture medium but *L. casei* was identified through observation and plates prepared directly from salivary samples. Molecular analyses of lactobacilli species were done by method previously described [25,26]. To culture *Enterococci* spp., blood agar containing sheep blood was used at 37°C. All the culture media were incubated for 2 days at 37°C for identification procedures [19,25].

Data Analysis

Data were analyzed with descriptive statistics (frequencies and percentage) and Chi-squared test, using SPSS Statistics for Windows Software, version 17 (IBM Corp., Armonk, NY, USA). Statistical significance was defined at $p<0.05$.

Ethical Aspects

All the ethical aspects of the present study were evaluated and confirmed by the Ethics Committee of Tabriz University of Medical Sciences. At the beginning of the study, an informed consent form was explained to the subjects by the executive manager of the study to solve the problem of subjects who were unable to read or write.

Results

In the present study, 100 patients with a mean age of 37.4±11.6 years were evaluated; 50 subjects had hiatal hernia, with a mean age of 41.6±13.05 years. Comparison of colonization of *Lactobacilli* in the study groups showed a higher rate of *Lactobacillus gasseri* colonization after culturing compared to *L. fermentum* and *L. casei* group.

L. fermentum, *L. gasseri* and *L. casei* group were identified in 14%, 34% and 12% of the subjects with hiatal hernia and in 12%, 26% and 20% of the healthy subjects, respectively. In addition, 22% of subjects with hiatal hernia and 26% of healthy subjects exhibited both *L. fermentum*

and *L. gasseri* in their salivary samples. Furthermore, 16% and 16% of subjects in both groups did not exhibit any *Lactobacilli* in their salivary samples, with no significant differences between the two groups (p>0.05) (Table1).

Table 1. Distribution (absolute and relative) of *Lactobacilli* in the healthy and afflicted groups.

Bacterial Species	Total		Afflicted		Healthy		
	N	%	N	%	N	%	p-value
Lactobacillus fermentum	13	13.0	7	14.0	6	12.0	0.67
Lactobacillus gasseri	30	30.0	17	34.0	13	26.0	0.21
Lactobacillus casei	16	16.0	6	12.0	10	20.0	0.12
L.Gasseri +*L. fermentum*	24	24.0	11	22.0	13	26.0	0.50
L.casei + *L. fermentum*	1	1.0	1	2.0	0	0.0	-
without *Lactobacillus* spp.	16	16.0	8	16.0	8	16.0	-

Comparison of colonization of *Enterococci* spp. in the study groups showed that 16% of subjects with hiatal hernia and 6% of healthy subjects exhibited *Enterococci* spp. in their salivary samples, with no significant difference between the two groups (p=0.11).

Comparison of colonization of *Streptococci* in the study groups showed higher colonization of *S. mutans* compared to other *Streptococci. S. mutans, S. sanguis, S. mitis, S. salivarius* and *S. milleri* were indentified in 82%, 58%, 24%, 2% and 2% of salivary samples in subjects with hiatal hernia and 72%, 54%, 26%, 6% and 2% of salivary samples of healthy subjects, respectively; however, the differences were not significant (p>0.05) (Table 2).

Table 2. Distribution (absolute and relative) of Streptococci in the healthy and afflicted groups.

	Total		Afflicted		Healthy		
Bacterial Species	N	%	N	%	N	%	p-value
S. mutans	77	77.0	41	82.0	36	72.0	0.23
S. sanguis	56	56.0	29	58.0	27	54.0	0.68
S. mitis	25	25.0	12	24.0	13	26.0	0.81
S. salivarius	4	4.0	1	2.0	3	6.0	0.14
S. milleri	2	2.0	1	2.0	1	2.0	1

Evaluation of the frequency of gram staining and morphologies showed that 74% and 4% of subjects with hiatal hernia and 76% and 4% of healthy subjects exhibited gram-positive and gram-negative bacilli in their salivary samples; 14% of subjects with hiatal hernia and 12% of healthy subjects did not exhibit any bacilli in their salivary samples. The results of chi-squared test did not reveal any significant differences between the two groups (p=0.99) (Table 3).

Based on the results, 98% of subjects with hiatal hernia and 86% of healthy subjects exhibited gram-positive cocci in their salivary samples; 2% of subjects with hiatal hernia and 4% of healthy subjects had both gram-positive and gram-negative cocci in their salivary samples. All the subjects with hiatal hernia exhibited cocci in their salivary samples; however, 4% of healthy subjects had no cocci in their salivary samples, with no significant difference between the two groups (p=0.19).

Table 3. Distribution (absolute and relative) of gram-positive and gram-negative bacilli and cocci in the groups.

Gram Reaction	*Bacilli*			*Cocci*		
	Total	Afflicted	Healthy	Total	Afflicted	Healthy
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Gram Positive	75 (75.0)	37 (74.0)	38 (76.0)	92 (92.0)	49 (98.0)	43 (86.0)
Gram Negative	4 (4.0)	2 (4.0)	2 (4.0)	-	-	-
Gram Positive and Gram Negative	8 (8.0)	4 (8.0)	4 (8.0)	3 (3.0)	1 (2.0)	2 (4.0)
No	13 (13.0)	7 (14.0)	6 (12.0)	2 (2.0)	0 (0.0)	2 (4.0)

Discussion

The oral microbial flora is involved in oral diseases such as dental caries and periodontitis and in systemic conditions such as bacterial endocarditis and cardiovascular diseases [14-16]. On the other hand, the presence of resident microflora results in prevention of some diseases by decreasing the odds of colonization of exogenous species; therefore, preservation and control of oral microflora is useful [27]. As a result, understanding changes in the oral environment over time or due to diseases or other host-related disorders might help researchers discover new interventions for the diagnosis and better treatment of diseases and their complications [18].

The present study was undertaken to compare the microbial flora in the salivary samples of patients with hiatal hernia and healthy subjects. The results showed that the colonization of *S. mutans* (77%) and *L. gasseri* (30%) was higher in the both group compared to other *Lactobacilli* and *Streptococci.* Some authors, in a previous study using Pyro sequencing Analysis, evaluated the salivary microflora and reported that Firmicutes (genus Streptococcus and Veillonella) and *Bacteroidetes* spp. (genus Prevotella) were the predominant phyla in saliva [28]. It was reported that the most microbial composition in brush and biopsy samples from cheek were *Streptococcus viridans* (98%) and fusiform rods such as *Fusobacterium nucleatum*, followed by *Neisseria* spp., *Haemophilus* spp., and *Prevotella* species [19,29].

In the present study there were no significant differences in the presence of *Lactobacilli*, *viridans streptococci* and *Enterococci* between the salivary samples of patients and healthy subjects. In addition, there were no significant differences in gram-positive and gram-negative bacteria and their morphologies (bacilli and cocci) between the two groups.

A search in databases and academic sources such as Pubmed, Google Scholar and Medline brought up no similar study and only a limited number of studies are available on comparison of the oral flora of patients with GERD or Crohn's disease and healthy subjects. Consistent with the results of the present study, it was previously demonstrated that although the counts of *S. mutans* in the salivary samples of children with GERD was significantly higher than that in the control group, the difference was not significant [10]. In contrast, in a previous study *S. mutans* counts in subjects with GERD were significantly higher than those in the control group. It was concluded that the acidic environment that is created due to GERD possibly results in the induction of proliferation of acidophilic *S. mutans* [11]. Another study showed that the counts of *Lactobacilli* and *Streptococci* in the saliva of subjects with GERD were significantly lower than those in the control group [14]. The

counts of *Streptococci* spp. and *Lactobacilli* spp. in patients with active GERD were lower than those in the control group, which was attributed to the very low pH of saliva in subjects with active GERD [10]. The oral microbiome is exclusively altered in children's with Crohn's disease [20].

As discussed above, apart from the availability of a limited number of studies on the subject, the results of studies are very divergent, which might be attributed to differences in study designs, the age of the participants, differences in the genetic make-up of the subjects, differences in the duration of affliction with the disease, among other factors. On the other hand, it has been reported that salivary factors such as its flow rate, pH and buffering capacity are some of the effective factors in such patients. Therefore, it is suggested that another study be designed by considering salivary factors and their effect on the colonization of different bacterial species.

Although in the present study, patients with severe oral disease such as generalized periodontitis, rampart caries, fistula with active drainage of pus, any advanced mucosal disease such as rubor and inflammation were excluded from the study, but it is suggested that in subsequent studies, patients should be closely matched for oral health, DMFT, and periodontal status. Also, it is suggested that in future studies, in patients with hiatal hernia a distinction be made between subjects with reflux and those without it with the use of accurate techniques.

In addition, the differences might become statistically significant with larger sample sizes. Use of newer techniques for isolation and identification of bacteria (such as next generation sequencing-NGS) might yield more accurate results and should be considered in future studies. In addition, due to limitations in time and budget, in the present study only a small number of salivary bacteria were evaluated. Therefore, it is suggested that other bacterial species be evaluated and compared in patients with hiatal hernia and healthy subjects.

Conclusion

No significant differences were observed in the counts of *Lactobacilli*, *Enterococcus* spp., *S. viridians* and gram-positive and gram-negative bacteria in the salivary samples of patients with hiatal hernia and the healthy controls.

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