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| ORIGINAL

Microbiological analysis of tongue dorsum coating in patients hospitalized in ICU

Análise microbiológica da saburra em dorso lingual de pacientes internados em UTI

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ABSTRACT

Objective: Assess quantitatively and qualitatively tongue coating microbiota in ICU patients. **Methods**: Analytical observational study, convenience sample comprising 65 patients was included for medical report analysis and collection of general data, tongue coating assessment through visual inspection and microbiological sample collection for further laboratory analysis. The collection was performed by a single examiner using a sterile swab introduced and rubbing the posterior portion of the tongue close to the oropharynx. **Results**: Most patients (60%) belonged to the female sex, at mean age of 74.2 years. The main reasons for hospitalization were lung issues (26.2%) - prevailing associated comorbidities were diabetes (43.1%) and high blood pressure (66.2%). The mean length of stay in the ICU was one day. All patients presented tongue dorsum coating. There were Candida albicans (37%), Streptococcus parasanguinis (26.1%) and Streptococcus mitis (32.6%) in 1/3 of lingual extension. Streptococcus mitis (p=0,0265) was the most prevalent species. **Conclusion**: There was no significance between the amount of coating and number of observed species, although all assessed patients had presented coating. The most prevalent microorganisms were Candida albicans, Streptococcus parasanguinis and Streptococcus mitis.

Indexing terms: Biofilms. Hospital assistance. Intensive care unit. Microbiology. Oral health.

RESUMO

***Objetivo**: Avaliar quantitativa e qualitativamente a microbiota da saburra lingual em pacientes internados em UTI. **Métodos**: Estudo observacional analítico, amostra de conveniência composta por 65 pacientes para análise de laudo médico e coleta de dados gerais, avaliação da saburra lingual por inspeção visual e coleta de amostra microbiológica para posterior análise laboratorial. A coleta foi realizada por um único examinador por meio de swab estéril introduzida e fricção na porção posterior de língua próxima à orofaringe. **Resultados**: A maioria dos pacientes (60%) pertencia ao sexo feminino, com média de idade de 74,2 anos. Os principais motivos de*

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internação foram problemas pulmonares (26,2%) - as comorbidades associadas predominantes foram diabetes (43,1%) e hipertensão arterial (66,2%). O tempo de internação médio na UTI foi de um dia. Todos os pacientes apresentavam saburra do dorso da língua. Havia Candida albicans (37%), Streptococcus parasanguinis (26,1%) e Streptococcus mitis (32,6%) em 1/3 da extensão lingual. Streptococcus mitis (p=0,0265) foi a espécie mais prevalente. ***Conclusões****: Não houve significância entre a quantidade de recobrimento e o número de espécies observadas, embora todos os pacientes avaliados tenham apresentado recobrimento. Os microrganismos mais prevalentes foram Candida albicans, Streptococcus parasanguinis e Streptococcus mitis.*

Termos de indexação: *Biofilmes. Assistência hospitalar. Unidades de terapia intensiva. Microbiologia. Saúde bucal.*

INTRODUCTION

Lack of oral care remains a great challenge, as well as its implementation in the assistance routine available for patients in intensive care units (ICU), mainly when it comes to actions focusing on biofilm cleaning and disorganization [1,2].

Oral biofilm formation - with emphasis on teeth, tongue dorsum (coating) and artificial respirator tube (ventilator) - associated with mechanical ventilation in critical patients is closely related to lack of care, cleaning frequency and time in hospital [3,4]. Tongue coating is considered a microbial reservoir of gram-positive and gram-negative bacteria, fungi and viruses. Thus, the efficient investigation of this biofilm allows greater microbial knowledge, a fact that favors the proper use of medication, and helps preventing opportunistic diseases and hospital infections [5-8].

It is important emphasizing that oral microbial changes take place from 48 to 72 hours after the patient is referred to ICU, and it favors the emergence of diseases such as nosocomial pneumonia (acquired after hospitalization), pneumonia associated with mechanical ventilation (PAV) and opportunistic diseases, mainly the ones of fungal origin [9,10]. Oral medium promotion and adjustment, as well as a necessary care in hospital daily routines, are essential actions to prevent systemic diseases [11,12].

Accurate systemic investigations carried out by a multidisciplinary team lead to correct diagnostics and treatment plan. Tongue coating microbiological analysis can be an important strategy, but it is not performed as routine in ICU, be it due to lack of knowledge by hospital teams or to the cost of performing such an investigation for each hospitalized patient, since its hospitalization [4,13,14].

The aim of the current study was to investigate the oral microbiota in tongue dorsum coating of ICU patients and verify whether there is an association between microorganisms (type and number) and the amount of tongue coating.

METHODS

Ethical aspects

Research project was approved by the Ethics Committee in Research of Dentistry Faculty São Leopoldo Mandic (Dental Research Center), Opinion n. 2.544.250 (CAAE 78245917.0.3006.5374).

All awaken patients (without any sedation type) and the legal guardians of patients under sedation were informed about the study and signed the Informed Consent Form (ICF) when they agreed on participating in the study.

Collected general data and microbiological analysis results were ethically preserved in order not to cause any sort of embarrassment. Individual information of each patient was provided to the medical team in charge of the study, in case it was necessary.

Experimental design

Observational analytical study about the microbiota in tongue dorsum coating of ICU patients in a reference hospital on care provided to systematically compromised and high systemic complexity patients in Brasília City (DF – Brazil).

Sample features

The study included a convenience sample of 65 ICU patients undergoing high-complexity treatment. The coating of the dorsum of the tongue was collected by the calibrated researcher.

Patients diagnosed with COVID were also evaluated.

It is important to emphasize that in the evaluated ICU there is no routine and effective oral hygiene measures.

Because the study was carried out in the ICU, the convenience sample was adopted because the patients have several associated medical complexities and specific hospitalization conditions, not favoring the organization of standardized patient groups.

Inclusion and exclusion criteria

Inclusion criteria were ICU patients up to 24-96 hours after admission; awaken patients (without any sort of sedation) who agreed in participating in the study and signed the Informed Consent Form (ICF); legal guardians of sedated ICU patients, or who needed ventilatory support (tracheostomy and mechanical ventilation); and who were instructed about the coating collection, agreed on participating and signed the ICF for such a purpose.

Exclusion criteria were patients who presented severe systemic condition, which did not make the clinical evaluation possible. And patients submitted to oral hygiene before evaluation and microbiological collection.

Clinical procedures

Lingual coating collection was carried out with the aid of a single previously-calibrated examiner in order not to impair the necessary-care routine and ICU's logistics, from July 2020 to March 2021. The calibration of the tongue coating sample was previously performed with 15 patients admitted to the ICU - patients who were not part of the study sample. Patients' general data were collected from their medical reports and through clinical evaluations. The following data were collected: sex, hospitalization reason, presence of associated comorbidities; visual inspection over the presence and extension (amount) of tongue dorsum coating [5,6]: 0 - absence/subclinical; 1 - 1/3 of lingual extension; 2 - 2/3 of lingual extension; 3 - whole lingual extension; age and hospitalization time (1 day = 24 hours).

Methods that respect biosafety, and infection control methods of the hospital, were adopted for coating collection. Sterile swab (medium Stuart – 4ml, Absorve) – provided by the specialized hospital – was introduced in the posterior portion of the tongue (vallate papilla region) close to the oropharynx through friction and further stored in test tube filled with reagent solution – performed by a single examiner. Each collection was stored in protection container (Styrofoam box), which was taken to specialized laboratory within 1 hour after preparation, at most.

The method of collection, storage and sending for microbiological analysis were done correctly, contributing to the non-loss of the sample.

Laboratory procedure

The microbiological analysis applied to tongue coating followed the specific cultivation method by mass spectrometry (MALDI-TOF) and automated antibiogram [Vitek-2® (bioMérieux, Hazelwood, MO)].

In the specific mass spectrometry cultivation method, microorganisms are placed on a plate that contains a polymer matrix. The plate is irradiated with a laser that vaporizes the sample, ionizing the molecules that will be aspirated and elevated to a detector. Depending on the molecule, the time of arrival will be different (time of flight). The data obtained through graphs representing these readings will be compared to an algorithmic database that contains a

large number of species of clinical relevance – including aerobic microorganisms, anaerobes, mycobacteria, yeasts and filamentous fungi. The procedure is very quick and results are obtained in minutes.

The application of mass spectrometry using the MALDI-TOF technique to clinical microbiology is undeniable. It is a simple, fast and highly reliable tool that replaces conventional phenotypic methods for bacterial and fungal identification in the clinical laboratory routine, minimizing the time to perform fundamental diagnoses and optimizing antimicrobial therapy.

The automated antibiogram is a system that guarantees excellence in routine microbial identification and antimicrobial susceptibility testing (ATS) in microorganisms isolated from clinical samples.

Through this examination, it is possible to observe which antibiotics the bacteria found in the analyzed material are sensitive or resistant to, that is: the antibiogram will allow the identification of the most appropriate antibiotic for the treatment of the infection presented by the patient.

This analysis was performed in a specialized private laboratory with the support of funding from the development study - a condition of greater logistical ease and support for the results in less time.

Statistical analysis

All data were collected from both the medical reports of patients and of tongue coating microbiological analysis. They were then organized in spreadsheet (Excel software) for further statistical analysis.

Data were analyzed through Chi-square non-parametric and Fisher's exact tests at 5% significance level. The dependent variable was the amount of tongue coating and the independent ones were sex, chronic disease and type of microorganism found in the coating.

Descriptive analysis of all data were carried out. Subsequently, the prevalence of each bacterial species in the total sample and in the group presenting the least and largest amount of coating was calculated. Chi-square test and Fisher's exact tests were used to analyze the association between the presence of species and the amount of tongue coating. The amount of coating was also compared between groups with and without each bacterial species; it was done through Mann Whitney test. Kruskal Wallis test was applied to compare the amount of coating based on the number of observed bacterial species. All analyses were carried out in R statistical software, at 5% significance level. R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

RESULTS

The sample comprised 65 patients at mean age 74.2 years (43 years old, minimum and 97 years old, maximum), and 60% of it was formed by individuals of the female sex (table 1). Mean hospitalization time was one day, and it could vary from less than one day, up to four days. The main reasons for hospitalization were associated with lung issues. All patients had tongue dorsum coating, and it prevailed in 1/3 of the lingual extension (70.8%), in 2/3 of the lingual extension (24.6%) and in the whole lingual extension (4.6%).

There was no significant association between the amount of coating and the number of observed bacterial species ($p>0.05$) (table 2). The prevalence of patients with at least one species reached 83.1%. The prevalence of patients with two species, or more, recorded 44.6%. The prevalence of three, or four species, was 6.2%.

Table 3 shows the prevalence of bacterial species in the total sample, based on the amount of coating. In total, 15 different bacterial species were found in the coating (figure 3), and the most common ones were Candida albicans (38.5% of patients), *Streptococcus parasanguinis* (26.2% of patients) and *Streptococcus mitis* (23.1% of patients). The following species were also found: *Streptococcus vestibularis*, *Staphylococcus aureus*, *Kleibsella pneumoniae*, *Candida tropicalis* and *Streptococcus Salivarius* in more than 5% of patients. *Species Streptococcus* mitis was only found in patients recording the lowest amount of coating (1/3 of the lingual extension); it was observed in 32.6% of them ($p<0.05$).

Table 1. Descriptive analysis of features recorded for participants in the sample (n=65).

Variable	Category	Frequency (%)
Sex	Male	26 (40.0%)
	Female	39 (60.0%)
Reason for hospitalization	Heart Disease	10 (15.4%)
	Lung Disease	17 (26.2%)
	Cerebrovascular Diseases	8 (12.3%)
	Traumas	7 (10.8%)
	General profile postoperative	17 (26.2%)
	clinical follow-up	6 (9.2%)
Comorbidities	Diabetes	28 (43.1%)
	High blood pressure	43 (66.2%)
	Chronic kidney failure	10 (15.4%)
	Infarction and Cardiac Issues	23 (35.4%)
	Pneumonia and breathing issues	15 (23.1%)
	Liver complications	4 (6.2%)
Tongue dorsum coating	Lack of/subclinical	0 (0.0%)
	1/3 of the lingual extension	46 (70.8%)
	2/3 of the lingual extension	16 (24.6%)
	The whole lingual extension	3 (4.6%)
	Mean (Standard deviation)	Median (Minimum and maximum value)
Age (years)	74.2 (12.4)	76.0 (43.0-97.0)
Hospitalization time (days)	1.0 (0.9)	1.0 (0.0-4.0)

Table 2. Prevalence of the number of bacterial species in the microbiological analysis of participant's tongue coating (n=65).

Number of species	Amount of coating		
	Total sample	1/3 of the lingual extension	At least 2/3 of lingual extension
	Frequency (%)		
0	11 (16.9%)	7 (15.2%)	4 (21.0%)
1	25 (38.5%)	19 (41.3%)	6 (31.6%)
2	25 (38.5%)	16 (34.8%)	9 (47.4%
3	3 (4.6%)	3 (6.5%)	0 (0.0%
4	1 (1.5%)	1 (2.2%)	0 (0.0%

p=0.7483 (Fisher's exact test).

There was no significant difference in the amount of coating due to the number of observed species (p>0.05) (table 4).

The amount of coating was significantly lower in patients with Streptococcus mitis than in the ones lacking this bacterium (p<005) (table 5). It is important reinforcing the fact that this species was more common in patients recording the lowest amount of coating (p<0.05).

DISCUSSION

Hospitalization is a huge issue for critical hospitalized patients who suffer with some sort of systemic disorganization or with changes in control of existing comorbidities. It is essential pointing out that most patients in intensive care units are elderlies with some type of systemic complexity who need full support in order to help treatment and recovery [15,16], as shown in the current study.

Table 3. Prevalence of bacterial species detected in the microbiological analysis of patients' tongue coating (n=65).

Species	Total Sample	Amount of coating		p-value
		1/3 of lingual extension	At least 2/3 of lingual extension	
		Frequency (%)		
Candida albicans	25 (38.5%)	17 (37.0%)	8 (42.1%)	[2]0.6980
Streptococcus parasanguinis	17 (26.2%)	12 (26.1%)	5 (26.3%)	[1]1.0000
Streptococcus mitis	15 (23.1%)	15 (32.6%)	0 (0.0%)	[1]0.0031
Streptococcus vestibularis	6 (9.2%)	1 (6.5%)	3 (15.8%)	[1]0.3469
Staphylococcus aureus	5 (7.7%)	2 (4.4%)	3 (15.8%)	[1]0.1444
Kleibsella pneumoniae	4 (6.2%)	4 (8.7%)	0 (0.0%)	[1]0.3126
Candida tropicalis	4 (6.2%)	2 (4.4%)	2 (10.5%)	[1]0.5740
Streptococcus Salivarius	4 (6.2%)	3 (6.5%)	1 (5.3%)	[1]1.0000
Enterobacter cloacae	2 (3.1%)	1 (2.2%)	1 (5.3%)	[1]0.5024
Pseudomonas aeruginosa	1 (1.5%)	1 (2.2%)	0 (0.0%)	[1]1.0000
Streptococcus sp.	1 (1.5%)	1 (2.2%)	0 (0.0%)	[1]1.0000
Serratia marcescens	1 (1.5%)	1 (2.2%)	0 (0.0%)	[1]1.0000
Eschericia coli	1 (1.5%)	0 (0.0%)	1 (5.3%)	[1]0.2923
Candida glabrata	1 (1.5%)	1 (2.2%)	0 (0.0%)	[1]1.0000
Streptococcus gordonii	1 (1.5%)	1 (2.2%)	0 (0.0%)	[1]1.0000
Stenotrophomas maltophilia	0 (0.0%)	0 (0.0%)	0 (0.0%)	-
Acinetobacter calticusbau	0 (0.0%)	0 (0.0%)	0 (0.0%)	-
Staphylococcus sp.	0 (0.0%)	0 (0.0%)	0 (0.0%)	-
Actinobacter calcoaceticus bio anitratus	0 (0.0%)	0 (0.0%)	0 (0.0%)	-
Corynebacterium sp.	0 (0.0%)	0 (0.0%)	0 (0.0%)	-
Streptococcus pneumoniae	0 (0.0%)	0 (0.0%)	0 (0.0%)	-
Streptococcus cristatus	0 (0.0%)	0 (0.0%)	0 (0.0%)	-

[1] Fisher's exact test; [2]Chi-square test.

Table 4. Amount of tongue coating due to the number of bacterial species (n=65).

Number of species	Amount of coating on tongue dorsum
	Median (minimum and maximum)
0	1.0 (1.0-3.0)
1	1.0 (1.0-2.0)
2 or more	1.0 (1.0-3.0)

p=0.6418 (Kruskal Wallis). Amount of coating: 0 – lack of/subclinical; 1 – 1/3 of lingual extension; 2 - 2/3 of lingual extension; 3 – whole lingual extension.

Table 5. Amount of tongue coating due to the presence of bacteria (n=65).

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Species	Presence of the species in the coating		[1]p-value
	Absence	Presence	
	Median (Minimum and maximum)		
Candida albicans	1.0 (1.0-3.0)	1.0 (1.0-3.0)	0,7874
Streptococcus parasanguinis	1.0 (1.0-3.0)	1.0 (1.0-3.0)	0,9643
Streptococcus mitis	1.0 (1.0-3.0)	1.0 (1.0-1.0)	0,0265
Streptococcus vestibularis	1.0 (1.0-3.0)	1.0 (1.0-2.0)	0,4146
Staphylococcus aureus	1.0 (1.0-3.0)	2.0 (1.0-3.0)	0,1757
Kleibsella pneumoniae	1.0 (1.0-3.0)	1.0 (1.0-1.0)	0.2996

Table 5. Amount of tongue coating due to the presence of bacteria (n=65).

Species	Presence of the species in the coating		[1]p-value
	Absence	Presence	
	Median (Minimum and maximum)		
Candida tropicalis	1.0 (1.0-3.0)	1.5 (1.0-3.0)	0,605
Streptococcus Salivarius	1.0 (1.0-3.0)	1.0 (1.0-2.0)	0,8485
Enterobacter cloacae	1.0 (1.0-3.0)	1.5 (1.0-2.0)	-
Pseudomonas aeruginosa	1.0 (1.0-3.0)	1.0 (1.0-1.0)	-
Streptococcus sp.	1.0 (1.0-3.0)	1.0 (1.0-1.0)	-
Serratia marcescens	1.0 (1.0-3.0)	1.0 (1.0-1.0)	-
Eschericia coli	1.0 (1.0-3.0)	2.0 (2.0-2.0)	-
Candida glabrata	1.0 (1.0-3.0)	1.0 (1.0-1.0)	-
Streptococcus gordonii	1.0 (1.0-3.0)	1.0 (1.0-1.0)	-
Stenotrophomas maltophilia	1.0 (1.0-3.0)	-	-
Acinetobacter calticusbau	1.0 (1.0-3.0)	-	-
Staphylococcus sp.	1.0 (1.0-3.0)	-	-
Actinobacter calcoaceticus bio anitratus	1.0 (1.0-3.0)	-	-
Corynebacterium sp.	1.0 (1.0-3.0)	-	-
Streptococcus pneumoniae	1.0 (1.0-3.0)	-	-
Streptococcus cristatus	1.0 (1.0-3.0)	-	-

[1]Mann Whitney. Median (minimum and maximum). Amount of coating: 0 – lack of/subclinical; 1 – 1/3 of lingual extension; 2 - 2/3 of lingual extension; 3 – Whole lingual extension.

The difficulty in having a standardized oral hygiene routine in ICU, with emphasis on tongue dorsum associated with hospitalization time, favors coating accumulation, which is seen as a complex bacterial niche closely associated with hospital infections [5,6,9,11,13].

After 24 to 72-hour hospitalization in ICU, one finds oral microbiological change due to the prevalence of gram-negative bacteria and to bacteria associated with systemic conditions featured by respiratory-profile infections. Therefore, it is necessary intensifying oral healthcare right at patient's admission or, mainly, when they are referred to ICU [17-20].

The presence of associated comorbidities is another relevant factor for critical and elderly patients, since any sort of disorganization can lead to new systemic losses and impair their recovery process [16-19].

As for the current study, the total sample comprised elderly, it emphasized the main systemic issues associated with diabetes and high blood pressure, which are the most prevalent conditions in the age group and they need interdisciplinary and personal assistance, mainly in ICU [6,9,18].

Since the study was carried out within the most critical period of the pandemic (COVID-19), the access to intensive therapy units and to critical patients was limited and biosafety strategies were strict [21]. However, it is very difficult measuring the oral hygiene of infected patients, since lack of proper cleaning was evidenced by coating accumulation on tongue dorsum (2 patients).

The proper microbiological investigation of the oral biofilm as hospital routine, mainly in ICU patients, is not yet a routine. It is only requested when there is any doubt about the diagnostic or the need of specific investigation [4,13,19]. Thus, this microbiological investigation strategy can contribute to better pharmacological management and treatment strategies.

Biofilm complexity, as well as the performance of conducts aimed at being less harming to intensive care unit patients is a valuable path for clinical and research activities [7-10,19,22]. It is so, because only few studies have emphasized such an oral association with hospital infections, mainly in Brazil.

The possibility of using high diagnostic power techniques and resources, at short response time, allows more accuracy and evidence in microbiological results of the investigated patients. The use of specific culture through spectrometry and the evaluation of anti-microbial sensitivity are effective in the general analyses of bacterial species, and can be investments in hospitals, as well as used based on the support by specialized laboratory [23-25].

The routine performance of tongue coating microbiological analysis can help changing the provided care, innovate accurate diagnostic and the implementation of individual hygiene routines focused on excellent care provided to ICU patients – this condition is not observed in the current study, because all assessed patients had tongue coating [4,9].

The presence of opportunistic microorganisms, such as Candida albicans - which was found in most of the assessed patients [26], Candida tropicalis and Candida Glabrata - in ICU patients due to low immunity and to the use of medication with high modification power are strategies to defend the human body.

Many microorganisms belong to the oral ecosystem, such as Streptococcus parasanguinis, Streptococcus vestibularis; Streptococcus salivarius; Streptococcus gordoni; Streptococcus cristatus, were found in patients in the present study [2]. They must be there, since they allow greater balance and favor biofilm formation (Streptococcus parasanguinis, Streptococcus vestibularis; Streptococcus salivarius; Streptococcus gordoni; Streptococcus cristatus - all found in patients in the present study) [27].

The biggest concern must be oral microorganisms that can cause systemic diseases, hospital infections, meningitis and sepsis such as Streptococcus mitis, Staphylococcus aureus, Kleibsella pneumoniae, Streptococcus (αhemolico group viridans) and Pseudomonas aeruginosa, which were found in some of the assessed patients. This factor favors the need of investigative and preventive microbiological actions to allow providing the necessary treatment right after referral to ICU [9,17-29].

Nosocomial pneumonia is the most common hospital infection, it is acquired after hospitalization and is associated with mechanical ventilation (PAV), which are mainly related to bronchoaspiration of gram-negative microorganisms found in biofilm and in tongue coating. Therefore, actions focused on adjusting the oral medium and biofilm disorganization, such as using chlorhexidine 0.12% as routine during hospitalization, can help improving the care provided to ICU patients and decreasing mortality rates [3,11,12,22,28,30].

The complexity of tongue coating is featured by the variety of microorganisms in it, with emphasis on the need of specific investigations, as approached in specific study to help improving the oral care provided to ICU patients. It can avoid infection conditions, opportunistic diseases and the worsened systemic conditions of these patients [6,7,19].

Deficiency in hygiene contributes to the accumulation of biofilm and microbial reservoir associated with nosocomial infections. It is important to emphasize that there are professionals who are unprepared to perform this activity, requiring effective educational actions and constant training in the hospital [4,6,9,22,29].

Based on the herein observed microbiological overview of the oral cavity, it is important emphasizing the need of implementing more investigative measures regarding tongue coating microbiology, as well as of relating the need of implementing guidelines/orientation concerning activities aimed at the correct cleaning of critical patients' tongue dorsum.

The specific study presented some limitations such as the moment of the pandemic in which it was carried out, the difficulty of access to patients admitted to the ICU for a larger sample, the acceptance of to participate in the study because they thought that the microbiological analysis could be a procedure more invasive and the length of stay in the ICU, considering that the most important changes in oral microbiology are observed with prolonged hospitalizations.

CONCLUSION

All assessed patients presented tongue coating on the dorsum, but its extension and location have varied. However, there was no significance between the amount of coating and the number of observed species.

The most prevalent microorganisms found in tongue coating of the assessed patients were Candida albicans, Streptococcus parasanguinis and Streptococcus mitis. This last species was found in 1/3 of the lingual extension.

Collaborators

AF Miranda and ALF Arruda, methodological organization, data collection, interpretation of statistical analyses and manuscript writing. DC Peruzzo methodological organization, interpretation of statistical analyses and manuscript writing.

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