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Salivary level of *Streptococcus mutans* and *Lactobacillus spp.* related to a high a risk of caries disease

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Abstract

Dental caries is currently the most widespread infectious disease among children and adults. This study aimed to establish the correlations between the salivary levels of Streptococcus mutans and Lactobacillus and the degree of caries activity in children. The study was performed on a sample of 60 children, selected after examination of a total of 144 children, age between 6-11 years. They were divided in two main groups: children from urban community (n=30) and children from rural community (n=30), each with 3 subgroups, depending on the degree of caries-activity. In order to determine the level of the salivary Streptococcus mutans and Lactobacillus, CRT Bacteria test was performed. The results emphasizeda statistically significant association between the degree of caries activity in children and the salivary levels of Streptococcus mutans, with specific variations depending on age, gender and living environment of the children examined. No statistically significant association was observed between the salivary levels of Lactobacillus and the degree of caries-activity. The salivary level of cariogene microorganisms represents an efficient marker of early detecting risk in the occurrence of caries disease, providing an opportunity to adopt a series of specific oral preventive measures.

Keywords: dental caries, bacteria test, salivary microorganisms

1. Introduction

Although it is currently considered the most widespread infectious disease among children and adults, dental caries raise multiple questions regarding the mechanism of initiation. The appearance of carious lesions in children presents a wide variety of issues and the carious process is the result of the imbalance between the risk factors, represented in particular by the pathogenic micro-organisms, and nutritional and protection factors, such as additional measures of prevention and oral hygiene. The first micro-organism incriminated in the genesis of dental caries was *Lactobacillus acidophilus*, normally present in the flora of the oral cavity but whose number increases significantly with 2-3 months before the occurrence of an caries injury, a phenomenon called "explosion of *Lactobacillus*" and decreases after the appearance of the dental injury (M. ROGOSA & al. [1]). However, the acid produced by the *Lactobacillus* represents only 0.025%, which means it is playing only a secondary role in initiating the caries process while its appearance is related to the decrease of the salivary pH in oral environment. Thus, the number of *Lactobacillus* was related to the activity of another micro-organism, *Streptococcus mutans (S. mutans)*, whose major role in initiating the caries process by hard tissues demineralization is incontestable (W.J. LOESCHE [2]). Unlike *S. mutans*, *Lactobacillus* do not adhere to the tooth surface on their own, therefore they need

natural or iatrogenic retention niches, such as pits and fissures of oclussal surface, cavities, marginal gaps of incorrect restorations or brackets of fixed appliances. *S. mutans*is a Gram-positive bacteria that through the ability to produce extracellular polysaccharides has the capacity to adhere to the tooth structure and through intracellular polysaccharides it creates energy reserves, so that the level of produced acids, mainly lactic acid, remain constant even under an external low intake of sugar. The main feature of *S. mutans* is its acidophilicity so that under acidic conditions it thrives and become the dominant bacteria of oral cavity. On the other hand, falling pH-levels prevents many oral strains from growing whereas the *S. mutans* counts increase (D.S. HARPER & al. [3]). Quantitative analysis of *S. mutans* in saliva was proposed first by Klock and Krasse, who found that the salivary concentration of these microorganisms is directly correlated to their quantity in dental bacterial plaque. Moreover, saliva is sampled more easily than dental plaque, which should be collected from many teeth in order to be representative of the whole mouth of an individual (S. PETTI [4]). Previous studies have shown that an increased salivary level of *S. mutans* alone is not a decisive indicator for a high caries risk and thus determination of both the *S. mutans* and the *Lactobacillus* counts, increases accuracy of microbiological evaluation. The objective of this study is to determine the positive or negative correlation that exists between the salivary level of *S. mutans* and *Lactobacillus* and the degree of caries-activity on a sample of children with different ages and socio-economic conditions, selection based on the values of DMFT caries index (**D** = decay, **M** = missing, **F** = filling, **T** = teeth), that is a marker of present and past degree of caries-activity of an individual.

2. Materials and Methods

2.1. Selection of subjects

For the selection of the main groups, a total of cooperative 144 children aged 6-11 years were examined, coming from two primary schools, from urban (n=88) and rural (n=56) communities. For examination, the informed consent was obtained from both the management of their schools and from their parents. Examination of the children was done in the medical office of their schools with disposable gloves, in natural light. Parallel to the examination of the dental formula, the children were questioned about oral hygiene habits, especially the frequency of dental brush, mouthwash rinsing and eating habits (use of sweets and carbonated drinks between meals). To correlate statistically the degree of hygiene with other analysis parameters, we used “0” for brushing teeth occasionally, “1” for brushing teeth only once a day, “2” for brushing teeth two times/day and “3” for brushing three times/day. The degree of caries-activity of each child was evaluated using DMFT index (Decay-Missing-Filling-Teeth) according to the dental caries diagnostic criteria WHO (World Health Organization [5]). The DMFT score is defined as the total number of teeth with caries, missing teeth or number of fillings for an individual. When calculating the DMFT index, teeth extracted for orthodontic reasons, or, primary teeth lost as a result of the physiological process of resorption, were no taken into account. Also, restored teeth with recurrent caries were considered as decayed.

2.2. Criteria selection for subjects

After the DMFT score was calculated, a sample of 60 children was selected from both areas and divided in two main groups: 30 children from urban (15 girls and 15 boys) and 30 children from rural (15 girls and 15 boys), each with 3 subgroups (n =10), depending on the degree of caries-activity as follows:

1. low caries-activity (DMFT = 0- 3)
2. medium caries-activity (DMFT = 4- 6)
3. high caries-activity (DMFT > 7).

From the total number of children examined we selected the subjects that had the most significant values of the caries index, the lowest values in case of those with low caries-activity and the highest in case of those with high caries-activity. None of the selected children in this study had received any antibiotic therapy during the last 2 weeks before the saliva sampling.

2.3. Microbiological examination

Semi-quantitative determination of *S. mutans* and *Lactobacillus* was carried out using the *CRT® Bacteria test* (Ivoclar-Vivadent). This test enables the simultaneous determination of salivary level of cariogenic bacteria and the assessment of caries risk by including in one of the two risk groups regarding the appearance of caries lesions, respectively a low caries risk and a high caries risk. The *CRT ® Bacteria kit* test contains a pipette used to covered the culture media with saliva, a $NaHCO_3$ (sodium hydrogen carbonate) tablet to ensure appropriate conditions for growth and bacterial multiplication by releasing CO_2 (carbon dioxide) when it comes in contact with moisture and the specific selective medium for the two cariogenic micro-organisms. *S. mutans* was identified by specific procedures that involve mitis-salivarius blue agar with bacitracin; *Lactobacillus* was identified on Rogosa agar medium.

2.4. Saliva sampling and medium cultivation

Before saliva collection, no food was allowed 1h before the test as well as no antibacterial mouthwash use 12-24 h before the test. To stimulate salivation and to transfer bacteria from the dental surfaces to the saliva, the subjects chewed 1-2 minutes a paraffin pellet enclosed in the test kit and then the saliva was collected in a suitable sterile container. After the protective foil has been removed, using a pipette, both agar culture media were entirely covered with saliva, carefully without scratching the surfaces, holding the agar carrier slightly oblique to prevent saliva from flowing off too quickly and thus favoring the bacterial growth. The bacteria will grow only in areas that have come in contact with saliva. The agar carrier was closed tightly by placing it back immediately into the vial, with previous addition of $NaHCO_3$ - tablet at the bottom of the vial. After cultivation, the agar carrier was placed upright in an incubator (Mini-incubator – Cultura from Ivoclar-Vivadent) and incubated at 37ºC/ 99F for 48 hours, sufficiently to allow the bacterial colonies to grow. After agar medium cultivation, the determination of the salivary pH for each sample was performed, using a special test paper (*Paper pH-test).* The results obtained by comparison with the standard color map for pH have been reported to normal average children's pH, which is 7.5, higher than the average in adults (6.7).

2.5. Interpretation of microbiological results

Semi-quantitative determination of salivary level of cariogenic bacteria was obtained for each agar medium by comparing the density of *S.mutans*and *Lactobacillus* colonies with the corresponding images in the enclosed model chart. *S. mutans* shows as small blue colonies with a diameter of < 1 mm on the blue agar, while *Lactobacillus* grows as white colonies on the transparent agar. Frequently, on the specific *Lactobacillus* medium, yeasts may also grow, especially *Candida albicans,* whose appearance is distinguished by larger size and cream-coloured colonies. Thus, findings a salivary level of *S. mutans* and *Lactobacillus* of $< 10^5$ CFU per milliliter of saliva indicates a low risk for caries occurrence and a level of $\geq 10^5$ CFU framed the subjects in a high risk group for caries development. In order to facilitate the statistical analysis, these groups have been quantified with 1 and 2 for the low-risk group ($< 10^5$ CFU), respectively with 3 and 4 for the high-risk group ($\geq 10^5$ CFU).

2.6. Statistical procedures

The resulting data in the context of this study, specifically the patient demographics data (age, sex and living environment) and the clinical data (oral hygiene, DMFT index, salivary pH and salivary level of *S. mutans* and *Lactobacillus*) were entered in a database. As a reference to the nominal variables percentages were used; for numeric variables, mean (M) and standard deviation (SD) were used. The statistical analysis was performed using *GraphPadInstad* 3 and *NCSS*/*PASSDowsonEdition* programs. The results were considered statistical significant at $P < 0.05$. Within the study we had many nominal values and small subject groups, thus we decided to apply non-parametric tests for the statistical analysis. Multiple comparisons between the study groups were performed using Mann-Whitney U test; the associations among groups were tested with the chi square test (X^2 test).

3. Results and Conclusions

3.1. Results and Discussions

The results achieved in the present study showed that the mean of DMFT caries index for the group of children in urban communities was 4.93 (± 3.88) and for those from rural communities was 5.96 (± 3.78). The difference between the two main groups was not statistically significant (p=0,288), instead there was a statistically significant difference (p=0,041) between urban and rural communities concerning the D component of DMFT index. Also, by comparing the mean salivary level of *S. mutans* between urban and rural communities, a lower mean in urban (2.76 ± 1.19) than in rural (3.06 ± 0.86) community was observed, but the differences were not statistically significant (p=0,433). The mean values of the compared parameters between urban and rural areas are presented in Table 1. Regarding the correlation between the degree of caries-activity and the salivary level of cariogene micro-organisms on the entire sample of subjects, a statistically significant association between DMFT index and salivary level of *S. mutans* (p=0,008) was observed, but the cause-effect type association was not present between the salivary level of *Lactobacillus* and DMFT index (p=0,131). From the three components of the caries index (D, M, F), the greatest influence on the salivary level of *S. mutans* is exercised by the number of decayed teeth (D), with a statistically significant association (p=0,002). Regarding the salivary level of *Lactobacillus*, a statistically significant association (p=0,516) with the number of decayed teeth (D) has not been found. There was no statistically significant association between the number of extracted (M) or restored (F) teeth and the salivary level of *S. mutans* or *Lactobacillus*.

Table 1. Means and standard deviation for compared parameters between urban and rural communities.

Parameters	*Urban*	*Rural*	*p-value*
DMFT index	4,93 ± 3,88	5,96 ± 3,78	0,288
D (decay)	3,96 ± 3,47	5,86 ± 3,80	**0,041 ***
M (missing)	0,23 ± 0,89	3,33 ± 0,18	0,544
F (filling)	0,73 ± 1,31	6,66 ± 0,36	**0,006 ***
S. mutans	2,76 ± 1,19	3,06 ± 0,86	0,433
Lactobacillus	2,36 ± 1,12	1,81 ± 0,88	**0,048 ***
Oral hygiene	1,81 ± 0,71	1,16 ± 0,94	**0,009 ***
Salivary pH	6,78 ± 0,46	7,16 ± 0,27	**0,0004 ***

* statistically significant association ($p < 0,05$)

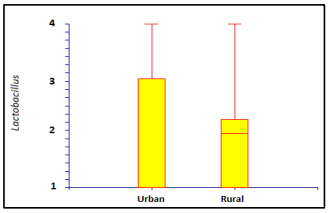

Fig. 1. Semi-quantitative salivary level of Lactobacillus in urban community group compared with rural community group.

The salivary level of *Lactobacillus* in the group of children from urban community (2.36 ± 1.12) is higher than in rural area group (1.8 ± 0.88), with a statistically significant difference (p= 0,047) (Fig. 1). Comparing the salivary pH values between the groups of children from urban and rural communities, a statistically significant difference was found (p=0,0004) (Fig. 2). Thus, in the group of children from rural community, the mean value of pH was 7.16 (± 0.27), closer to the normal mean value of pH, in contrast to the group from urban community with a mean of 6.78 (± 0.46). Comparing the degree of oral hygiene of children in urban and rural communities, a statistically significant difference (p=0,009) between the two groups of children was found, with a mean value of 1.8 (± 0.71) in urban community versus 1.16 (± 0.94) in rural community. A statistically significant association between gender and salivary level of *S. mutans* (P= 0,441) or *Lactobacillus* (p=0,189) was not found. No statistically significant differences was found between age and salivary levels of *S. mutans* (p=0,627) or *Lactobacillus* (p=0,211), thus the age did not influence the salivary level of these microorganisms (Table 2).

Table 2. Statistically association (p-value) between salivary level of S. mutans and Lactobacillus and other parameters.

Parameters	***p value***	
	S. mutans	***Lactobacillus***
DMFT index	**0,008 ***	0,131
D (decay)	**0,002 ***	0,516
M (missing)	0,769	0,351
F (filling)	0,592	0,344
Salivary pH	0,588	0,057
Oral hygiene	0,449	0,678
Environment	0,122	0,081
Gender	0,441	0,189
Age	0,627	0,211

* statistically significant association (p < 0,05)

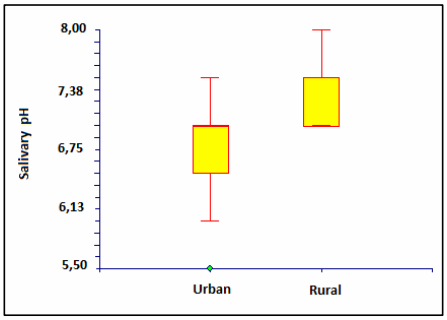

Fig. 2. Comparative values of salivary pH between urban and rural community groups.

The possibility of semi-quantitative determination of the salivary level of *S. mutans* and *Lactobacillus* represents a major progress in the current dental practice. O.G. GOLD & al. [6] have used the agar blue mitis-salivarius medium with bacitracin for the first time in 1973 and it is still used because of the selective development of *S. mutans* strains, while other microorganisms are inhibited. Similarly, the specific medium for the *Lactobacillus* has been used for the first time in 1951 by M. ROGOSA & al. [1] and it is still being used today because of the facile cultivation and identification of *Lactobacillus* strains. An increased salivary level of cariogene microorganisms indicates a latent risk of developing caries sooner or later under the terms of the existence or absence of the protective factors. The results of this study underline once again the major role played by *S. mutans* in the formation and the progression of dental caries. This is enhanced by statistically significant association found between DMFT index of caries, more precisely of D (decay) component and the salivary level of *S. mutans.* This association is not found yet in the relationship between DMFT index and the salivary level of *Lactobacillus,* which indicates that *Lactobacillus* are not essential for caries development. Other previous studies have shown that, at least in humans, the relationship *Lactobacillus* – caries was not proven to be cause-and-effect

(I. KLEINBERG [7]). The DMFT caries index value, obtained by the summation of its components, does not provide information about the contribution of each element, which can explain why there is no clear correlation between DMFT data and cariogene bacterial counts (W.J. LOESCHE [2]). The situation changes however if we analyze separately the relationship between the number of carious teeth and the salivary level of cariogene bacteria. In the present study, no statistically significant associations were found between the salivary level of *S. mutans* and components M and F of DMFT index, due to the low values of these components in the children groups from both rural and urban communities. More recently, it was found that negative correlation with caries incidence (the probability that *S. mutans* free subjects do not develop caries) was more reliable and predictive than positive correlation (probability that subjects with high *S. mutans* counts develop caries) (S. PETTI [4]). In another study, M.I. MATEE & al. [8] found a significant relationship between *S. mutans* level and dental caries index, but they also observed high levels of this microorganism in children who did not present carious lesions, which suggests that the presence of cariogenic bacteria does not necessarily mean high caries activity and the presence of protective factors such as fissure sealing, topical fluoride application, mouthwash rinsing, may reduce the risk of caries development in children. We did not find a statistically significant association between the salivary level of *Lactobacillus* and the DMFT index or component D of this index, neither in the urban nor in the rural communities. M. AHUMADA & al. [9] have analyzed the origin of *Lactobacillus* in children with dental decay and without decay; they have noticed that in children with decays, 78% of the lactobacillicame from the tongue and 22% from the gums, while in children without dental decays, 42% of lactobacillicame from the tongue and 12% from the gums. This research suggests that these oral mucous surfaces are reservoirs for *Lactobacillus.* Also in our study we found that there is no statistically significant association between the salivary level of *S. mutans* or *Lactobacillus* and the oral hygiene. Other studies have shown that tooth brushing does not have a significant effect on salivary level of cariogenic bacteria (M.A. EL-NADEEF & al. [10]), but can balance the negative effect of these microorganisms on dental hard tissues. A high *Lactobacillus* count is an indicator of high sugar intake (C.G. CROSSNER [11]). This hypothesis can be supported in the current study by comparing the salivary level of *Lactobacillus* in groups from urban and rural communities. Thus, in the group of children from urban community the salivary level of *Lactobacillus* is higher compared to those in rural community, furthermore, this aspect is emphasized by the different mean values of salivary pH in children from the two communities. However, a statistically significant association between the salivary pH and the salivary level of studied microorganisms was not found. J.H. SULLIVAN & al. [12] also showed that there is no statistically significant association between the salivary pH and *Lactobacillus* count. In another study, T. PARVINEN& al. [13] have found that the pH plays an important role in primary colonization of saliva with *Lactobacillus* and that a low pH increases the number of bacteria. In our study we observed that the mean value of the salivary pH is higher in rural community group compared with the group from urban community, which can be explained by the higher intake of sugar in urban compared to rural community. The average value of children's salivary pH is approximately 7.5, higher than the salivary pH in adults (6.7), due to a higher calcium ions concentration in the saliva of children. A remarkable example of the balanced relationship between salivary and oral microbiota is the fact that saliva is supersaturated with calcium and phosphate ions, which precipitate to form hydroxyapatite and re-mineralize the teeth (H. AIUCHI & al. [14], F. CHEN & al. [15]). This supersaturated solution should theoretically result in uncontrollable tooth growth as a result of constant precipitation of calcium phosphate onto the teeth. However, proteins present in

saliva, especially those containing proline and a peptide called statherin, have been shown to slow the rate of precipitation of these ions to a rate that perfectly matches the rate of decay induced by bacteria during normal lactic acid formation. Under these conditions, teeth should remain caries-free while the mouth is colonized with a very wide range of microorganisms, 400 species on average each with specific location. However this is not happening simply because of the imbalance between risk factors and protection factors. (M. AHUMADA & al. [9], I. BADEA & al. [16], M. GIURGIU & al. [17], R. SULLIVAN & al. [18]). Concerning the association between the age and salivary level of *S. mutans* and *Lactobacillus,* in the present study no statistically significant association between the two parameters was found which may be due to restricted age range within the studied group of children. Previous studies have shown that oral microflora in children differs from that in teenagers and in adults. The colonization of oral cavity starts at birth and the mother is considered to be the main source. A recent study has shown that the prevalence of certain strains of *S. mutans* and *Lactobacillus* is significantly higher in children delivered vaginally compared with infants delivered by C-section (M.N. BARFOD & al. [19], R. ECKERT & al. [20]). Previously, it was believed that *S. mutans* colonized oral cavity after the eruption of teeth (W.J. LOESCHE [2]), but recent studies have found the presence of this bacteria in children smaller than 2 months (S. TANKKUNNASOMBUT & al. [21]).

3.2. Conclusions

The results of this study emphasize the primary role played by the microbial factor in etiology of dental caries. Although many factors influence dental health, the results of this research demonstrate once again that there is a strong association between the degree of caries-activity, represented mainly by the number of decayed teeth and the salivary level of *S. mutans*. The salivary level of cariogenic microorganisms represents an efficient marker of early detecting risk in the occurrence of caries disease, providing an opportunity to adopt a series of specific oral preventive measures. An early assessment of the caries risk group assures a balance between the risk factors and the protective factors, appropriately adapted to age and socio-economic conditions.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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