

INTENSITY OF DENTAL CARIES AND BACTERIAL STRAINS *S. MUTANS*, *S. SOBRINUS*, *S. SALIVARIUS*, *S. MITIS* IN SALIVA OF CHILDREN WITH PERMANENT DENTITION

ИНТЕНЗИТЕТ НА ДЕНТАЛЕН КАРИЕС И БАКТЕРИСКИТЕ СОЕБИ *S. MUTANS*, *S. SOBRINUS*, *S. SALIVARIUS*, *S. MITIS* ВО ПЛУНКА НА ДЕЦА СО ТРАЈНА ДЕНТИЦИЈА

Nashkova S.¹, Zlatanovska K¹., Atanasova S.¹

¹ Faculty of Medical Sciences, Goce Delcev University, Stip, North Macedonia

Abstract

Introduction: Dental caries is one of the most common chronic and multifactorial disease affecting the human population. The appearance of a caries lesion is determined by the coexistence of three main factors: acidogenic and acidophilic microorganisms, carbohydrates derived from the diet and host factors. Caries develops as a result of an ecological imbalance in the stable oral microbiom. *Streptococcus mutans*, other *Streptococci* of the so-called non-*Mutans streptococci* group, *Actinomyces* and *Lactobacillus* play a key role in this process. The aim of our research is to detect the bacteria *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus salivarius*, *Streptococcus mitis* in saliva and to analyze the interdependence of dental status and the degree of dental caries activity with each examined parameter separately. **Material and method:** The study included 71 children (26 females and 45 males) aged 12 years. We selected the 12-year-old age group based on WHO recommendations, which recommends that age for global monitoring of dental caries and applies only to children with permanent dentition. **Results:** The data relating to the detection of *Streptococcus mutans* in the saliva of children with permanent teeth showed that in the experimental group of 40 (56.30%) children in total, *Streptococcus mutans* were isolated in 28 (39.40%) children and in 12 (16.90%) children were not isolated from the saliva. In the presented distribution of data relating to the detection of *Streptococcus mutans* in the saliva of children with permanent teeth there is a significant difference between the two groups ($p < 0.01$). **Conclusions:** *Streptococcus mutans* and *Streptococcus sobrinus* are considered the main etiological factors for the occurrence of dental caries. **Keywords:** *Streptococcus mutans*, dental caries, saliva, permanent dentition.

Апстракт

Вовед: Забниот кариес е едно од најчестите хронични и мултифакторни заболувања кои ја зафаќаат човечката популација. Појавата на кариесната лезија е определена од коегзистенцијата на три главни фактори: ацидогени и ацидофилни микроорганизми, јаглехидрати добиени од исхраната и фактори поврзани со домаќинот. Кариесот се развива како резултат на еколошка нерамнотежа во стабилниот орален микробиом. *Streptococcus mutans*, други стрептококи од т.н. група на не-*Mutans streptococci*, *Actinomyces* и *Lactobacillus* играат клучна улога во овој процес. Целта на нашето истражување е да ги

детектираме бактериите *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus salivarius*, *Streptococcus mitis* во плунката и да направиме анализа за меѓузависноста на денталниот статус и степенот на активност на денталниот кариес со секој испитуван параметар поодделно. **Материјал и метод:** Во истражувањето се вклучени 71 дете (26 од женски и 45 од машки пол) на возраст од 12 години. Возрасната група од 12 години ја избравме по препораките на СЗО која ја препорачува таа возраст за глобален мониторинг за забниот кариес и се однесува само на деца со трајна дентиција. **Резултати:** Податоците кои се однесуваат на детекцијата на *Streptococcus mutans* во плунката на децата со трајни заби, покажаа дека во експерименталната група од вкупно 40(56,30%) деца, кај 28(39,40%) деца е изолирана е *Streptococcus mutans* и кај 12(16,90%) деца од плунката не е изолирана. Во прикажаната дистрибуција на податоци кои се однесуваат на детекцијата на *Streptococcus mutans* во плунката кај децата со трајни заби постои значајна разлика помеѓу двете групи ($p < 0,01$). **Заклучоци:** *Streptococcus mutans* и *Streptococcus sobrinus* се сметаат за главен етиолошки фактор за појава на забен кариес. **Клучни зборови:** *Streptococcus mutans*, дентален кариес, плунка, трајна дентиција

Introduction

Dental caries is one of the most common chronic and multifactorial disease affecting the human population. The appearance of a caries lesion is determined by the coexistence of three main factors: acidogenic and acidophilic microorganisms, carbohydrates derived from the diet, and host factors. Caries develops as a result of an ecological imbalance in the stable oral microbiom. Oral microorganisms form dental plaque on the surfaces of teeth, which is the cause of the caries process, and shows features of the classic biofilm. *Streptococcus mutans*, other *streptococci* of the so-called non-*Mutans streptococci* group, *Actinomyces* and *Lactobacillus* play a key role in this process.¹ Dental caries remains the most common chronic disease in children and the respective etiology is not fully understood. Though *Streptococcus mutans* is an important factor in the initiation and progression of caries, its presence is not always associated with the disease. The existence of caries, in which *Streptococcus mutans* counts does correlate with caries experience, poses a challenging problem.² Dental caries is an infectious, transmissible disease caused by a group of phenotypically similar bacteria collectively known as *Mutans streptococci*, which are the main bacterial components responsible for the initiation and development of caries. The earliest time at which cariogenic *Mutans streptococci* can appear and act is during tooth eruption, which requires hard tooth surfaces, streptococcal colonization, and their multiplication.³ Current concepts of the etiology of dental caries have focused on the fermentation of carbohydrates by cariogenic bacteria into organic acids. Plaque bacteria produce a variety of end products that may vary depending on the diet. In the presence of fermentable carbohydrates, the most common organic acids produced are lactic, formic and acetic acids. These acids cause a drop in plaque pH, resulting in demineralization of the tooth and the creation of an environment favorable for further growth of *Mutans streptococci*. In addition to acid production, *Mutans streptococci* contain a wide range of virulence factors that are responsible for the cariogenicity of dental plaque.^{4,5,6,7,8}

Microorganisms play an important role in the development and progression of caries. Bacteria in the oral cavity are in balance, but the risk of caries increases if the number of certain bacteria (*Streptococcus mutans*, *Lactobacilli*) increases significantly while protective factors do not function normally. It has been established that the largest number of cariogenic bacteria are from the group of *Streptococci*, especially *Streptococcus mutans* and *Streptococcus sobrinus* and *Lactobacilli*. *Mutans streptococci* are considered significant determinants of plaque cariogenicity and are associated with the initial development of caries, while the number of *Lactobacilli* increases during the progression of caries.^{9,10} The aim of our research is to detect the bacteria *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus salivarius*, *Streptococcus mitis* in saliva and to analyze the interdependence of dental status and the degree of dental caries activity with each examined parameter separately.

Material and method

To achieve the set goal, children were randomly selected from primary schools in the city of Stip. The study included 71 children (26 females and 45 males) aged 12 years. We selected the 12-year-old age group based on the recommendations of the WHO, which recommends that age for global monitoring of dental caries and applies only to children with permanent dentition.¹¹ Dental examinations were performed using portable 60 W white-blue light bulbs and sterilized periodontal probes No. 5 and a mirror. To avoid visual fatigue, a maximum of 15 children were observed during one day. The examinations were performed after verbal consent of the subjects and their parents and they were divided into two groups, a control group (31 subjects without caries, fillings and extractions, DMFT=0) and an experimental group (40 subjects with caries, fillings and extractions of the teeth). In the study, we used the generally accepted index of the presence or absence of carious process Klein – Palmer – this index is designated as DMFT which is a sum of Decayed (carious), Missing (extracted) and Filled (filled) teeth. We registered clearly visible lesions on the surfaces of the teeth as caries, while changes in transparency and initial demineralization with an intact surface, without cavitation, we noted as a healthy tooth. According to the data obtained from the clinical examination, we determined the intensity (presence/absence) of dental caries, we noted and interpreted them as follows:¹²

- a. 0.0-0.9- very low caries risk
- b. 1.0-2.4- low caries risk
- c. 2.5-3.8- moderate caries risk
- d. 3.9-5.5- high caries risk
- e. ≥ 5.6 - very high caries risk

The assessment of *Streptococcus mutans*, *sobrinus*, *salivarius* and *mitis* in saliva was performed by applying saliva to sterile paper, No. 50, which was held with sterile beaked tweezers and placed under the tip of the child's tongue for 1 min. to soak in saliva. Each paper was then transferred to

sterile Eppendorf tubes with sterile tweezers, which were transported to a portable refrigerator with a freezer at a temperature of -80°C. Undiluted saliva samples with sterile swabs were plated on Mitis Salivarius Agar (Fluka, a medium with sucrose, glucose, tryptan blue and crystal violet) which is recommended for the isolation of mixed cultures of *streptococci* such as *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus salivarius*, *Enterococcus faecalis*, etc. The plates were incubated under microaerophilic conditions (5-10% CO₂) for 48 hours at 37°C. The characteristic features of the colonies and standard microbiological techniques were used to identify the isolates. Specific features of Mitis Salivarius Agar: colonies grown on this medium appear blue due to absorption of tryptan blue dye. *Streptococcus salivarius* is characterized by large, pale blue, smooth colonies with a diameter of 1-5 mm, which resemble “chewing gum” due to the production of levan from sucrose. Colonies of *Streptococcus mutans* were raised, convex, pale blue and granular like “ground glass”. Sometimes the surface of the colony had a “meshy” appearance, due to the synthesis of glucan from sucrose. *Streptococcus mitis* forms small, tiny colonies. Suspect colonies were sub-cultured on blood medium and finally identified with the Vitec 2 system, especially *Streptococcus salivarius*. DNA Isolation of *Streptococcus mutans* and *Streptococcus sobrinus* from saliva samples was done with the genomic Charge Switch Forensic DNA Purification kit, (Invitrogen Corporation 1600 Faraday Avenue Carlsbad).

Results

A total of 71 (100.00%) children make up the group with permanent dentition. Of these, 40 (56.30%) belong to the experimental group in which 16 (22.50%) children are female and 24 (33.80%) children are male. The data relating to the detection of *Streptococcus mutans* in the saliva of children with permanent teeth showed that in the experimental group of a total of 40 (56.30%) children, *Streptococcus mutans* was isolated in 28 (39.40%) children and in 12 (16.90%) children it was not isolated from the saliva. In the control group of a total of 31 (43.70%) children, *Streptococcus mutans* was isolated from the saliva of 12 (16.90%) children and in 19 (26.80%) children *Streptococcus mutans* was not isolated from the saliva. In the presented distribution of data relating to the detection of *Streptococcus mutans* in saliva in children with permanent teeth, there is a significant difference between the two groups for Pearson Chi Square=6.95 and $p < 0.01$ ($p = 0.008$). (Table 1)

Table 1. Group / *Streptococcus mutans*

			<i>Streptococcus mutans</i>		Total
			Yes	No	
Group	Experimental	Count	28	12	40

		% of Total	39,4%	16,9%	56,3%
	Control	Count	12	19	31
		% of Total	16,9%	26,8%	43,7%
Total		Count	40	31	71
		% of Total	56,3%	43,7%	100,0%

The data relating to the detection of *Streptococcus sobrinus* in saliva in children with permanent teeth for the experimental group of 40 (56.30%) children, in 30 (42.30%) children *Streptococcus sobrinus* was isolated from the saliva and in 10 (14.10%) *Streptococcus sobrinus* was not isolated. In the control group of 31 (43.70%) children, in 12 (16.90%) children *Streptococcus sobrinus* was isolated from the saliva and in 19 (26.80%) children *Streptococcus sobrinus* was not isolated from the saliva. In the presented distribution of data relating to the detection of *Streptococcus sobrinus* in saliva in children with permanent teeth, for Pearson Chi Square=9.53 and $p < 0.01$ ($p = 0.002$) there is a significant difference between the two groups. (Table 2)

Table 2. Group / *Streptococcus sobrinus*

		<i>Streptococcus sobrinus</i>		Total	
			Yes	No	
Group	Experimental	Count	30	10	40
		% of Total	42,3%	14,1%	56,3%
	Control	Count	12	19	31
		% of Total	16,9%	26,8%	43,7%
Total		Count	42	29	71
		% of Total	59,2%	40,8%	100,0%

The data relating to the detection of *Streptococcus salivarius* in the saliva of children with permanent teeth indicate that in the experimental group of 40 (56.30%) children, *Streptococcus salivarius* was isolated from the saliva of 35 (49.30%) children and not isolated in 5 (7.00%). In the control group of 31 (43.70%) children, *Streptococcus salivarius* was isolated from the saliva of 10 (14.10%) children and not isolated in 21 (29.60%). In the presented distribution of data relating to the detection of *Streptococcus salivarius* in the saliva of children with permanent teeth for Pearson Chi Square=22.96 and $p < 0.001$ ($p = 0.000$) there is a significant difference between the two groups. (Table 3)

Table 3. Group / *Streptococcus salivarius*

		<i>Streptococcus salivarius</i>		Total	
		Yes	No		
Group	Experimental	Count	35	5	40
		% of Total	49,3%	7,0%	56,3%
	Control	Count	10	21	31
		% of Total	14,1%	29,6%	43,7%
Total		Count	45	26	71
		% of Total	63,4%	36,6%	100,0%

The results of the detection of *Streptococcus mitis* in the saliva of children with permanent teeth indicate that in the experimental group of 40 (56.30%) children, *Streptococcus mitis* was isolated from the saliva of 37 (52.90%) children and *Streptococcus mitis* was not isolated from 2 (2.90%) children. In the control group of 31 (43.70%) children, *Streptococcus mitis* was isolated from the saliva of 8 (11.40%) children and *Streptococcus salivarius* was not isolated from the saliva of 23 (32.90%) children.

Table 4. Group / *Streptococcus mitis*

		<i>Streptococcus mitis</i>		Total	
		Yes	No		
Group	Experimental	Count	37	2	40
		% of Total	52,9%	2,9%	56,3%
	Control	Count	8	23	31
		% of Total	11,4%	32,9%	43,7%
Total		Count	45	25	71
		% of Total	64,3%	35,7%	100,0%

In the presented distribution of data relating to the detection of *Streptococcus mitis* in saliva in children with permanent teeth for Pearson Chi Square=16.14 and $p < 0.001$ ($p = 0.000$) there is a significant difference between the two groups. (Table 4)

The data relating to the predictive values of the detected *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus salivarius* and *Streptococcus mitis* in saliva in children with permanent teeth for the presence of dental caries (DMFT index) indicate that there is a significant association between the detected bacteria in saliva *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus salivarius*, *Streptococcus mitis* and the presence of dental caries for Pearson Chi-square=48.13 and $p < 0.001$ ($p = 0.000$). When determining the significance of the contribution to the

presence of dental caries of each bacteria, it was determined that the greatest influence has *Streptococcus mitis* (Wald=11.36 /p<0.01(p=0.001), *Streptococcus salivarius* (Wald=1.76 / p>0.05(p=0.19), while *Streptococcus mutans* (Wald=0.00 /p>0.05(p=0.99) and *Streptococcus sobrinus* (Wald=0.00 /p>0.05(p=0.99) have no predictive value for the presence of dental caries. Children in whose saliva *Streptococcus mitis* is detected compared to children in whose saliva *Streptococcus mitis* is not detected have a significantly higher probability of the presence of dental caries by 29.14 times (Exp(B)=29.14)(95%CI:4.10-207.00). Children in whose saliva *Streptococcus salivarius* was detected compared to children in whose saliva *Streptococcus salivarius* was not detected have a 3.27 times (Exp(B)=3.27)(95%CI:0.57-18.82) insignificantly higher probability of having dental caries. (Table 5)

Table 5. DMFT index / Cariogenic bacteria

Cariogenic bacteria								95% C.I.for EXP(B)	
Step 1 ^a		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Lower
		<i>S.mutans</i> (1)	18,76	28420,71	,000	1	,999	,000	,000
	<i>S.sobrinus</i> (1)	20,39	28420,71	,000	1	,999	7,180E8	,000	
	<i>S.salivarius</i> (1)	1,18	,89	1,76	1	,185	3,268	,57	18,82
	<i>S.mitis</i> (1)	3,37	1,00	11,36	1	,001	29,139	4,10	207,00
	Constant	-3,75	,99	14,07	1	,000	,024		

a. Variable(s) entered on step 1: *S.mutans*, *S.sobrinus*, *S.salivarius*, *S.mitis*

Discussion

Previous experimental and clinical research indicates that the occurrence of dental caries is responsible for several different risk factors that can act independently or in association at the same time.^{13,14} Yabao et al. emphasize the relatively well-known etiopathogenesis of dental caries, according to which the cariogenic flora in the presence of fermentable carbohydrates produces organic acids that reduce the pH of saliva and disrupt the complex dynamic balance of dental plaque, causing demineralization of enamel and the onset of initial caries and the formation of cavities in the teeth.¹⁵ Data regarding the intensity of dental caries in children with permanent dentition in the experimental group showed that out of a total of 40 (56.30%) children, 32 (45.10%) children had a low caries risk (1.0-2.4), 1 (1.40%) child had a moderate caries risk (2.5-3.8) and 7 (9.90%) children had a high caries risk (3.9-5.5) compared to the control group where the subjects were without dental caries and with a very low caries risk, there was a significant difference between the two groups (p<0.001).¹⁶ The number of bacteria in saliva and the amount of plaque are directly related to the occurrence of caries in each individual. Particularly strong indicators are the number of pathogenic *Mutans streptococci* mobilized in plaque, saliva, and fissures on the occlusal surfaces of the teeth, which place each patient at high risk for caries. Other important species of *Streptococcus* involved in the occurrence of caries are *Streptococcus mitis* and a group

of acidophilic *streptococci* that act only at "low pH" such as *Bifidobacterium* isolated from the white spot of teeth.¹⁷ In the presented distribution of data relating to the detection of *Streptococcus mutans* in saliva in children with permanent teeth, there is a significant difference between the two groups ($p < 0.01$).¹⁸ *Streptococcus mutans* and *Streptococcus sobrinus* are considered to be the main etiological factors for the occurrence of dental caries. Oda, using the polymerase chain reaction method, compared the levels of these bacteria in 145 patients aged 12 to 20 years and compared them with the occurrence of caries, and determined that children in whom *Streptococcus mutans* and *Streptococcus sobrinus* were isolated had a significantly higher incidence of dental caries than those with *Streptococcus mutans*.¹⁹

Conclusion

The number of bacteria in saliva and the amount of plaque are directly related to the occurrence of caries. Particularly strong indicators are the number of pathogenic *Mutans streptococci* mobilized in plaque, saliva and fissures on the occlusal surfaces of the teeth, which make each patient at high risk for the occurrence of caries. The data we obtained for the detection of *Streptococcus mutans* in saliva in children with permanent teeth in the experimental group out of a total of 40 (56.30%) children, in 28 (39.40%) children *Streptococcus mutans* was isolated from the saliva and in 12 (16.90%) children *Streptococcus mutans* was not isolated and in the control group out of a total of 31 (43.70%) children, in 12 (16.90%) children *Streptococcus mutans* was isolated from the saliva and in 19 (26.80%) children *Streptococcus mutans* was not isolated from the saliva. In the presented distribution of data relating to the detection of *Streptococcus mutans* in saliva in children with permanent teeth, there is a significant difference between the two groups ($p < 0.01$).

Data relating to the detection of *Streptococcus sobrinus* in saliva in children with permanent teeth, in the experimental group of 40 (56.30%) children in total, in 30 (42.30%) children *Streptococcus sobrinus* was isolated from saliva and in 10 (14.10%) children *Streptococcus sobrinus* was not isolated from saliva and in the control group of 31 (43.70%) children in total, in 12 (16.90%) children *Streptococcus sobrinus* was isolated from saliva and in 19 (26.80%) children it was not isolated from saliva. The distribution of data for the detection of *Streptococcus sobrinus* in saliva in children with permanent teeth indicates that there is a significant difference between the two groups ($p < 0.01$).

Data refer to the detection of *Streptococcus salivarius* in the saliva of children with permanent teeth, in the experimental group of a total of 40 (56.30%) children, in 35 (49.30%) children *Streptococcus salivarius* was isolated from the saliva and in 5 (7.00%) children *Streptococcus salivarius* was not isolated from the saliva and in the control group of a total of 31 (43.70%) children, in 10 (14.10%) children *Streptococcus salivarius* was isolated from the saliva and in 21 (29.60%) children *Streptococcus salivarius* was not isolated from the saliva.

The detection of *Streptococcus salivarius* in the saliva of children with permanent teeth indicated that there was a significant difference between the two groups ($p < 0.001$). The data regarding the detection of *Streptococcus mitis* in the saliva of children with permanent teeth, in the experimental

group of 40 (56.30%) children in total, in 37 (52.90%) children *Streptococcus mitis* was isolated from the saliva and in 2 (2.90%) children *Streptococcus mitis* was not isolated from the saliva and in the control group of 31 (43.70%) children in total, in 8 (11.40%) children *Streptococcus mitis* was isolated from the saliva and in 23 (32.90%) children *Streptococcus salivarius* was not isolated from the saliva.

Streptococcus salivarius in the control group of 31 (43.70%) children, in 10 (14.10%) children from *Streptococcus salivarius* and in the control group of 31 (43.70%) children, in 10 (14.10%) children from the saliva *Streptococcus salivarius* was isolated and in 21 (29.60%) it was not isolated. Data relating to the detection of *Streptococcus salivarius* in the saliva of children with permanent teeth, indicated that there is a significant difference between the two groups ($p < 0.001$).

The data relating to the detection of *Streptococcus mitis* in saliva in children with permanent teeth, in the experimental group of 40 (56.30%) children, in 37 (52.90%) children *Streptococcus mitis* was isolated from the saliva, in 2 (2.90%) children *Streptococcus mitis* was not isolated from the saliva and in the control group of 31 (43.70%) children, in 8 (11.40%) children *Streptococcus mitis* was isolated from the saliva and in 23 (32.90%) children *Streptococcus salivarius* was not isolated from the saliva.

The data relating to the detection of *Streptococcus mitis* in saliva in children with permanent teeth indicate a significant difference between the two groups of subjects ($p < 0.001$). When determining the significance of the contribution to the presence of dental caries of each bacterium, it was determined that *Streptococcus mitis* $p < 0.01$ had the greatest influence, followed by *Streptococcus salivarius* $p > 0.05$, while *Streptococcus mutans* $p > 0.05$ and *Streptococcus sobrinus* $p > 0.05$ had no predictive value for the presence of dental caries.

Children in whose saliva *Streptococcus mitis* was detected compared to children in whose saliva *Streptococcus mitis* was not detected had a 29.14 times significantly higher probability of the presence of dental caries. Children in whose saliva *Streptococcus salivarius* was detected compared to children in whose saliva *Streptococcus salivarius* was not detected had a 3.27 times insignificantly higher probability of the presence of dental caries.

This study provides evidence that *Streptococcus mutans* may play a role in shaping the salivary microbial community. Our results highlight that future caries research should consider additional species such as health/disease microbial markers in conjunction with *Streptococcus mutans* to improve diagnosis and caries management of the caries-discordant population.

References

1. Struzycka I. The oral microbiome in dental caries. Pol J Microbiol. 2014;63(2):127-35. [Pub Med]

2. Dinis M., Agnello M., Cen L., Shokeen B., He X., Shi W., Wong D.T.W., Lux R., Chaichanasakul Tral N. *Front Microbiol.* 2022 Feb 17;13:782825. doi: 10.3389/fmicb.2022.782825. e Collection 2022. [Pub Med]
3. Loesche W.J. Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev.* 1986, 50:353-380. [PubMed]
4. Geddes D.A.M. Studies on metabolism of dental plaque:diffusion and acid production in human dental plaque. *Front Oral Physiol.* 1981, 3:78-87.
5. Nyvad B., Fejerskov O. Development, structure, and pH of dental plaque. In: *Textbook of clinical cariology.* 1994, pp. 89-110.
6. Brandtzaeg P. Salivary immunoglobulins. In: *Human saliva:clinical chemistry and microbiology.* Vol. II. Tenovuo J, editor. Boca Raton, FL: CRC Press, 1989, pp. 1-54.
7. Dashper S.G., Reynolds E.C.of organic acid anions on growth, glycolysis and intracellular pH of oral streptococci. *Dent Res J.* 2000, 79:90-96.
8. Stookey G.K., Carlos González-Cabezas C. Emerging Methods of Caries Diagnosis. *Journal of dental education.* 2001, 01. October, 1001-1006.
9. Sawka M., Chevront S.N., Carter R. Human water needs. *Nutr Rev J.* 2005, 63:S30-9.
10. McCord M. The evolution of free radicals and oxidative stress. *Am J Med.* 2000,108:652–659.
11. Zickert I., Emilson C.G., Krasse B. *Streptococcus mutans*, lactobacilli and dental health in 13-14-year-old Swedish children. *Community Dent Oral Epidemiol.* 1982, 10: 77–81.
12. Granath L., Cleaton-Jones P., Fatti L.P., Grossman E.S. Salivary lactobacilli explain dental caries better than salivary mutants streptococci in 4-5-year-old children. *Scand J Dent Res.* 1994.
13. Federation Dentaire Internationale. Goals for oral health in the year, 2000.
14. *Oral Health Surveys: Basic methods.*4th ed.Geneva:World Health Organization WHO, 1997.
15. National Survey on Oral Health and Nutritional Status in the Philippines. Philippine Department of Education, 2006.
16. Cariño K.M., Shinada K., Kawaguchi Y. Early childhood caries in northern Philippines. *Community Dent Oral Epidemiol.* 2003, 31:81-89.
17. Yabao R.N., Duante C.A., Velandria F.V, Lucas M., Kassu A., Nakamori M., Yamamoto S. Prevalence of dental caries and sugar consumption among 6-12 year old schoolchildren in La Trinidad, Benquet, Philippines. *Eur J Clin Nutr.* 2005,59:1429-1438.
18. Fejerskov O. Concepts of dental caries and their consequences for understanding the disease.*Community Dent Oral Epidemiol.* 1997, 25(1):5–12. [PubMed]
19. Oda Y., Hayashi F., Okada M. Longitudinal study of dental caries incidence associated with *Streptococcus mutans* and *Streptococcus sobrinus* in patients. *BMC Oral Health.* 2015, Sep 2;15:102.