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Cross sectional study on the variation of plaque pH in diabetic patients. A clinical randomized trial on the capability of Probiotic (Lactobacillus Brevis CD2) to reduce plaque acidogenicity in a sample of diabetic children.

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ABSTRACTS

This thesis was planned as two different studies, the first one is a cross-sectional study and the second paper is a RCT longitudinal study. The abstracts show below:

ABSTRACT Cross-sectional study

Objective: The aim of this study was to evaluate the difference in caries experience and different caries-related variables between diabetic and non-diabetic children aged 5-13 years old. A further analysis was carried out on diabetic children after they were divided in two groups based on their metabolic control.
Material and Methods: We designed a case-control study on two categories of children: the first group consisted of 68 children diagnosed with type 1 diabetes and the second group consisted of 136 non-diabetic control children. The diabetic children were then divided into two subgroups: a) 20 children with adequate metabolic control (Hb1ac≤7.5) and b) 48 children with poor metabolic control (Hb1ac>7.5). Data on dietary and oral hygienic habits was obtained on all the subjects participating in the study. Collection of saliva was carried out after stimulating salivation by chewing on a piece of paraffin for 5 minutes. Microbial flora was analyzed using the checkerboard DNA-DNA hybridisation method. Plaque acidogenity was recorded using pH indicator strips up to 30 min after a sucrose rinse. Caries registration was performed using the ICDAS index.
Results: No statically significant difference in clinical data was found in the two study groups with similar caries status. No statistically significant difference was found for tooth brushing frequency, use of fluoridated toothpaste, mouthwash and other fluoride supplements and the pattern of dental check-ups between the groups examined. Statistically significant differences for plaque-pH when analyzed as minimum pH, $AUC_{6.2}$ and $AUC_{5.7}$ between all diabetic and the non-diabetic children ($p<0.01$ or $p<0.05$) were found. The bacterial counts differed significantly between diabetic and non-diabetic subjects regarding *S. Mutans, S. Sobrinus, L. Salivarius and L. Fermentum* ($p < 0.05$).
Conclusions: Type 1 Diabetes Mellitus patients showed a more cariogenic bacterial environment and a direct effect on plaque pH reducing it from normal levels was detected.

A 25-word summary of the abstract
Plaque acidogenicity and microbial flora were statistically different between diabetic and non-diabetic children and between subjects with a good-metabolic control and children with bad-metabolic control.

ABSTRACT RCT Longitudinal study

Objective: The aim of this study was to evaluate the effect of probiotics on the oral microbiotic flora and biofilm acidogenicity in children diagnosed with diabetes since at least two years. The null hypothesis is that the probiotic lozenge containing Lb CD2 would not reduce the pathogenic bacteria and modify the plaque-pH. Primary objective is the regular consumption of probiotic will lead to a significantly reduction of pathogenic bacteria compared to a placebo. Regular oral hygiene is permitted.

Material and Methods: A double-blind, longitudinal study was performed including 68 diabetic subjects shared into two group test control and control group each were 34 subjects. The dosage in the treatment phase subjects will use 2 oral tablets a day for 56 days. The inclusion criteria for the diabetic children were: 1) 5-13 years old, 2) diabetes diagnosed >2 years ago, 3) living in Sassari and surrounding region (rural area within a distance of 30-40 km from the city), 4) good general health, and 5) average oral hygiene (cleaning the teeth at least twice a day)

Result: The pH values recorded no statistically significant differences for maximun fall pH in each group after 90 days. No significant differences for control group when it evaluated the minimal pH. A significant association for the test group with p. <0.001 after 90 days when it considered minimal pH. Comparison between the two diabetes groups showed no statistically significant differences when compared each group at baseline and after 90 days A significant association for test group after intake of lozange of probiotic at 90 days (p.<0,001); for control group only *S. Sobribus* no one significant association.

The association between diabetic test group with diabetic control group showed a significant association at baseline only for the bacteria strain of *S. Salivarius* (p.<0.04) and *L. Casei* (p.<0.03);

after 90 days the comparison among diabetic test group and diabetic control group recorded a significant association for *S. Mutans, S. Sobrinus, L. Casei, L. Salivarius* (p. <0.01) and *S. Salivarius* (p.<0.02).

For other bacteria, no one statistically differences were found when comparing the groups in different time.

Conclusion: The results of this study show that use of probiotic may improve the minimal pH

in Diabetic children and may decrease the bacterial flora species as S. Mutans, S. Sobrinus, L. Casei, L. Salivarius decreasing the caries risk with good diet and oral hygiene.

CONTENT:

Preface

This thesis was written on the basis of my three years Ph.D studies at the Department of biomedical sciences, University of Sassari, Italy. The work was carried out under the supervision of Professor Guglielmo Campus.

The thesis is written as a general overview based in the following articles which will be referred below:

- Evaluation of the difference in caries experience in diabetic and non-diabetic children - A case control study
- Clinical and microbilogical evaluation of the effect of a probiotic lozenge (Inersan®) on caries-related variables in diabetics children
- A comparative assessment of gingivitis through reflectance spectrophotometry among Italian adolescents. A pilot study.

Background

Introduction

Diabetes mellitus is a chronic disease resulting from a relative or absolute deficiency of insulin, which affects the metabolism of carbohydrate, protein, and fat. The most obvious finding is a high level of blood glucose especially following a meal. [1, 2]

In Europe there is a north-south gradient in the disease incidence. The highest incidence is observed in Finland with 40.2 per 100,000 subjects and year, while the lowest incidence rates are reported by Balkan countries, particularly by Macedonia (3.2/100000/year). Finland, Sardinia (Italy) and Sweden are known to have the highest incidence of type 1 diabetes in the world. [3]

Diabetes mellitus can be divided into two main categories: insulin dependent (Type 1) and non insulin dependent (Type 2). The diagnosis of Type 1 diabetes mellitus in young patients is relatively easy, but in patients with adult-onset diabetes mellitus, the classification into either Type 1 or 2 can sometimes be difficult. [4] . Type 1 diabetes is mainly an autoimmunizing disease characterized by the specific destruction of pancreatic beta cells and the presence of specific autoantibodies. [5] [6]

The onset in children is on average under the age of fifteen years. Type 1 diabetes incidence increases as a child gets older and with an odd double at the age of 10-14 years old compared to the age of <5 years. This trend generally is not affected by gender. [3] [7] [8]

An expert panel was convened in October 2013 by the International Scientific Association for Probiotics and Prebiotics (ISAPP) to discuss the field of probiotics. It is now 13 years since the definition of probiotics and 12 years after guidelines were published for regulators, scientists

and industry by the Food and Agriculture Organization of the United Nations and the WHO (FAO/WHO). The FAO/WHO definition of a probiotic—"live microorganisms which when administered in adequate amounts confer a health benefit on the host"—was reinforced as relevant and sufficiently accommodating for current and anticipated applications. However, inconsistencies between the FAO/WHO Expert Consultation Report and the FAO/WHO Guidelines were clarified to take into account advances in science and applications. [9] The mechanisms of action are thought to be locally in the mouth by competing for adhesion sites and nutrients with the pathogens, and by inhibition of growth of pathogens by production of bacteriocins or other products. Thus, they modify the composition of the oral biofilm or the metabolic activity. Also, there is thought to be a regulation of the immune response [10], [11]. Generally, the effects of probiotic bacteria are strain specific and cannot be applied directly to other strains. Also, the same strains can have different effect in different individuals [12] .

Caries-risk in diabetic children

Dental caries risk factors include oral cariogenic bacteria, intake of fermentable carbohydrates as a substrate for cariogenic bacteria, and sufficient time allowed for caries formation. The protective factors against caries include the saliva, oral hygiene, and fluorides.
The research has shown that the levels of cariogenic bacteria, particularly of *Streptococcus mutans*, are higher in diabetic patients and the proportion of individuals with high levels of cariogenic bacteria, particularly *Streptococcus mutans*, is higher in the diabetic population.
Earlier studies have shown that although children with type 1 diabetes possess a lower caries risk, they have an increased risk of developing periodontal disease [13].

Various oral complications have been reported in diabetic patients including an increased presence of caries, even if no scientific evidence is described [10]. Changes in oral microflora of diabetics individuals with a poor glycaemic control may significantly influence the incidence of diseases caused by bacteria such as periodontal impairment and dental caries. [14] [15] Furthermore, diabetes may cause changes in salivary glands, which may contribute to a slow flow rate, prolonged oral clearance and altered salivary composition. [16] [17]. Other factors of diabetes associated with the cariogenic changes in the oral environment of diabetic children and

adolescents included less resting and stimulated whole saliva, lower saliva buffering capacity and acidic pH, higher salivary glucose, higher salivary albumin concentrations, high proportion of salivary Streptococcus mutans, and salivary yeast growth [18] [19] [20]. However, contrary findings with lower prevalence have also been reported [21] [22].

Dental plaque

Dental plaque has been defined as "a specific but highly variable structural entity consisting of micro-organisms and their products embedded in a highly organized intercellular matrix." It represents a true biofilm consisting of a variety of micro-organisms involved in a wide range of physical, metabolic and molecular interactions [23]. The cooperative nature of a microbial community provides advantages to the participating organisms such as a broader habitat range for growth, enhanced resistance to antimicrobial agents and host defenses and enhanced pathogenicity [23]

The oral microbiome includes hundreds of microorganisms which, as mentioned previously, colonise the oral surfaces and grow as the biofilm – dental plaque [24]. The majority of these microorganisms are bacteria originally identified and characterised using culture-dependant methods [25]. Recent culture-indipendent approaches have enhanced our knowledge of the complexity of the oral microflora. At present and based on these techniques, the human oral microbiota consist of more than 700 species, each composed of strain with different phenotypes and genotypes [26]. Oral infections are distinctive in the sense that bacteria commonly present in the resident oral flora are key players in disease initiation and progression [27].

Oral microbiological analyses

Fewer than 50% of the resident oral microflora can be cultivated, rendering culture-based analyses unsuitable for holistic studies [28] [25]. Microbiological analysis has witnessed a burst of culture-indipendent moleculartechnologies ranging from clone counting and

sequencing (16S ribosomal RNA analysis), fingerprinting of amplified polymerase chain reaction (PCR) products (a technique called "terminal restriction fragment length polymorphism"), quantitative PCR, pyrosequencing to higt-throughout microarrays and metagenomic and metatranscriptomic approaches [29] [28] [30]. Among the nucleic acid-based technologies that have revealed the complex microbiology of dental plaque, is the checkerboard DNA-DNA hybridasation technique [31]. This technique uses whole genomic DNA probes and give a simultaneous and quantitative analysis of up to 28 plaque samples against 40 key microbiol species [31]. For the analysis, 28 alkali lysates of dental plaque, and two DNA standards representing 10^5 and 10^6 cells per target species are fixed on a membrane in thin lanes. They are then simultaneously cross-hybridised with digoxigenin-labeled whole genome probes [32]. The technique is called "checkerboard" because the genomic probes are hybridised at right angles to the DNA of multiple oral samples, and the processed images of of the hybridisations resemble a checkerboard [28].

The checkerboard DNA-DNA hybridisation technique can be performed using one of three probe types, whole genomic probes, oligonucleotide probes (16S rRNA gene-based probes) or multiple displacement amplification-based probes [31], [33]. The technique offers ample advantages as it is rapid , sensitive, relatively inexpensive and permits the enumeration of a large number of species in large-scale studies with numerous samples [31] , [34] [29]

In these studies the analyses in the microbial flora was obtained by the checkerboard DNA-DNA hybridisation method [32]. And measurement of the bacterial count in the samples was performed by matching the obtained signals with the ones generated by the pooled standard samples containing a count of 10^6 and 10^5 of each bacterial species, respectively. The signals were coded on a scale from 0 to 5: 0 = no signal; 1 = a signal density weaker than that of the low standard ($<10^5$ bacteria); 2 = a signal density equal to that of the low standard ($=10^5$ bacteria); 3 = a signal density higher than that of the low standard but lower than that of the high standard ($>10^5$ but $<10^6$ bacteria); 4=a signal density equal to that of the high standard ($=10^6$ bacteria) and 5 = a signal density higher than that of the high standard ($>10^6$ bacteria). Other information regarding the technique are retrived in the article "Intra-familial comparison of supragingival dental plaque microflora using the checkerboard DNA–DNA hybridisation technique". [35] [36]

Studies on probiotics in children

Most clinical studies in ralation to caries preventive potential of probiotic bacteria have microbiological endpoints related to saliva or plaque. [37], [38], [39]. The sample sizes are generally small and the intervention periods were relatively short. A wide variety of delivering vehicles were used such as dairy products, ice cream, tablets and lozenges, and it seemed that the vehicle was of minor importance. There was seen a statiatical significant reduction of *S. mutans* in 12 out of 19 paper, but only an increase of lactobacilli.

Another randomized controlled trials (RCT) with caries as an endpoint has been published. The first study, investigated effects of probiotic bacteria given to 594 preschool children aged 1-6 years, and showed statistical significant less caries in the probiotic group after 7 months compared to the control group, but only in the subgroup of the 3-4 year olds [40]. Other studies used probiotic bacteria in combination with fluoride in milk and gained a reduction in in early childhood caries in 248 children [41].

The effect of the fluoride could not be separated from the possible effect of the probiotic bacteria. In the recent study on root caries in 160 older adults [42], different strategies have been suggested in order to prevent oral diseases. The effect of probiotics (Lb CD2), distributed in association with antibiotic on gingivitis, has been tested. In different fields of oral healthcare probiotics a clinical effect has been demonstrated on different oral conditions such as halitosis, oral candidiasis and dental caries. [43] [44] [45] [46] In order to prevent dental caries the effect of probiotics have been evaluated in different studies using various types of Lactobacilli strains. They have been proven to obtain a reduction in caries incidence, reduced number of mutans streptococci and lactobacilli, decrease plaque acidogenicity and a reversal of root caries lesions. [47] [48] [49] [50] [51] [52]

Gingival inflammation

Advances in research over recent years have led to a fundamental change in the understanding of the periodontal diseases process and in the assessment of the risk among adolescents or adults.

Gingival inflammation has been studied and assessed with several approaches. A variety of indices or schemes for evaluating the severity and extent on gingivitis have been formulated in the past. All are to some degree subjective in nature, depending on individual examiner skills, perceptions and judgments of gingival appearance. Among these scoring systems, the Gingival Index (GI), developed by Löe and Silness has gained the most widespread acceptance and use. Presence or absence of gingival bleeding has been associated with the visual clinical signs of gingivitis and with histologically detection of inflammation on gums. [53] [54] [55] [56]

Several investigations demonstrated also that gingival microcirculation exhibits a dramatic, dynamic change in response to the development and progression of gingivitis. In particular, they proved the growth of blood flow in inflamed gingiva in comparison with healthy gingiva both in human and in animal models [57] [58] Thus, all these morphologic alterations occurring in gingival inflammation and currently scored subjectively using the indices reported above may be related to functional changes in the gingival microcirculation, which can on the contrary, be mapped objectively.

Therefore, gingival microvascular function has been examined with a variety of techniques even if sometimes their invasive or toxic nature prevented their application for in vivo assessment in human volunteers. This is the case of infusion of labelled and unlabelled microspheres, electrical impendance plethysmography, high-speed cinematography, thermal clearance and radioisotope clearance [59] [60] [61]

As concerning non-invasive techniques, several authors have employed laser Doppler flowmetry as a method of studying gingival microvascular dynamic [62]

In human subjects, it has been reported laser Doppler flowmetrically was able to measure gingival blood flow in experimentally induced human gingivitis. Patients showed a reduction in gingival blood flow in labial marginal gingiva as compared with baseline measurements of healthy gingiva, indicating that human blood flow slows in the presence of inflammation [63]

Laser Doppler Flowmetry has been successfully used to determine gingival blood flow in normal human gingiva, dog gingiva with increasing and decreasing inflammation and in young humans with histories of various periodontal diseases [64] [65] [66]

Optical spectrophotometry proves itself to be able to simultaneously determine multiple inflammatory indices related to periodontal disease directly in gingival tissues in vivo [67] [68]

Reflectance spectrophotometry has been used as a noninvasive measure of microvascular function too, in particular to estimate the haemoglobin (Hb) concentration and Hb oxygen saturation and then to quantify severity and extent of gingival inflammation.

Many surveys showed that reflectance spectrophotometry can continuously measure the gingival Hb concentration and Hb oxygen saturation in situ and discovered an increase in Hb concentration and a decrease in Hb oxygen saturation with increasing inflammation both in dog and human gums, suggesting that the increase in blood supply may not be enough to meet the oxygen demand in inflamed gingiva. In addition, spectral ratios at specific wavelengths have been used to extract relevant information from the absorption haemoglobin spectra [69] [70] [71] [72]

It is mostly useful to distinguish two wavelength bands (A from 453 to 500 nm and B from 685 to 805 nm) in order to understand absorption coefficient for the oxygenated blood is higher than deoxygenated blood within Band A, and the opposite happens within Band B. Spectral ratio at wavelengths 460 and 615 from MHT Spectroshade™ Micro multispectral imaging system has been thus demonstrated to fall within bands A and B. It was especially assumed that comparing reflectance at these waveleghts would have provided accurate details about the blood content in the tissue [73]

Considering properly the variety of risk factors that can start occurring during the particular stage of adolescence, it seemed significant to estimate the feasibility of a method possibly able to: (1) help in measuring the smallest changes in periodontal inflammation, (2) monitor the progression of the disease longitudinally (3) detect as much preventively as possible the earliest conditions in population cross sectional studies.

Based on these previous considerations a clinical and experimental cross sectional pilot study was carried out as presented below.

Aim

The overall objective of the present thesis was to investigation the possible effect of probiotic on the oral microflora:

The specific aims of the studies are:

- to evaluate the difference in caries experience and different caries-related variables between diabetic and non-diabetic children aged 5-13 years old. A comparison in relation to metabolic control was also performed for the diabetic children. The null-hypothesis was that there was no difference regarding caries experience between the three groups.

- to evaluate the effect of probiotics on the oral microbiotic flora and biofilm acidogenicity in children diagnosed with diabetes since at least two years. The null hypothesis is that the probiotic lozenge containing Lb CD2 would not reduce the pathogenic bacteria and modify the plaque-pH. Primary objective is the regular consumption of probiotic will lead to a significantly reduction of pathogenic bacteria compared to a placebo. Regular oral hygiene is permitted.

- To assess gingival inflammation objectively through reflectance spectrophotometry in cross-sectional population studies.

Material and methods

Study desing and participants

The study desing, participants and investigations for each of the five studies included in the thesis are summarised the table below.

Table 1 . The desing, partecipants and investigations for each of the five studies included in the thesis.

STUDY	DESING	PARTICIPANTS	DATA COLLECTED
I	Cross-sectional Case control Rate 1:2 matched for age and gender	204 subjects shared: a) non-diabetic children 136 subjects b) 20 children with a good metabolic control (Hb1ac≤7.5) c) 48 children with bad metabolic control (Hb1ac>7.5)	-Questionnaire -ICDAS -"Checker DNA-DNA Hybridisation" analysis score -pH measurement
II	Longitudinal study (90 days)	68 subjects divided: a) 34 subjects control group b) 34 subjeces test group	Same as in Study I
III	Cross-sectional Pilot study	88 patients	- Gingival Index - Gingival Bleeding - Plaque Index - reflectance spectrophotometry

Study I

A cross-sectional case control (rate 1:2 matched for age and gender) study was designed in which two categories of subjects participated; diabetic and non-diabetic children aged 5-13 years old. The study protocol was approved by the Ethical Committee of Sassari University of Medicine, Sassari, Italy [authorisation number 133/2014] and conducted according to the

principles of the Helsinki Declaration II. The number of people affects by diabetes in Sardinia is estimated in almost 95,000 individuals. In Sassari area 1013 children affected by Type 1 diabetes is reported (https://www.regione.sardegna.it/documenti/1_19_20120702174709.pdf in paediatric age in Sardinia). Inclusion criteria to be enrolled into the study were: 5-13 years old, diabetes diagnosed more than 2 years before the start of the study, living in Sassari and surrounding region (within a distance of 30-40 km from the city), good general health apart from diabetes, reporting to clean the teeth at least twice a day.

Power analysis was performed before the start of the study using the web-based openepi platform (http:/openinfo.com), taking into account a caries prevalence of about 50 % [74] . The number was increased by 20% taking account a modification in caries prevalence and a high number of non-responders. The number of diabetic children needs to enrolled was fixed in 64 with an actual power of 0.95. Information of the study was mailed to 225 parents/guardians (related to 75 diabetic children and 150 non-diabetic children) asking consent for their children to participated to the study. Finally, 72 diabetic children agree to participate and 68 were enrolled, so from the 150 bunch of non-diabetic children 136 subjects matched by gender and age were selected. The study was carried out at the Peadiatric diabetic children and in the Dental Clinic of the School of Medicine, University of Sassari, Sassari, Italy. The study was conducted from January 2015 to September 2015.

The diabetic children were, according to information in their medical charts, divided into two subgroups: a) 20 children with a good metabolic control (Hb1ac≤7.5) and b) 48 children with bad metabolic control (Hb1ac>7.5). Subjects were instructed not to brush their teeth or to eat/drink anything during the last hour prior to examination.

Data collection

All subjects were examined in the Dental Clinic, of the University of Sassari. A plaque sample was collected for microbiological analyses, and acidogenicity of dental plaque was measured followed after a sucrose challenge. All parents/guardians fill in a questionnaire regarding oral hygiene and dietary habits of the children. For the diabetic children, data on their medical condition was also retrieved from their medical charts.

Questionnaire

A structured questionnaire with closed questions was used to elicit information of oral hygiene and fluoride habits as well as snacking and drinking habits. Each answer was assigned a code of intake frequency for drinking and snacking habits. The place of consumption of food and drink, at school (S) or outside school (OS), was registered. The answers were coded as never (Code 0), once a week (Code 1), two-four times a week (Code 2), five-six times a week (Code 3), once a day (Code 4), two-three times a day (Code 5) and over three times a day (Code 6). The answers of oral habits were obtained by questions focusing on regularity and frequency of toothbrushing, use of fluoridated toothpaste, the use of fluoride supplements and, frequency dental check-ups.

Clinical examination

The clinical examination was made under optimal lighting using a mirror and WHO-probe. For caries registration, the ICDAS II [22] index was used. The subjects were then merged and classified into the following four categories: caries-free (ICDAS 0), initial caries (ICDAS 1-2), moderate caries (ICDAS 3-4), and severe caries (ICDAS 5-6). No radiographic caries registrations were made. [75]

Saliva samples and Microbiological analyses

Saliva sample collection was made after stimulated saliva by chewing on a piece of paraffin during 5 min with continuously spitting into test tube. The saliva samples were sent to Department of Microbiology, University of Bologna for evaluation of bacterial pathogens.

The analysis of the microbial flora was made using the checkerboard DNA-DNA hybridisation method [32]. Whole genomic probes were matched from 15 bacterial strains known to be associated with caries. An evaluation of the bacterial count in the samples was performed by matching the obtained signals with the ones generated by the pooled standard samples containing a count of 10^6 and 10^5 of each bacterial species, respectively. The signals were coded on a scale from 0 to 5: 0 = no signal; 1 = a signal density weaker than that of the low standard ($<10^5$ bacteria); 2 = a signal density equal to that of the low standard ($=10^5$ bacteria); 3 = a signal density higher than that of the low standard but lower than that of the high standard ($>10^5$ but $<10^6$ bacteria); 4=a signal density equal to that of the high standard ($=10^6$ bacteria) and 5 = a signal density higher than that of the high standard ($>10^6$ bacteria).

Plaque acidogenicity

The plaque acidogenity was assessed using the pH indicator strips. The strip measure a pH value in the range of 4.0–7.0 (Spezialindikator, pH range 4.0–7.0; Merck, Darmstadt, Germany) [76]. Each strip was cut into 4 pieces (approx. 2 mm in width) in order to get a strip that more easily could be inserted into the interproximal space. The strip was held into the interdental space for 10 s after which it was removed and its colour compared to the colour index scheme supplied by the manufacturer. The pH was determined to one decimal of the value. For each subject, 3 measurements were carried out in 2 sites: 1) between the 2nd premolar and the 1st molar right or left upper jaw and 2) 2nd primary molar and 1st molar right or left upper jaw. In case the patient has not erupted 1st molar, the measurements were carried between 54/55 and 64/65. At each time point (t), measurements were performed before (0 min) and at 2, 5, 10, 15, 20 and 30 min after a mouth rinse with 10% sucrose.

Study II

Study design and study centre

A double-blind, longitudinal study was performed including 68 diabetic subjects. The dosage in the treatment phase subjects will use 2 oral tablets a day for 56 days. The inclusion criteria for the diabetic children were: 1) 5-13 years old, 2) diabetes diagnosed >2 years ago, 3) living in Sassari and surrounding region (rural area within a distance of 30-40 km from the city), 4) good general health, and 5) average oral hygiene (cleaning the teeth at least twice a day). Exclusion criteria were: 1) ongoing oral/dental treatment except for emergency treatment, 2) known allergic reactions to an oral hygiene product and/or medication and/or dental material previously used in the mouth or pharynx, 3) pathological changes of the oral mucosa, 4) use of fluoride-containing products (pastes, mouthrinses) within the 14 days prior to the introduction of the intraoral appliances, 5) antibiotic therapy within the past six months, 6) any non-permitted therapy. The study was carried out from in two centers: the Clinic of Pediatrics at the University of Sassari, School of Medicine, the University of Sassari, Sassari and in the Clinic of Dentistry at the University of Sassari, School of Medicine, the University of Sassari, Sassari, Italy. The study was carraied out from May 2014 to May 2015. Proposed start of the recruitment of test subjects was June 2014. The study protocol was approved by the Ethical Committee of Sassari University of Medicine, Sassari, Italy [authorisation number 133/2014] and conducted according to the principles of the Helsinki Declaration II. It was obtained from all study participants and their parents before to start.

Collection of data

All subjects arrived the Clinic of Dentistry, University of Sassari for a clinical examination. A plaque sample was collected for microbiological analyses, saliva test for microbiological analyses and acidogenicity of dental plaque was made after a sucrose challenge at baseline and then after 30, 60, 90 days. At baseline the subjects compiled with answer a questionnaire. For the diabetic children, data on their medical condition was also from found their medical journals.

Clinical examination

At baseline the clinical examination was made in the Clinic Pediatric and Clinic Dentistry, University of Sassari under optimal lighting using a mirror and WHO-probe. The ICDAS

index was used for caries registration. The subjects were classified into the following four categories: caries-free (ICDAS 0), initial caries (ICDAS 1-2), moderate caries (ICDAS 3-4), and severe caries (ICDAS 5-6). No radiographic caries registration was made. [75]

Saliva samples

The collection the samples of saliva was performed after stimulated saliva by chewing on a piece of paraffin during 5 min and continuously spitting the obtained saliva into test tube. The samples of saliva were sent to Department of Microbiology, University of Bologna for evaluation with DNA Checkerboard Method for Bacterial Pathogen Identification and count bacterial strain of oral microflora.

Microbiological analyses

The analysis of the microbial flora was obtained by the checkerboard DNA-DNA hybridisation method [32]. Whole genomic probes were matched from 10 bacterial strains known to be associated to caries (Table 1). And measurement of the bacterial count in the samples was performed by matching the obtained signals with the ones generated by the pooled standard samples containing a count of 10^6 and 10^5 of each bacterial species, respectively. The signals were coded on a scale from 0 to 5: 0 = no signal; 1 = a signal density weaker than that of the low standard ($<10^5$ bacteria); 2 = a signal density equal to that of the low standard ($=10^5$ bacteria); 3 = a signal density higher than that of the low standard but lower than that of the high standard ($>10^5$ but $<10^6$ bacteria); 4=a signal density equal to that of the high standard ($=10^6$ bacteria) and 5 = a signal density higher than that of the high standard ($>10^6$ bacteria). Other information regarding the technique are retrived in the article "Intra-familial comparison of supragingival dental plaque microflora using the checkerboard DNA–DNA hybridisation technique". [35] [36]

Plaque acidogenicity

The plaque acidogenity was followed with the method of pH indicator strips. The strips measuring a pH value in the range of 4.0–7.0 (Spezialindikator, pH range 4.0–7.0; Merck, Darmstadt, Germany) were used [76]. This method allows to determine changes in plaque pH, discriminating differences at the level of 0.2–0.5 pH units following a sugar challenge to the same extent as the microtouch method (correlation coefficient 0.99). The use of strips is

easily and performing for a chair-side clinical use [77]. Each strip was cut into 4 pieces (approx. 2 mm in width) in order to get a strip that could be more simple inserted into the interproximal space. The strip was held into the interdental space for 10 s, after which it was removed and its color compared to the color index scheme supplied by the manufacturer. The pH was determined to one decimal of the value. For each subject, 3 measurements were carried out on 2 sites, between the 2nd premolar and the 1st molar right and left of the upper jaw or 2nd primary molar and 1st molar right and left of the upper jaw; in the case the patient has not erupted 1st molar the measurements were carried between 54/55 and 64/65. At each time point (t), measurements were performed before 0 min and at 2, 5, 10, 15, 20 and 30 min after a mouth rinse with 10% sucrose.

Questionnaire

The questionnaire was organized in the Italian language with closed questions was used to elicit information of oral hygiene and fluoride habits and their snaking and drinking habits. The informations was collected from answers to questions. Every answer was corresponded a code of intake frequency for drinking and snacking habits. They were registered the place of consumption of food and drink if they occurred at school or no-school. The answers were: never (Code 0), once a week (Code 1), two-four times a week (Code 2), five-six times a week (Code 3), once a day (Code 4), two-three times a day (Code 5) and over three times a day (Code 6). The answers of oral habits was obtained by questions like regularity and frequency toothbrushing, if the children used the mouthrinse and toothpaste fluoridate, frequency dental check ups and to intake supplement fluoride.

Statistical analyses

The mean approximal plaque pH (±ES) for all participants at the different time points was calculated for the two interproximal sites. The maximum pH fall and minimum pH after a 10% sucrose rinse were also calculated.. The salivary ms concentrations were transformed to log10 values to normalise the data, and the mean and standard error (SE) was calculated for each group and time point.

Study III

Sampling selection

A cross sectional pilot study was designed and approved by the Ethics Committee of the University of Sassari (2014_23_0598) and performed in Sassari (Italy) during academic year 2014-2015.

Sample size for this pilot study was performed through the online sample size calculator for pilot studies setting confidence at 0.99 and probability at 0.05. Prevalence of gingival disease among 13-15 year adolescences derived from a previous epidemiological study not yet published.

The estimated sample size was of 89.8 patients, but since two of the patients missed the appointment several times and no extra agreements were obtained, our final sample was 88 patients, which was considered quite enough to perform our cross sectional pilot study as well [78].

The project was first discussed with the adolescents in a way to get their interest and a better collaboration, then a letter explaining the aim and purpose of the study was sent to their parents associated with a consent form to be signed in order to attest their understanding of the contents and to authorize to enrol their child in the study.

Children with systemic medicals problems or those undergoing orthodontic treatment with fixed appliances were excluded.

A cluster sampling was performed using the class as a cluster; the number of school classes was inserted into the list and then randomly selected. The design and procedure of the study is displayed in figure 1.

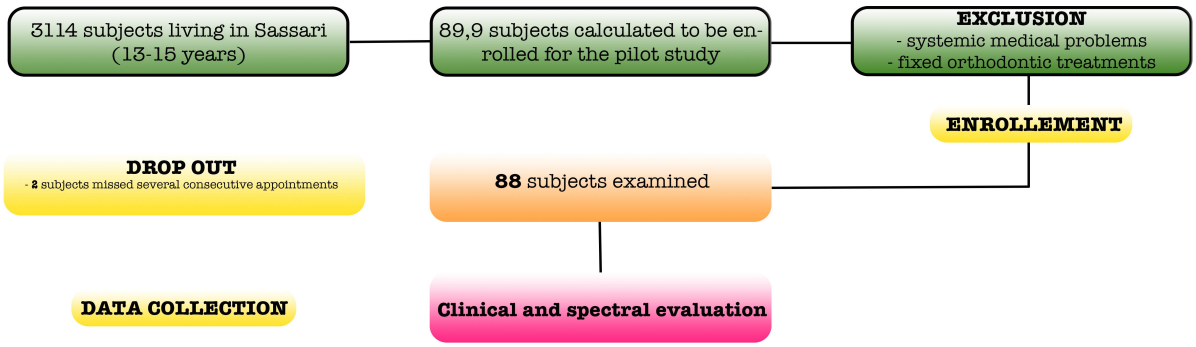

Figure 1. The design and procedure of the study III

Materials and measures

One single prior trained and calibrated examiner carried out spectral assessments assisted by a scribe across the whole study. All clinical examinations were performed under standard conditions in the infirmary of the high schools using natural daylight as a source of illumination. No radiographic examination was made.

To acquire spectra of gingival erythema a portable MHT Spectroshade™ Micro device was employed. This is a precision dental spectrophotometer largely used to evaluate the tooth colour and for this reason able also to be applied on soft tissues. Its illumination system consists in light emitting from 410 to 620 nm. It acquires calibrated images from 400 to 720 nm. A black and white charge-couple device (CCD) fitted with the system was used to capture the reflectance image.

Prior to the clinical assessment it was decided to consider the oral cavity as divided into sextants and to investigate just a tooth for each of them. Since the shape and the dimensions of the device enabled to detect gingival tissues from elements five to seven, a comparative assessment was completed including teeth 14, 11, 24, 34, 31, 44. That way each sextant was investigated anyway and two scans could be easily obtained.

Measurements of reflectance spectra were always performed with a special mouthpiece before clinical assessment of disease stage, as clinical examinations may disturb the sites causing bleeding, thereby interfering with the spectral measurements. Each data capture session was preceded by the calibration of the device through a green and white ceramic tile specific to the unit chosen. Once acquired each image captured was processed by a second examiner not

involved in the clinical session. Edge detection was applied to find the line contour corresponding to the gingival margin. To normalize the results, for each portion of gingival tissue selected spectral ratio (named below as Zn) was calculated considering the average with the two evaluations corresponding to the scans. The inflammation at the spectral reflectance ratio R(615)/(R460) was then calculated.

All patients were clinically assessed by recording the Gingival Index, the Plaque index and the Gingival bleeding after plaque removal scores. Superficial plaque was removed through a WHO ball ended probe. In view of the average age of the population, in order to avoid assessing false pockets associated with tooth eruption, no probe was inserted into the sulcus (World Health Organization, 2013).

Data analysis

All data, first collected in tables with Excel® software, were then moved and elaborated with Stata ® statistical software version 12. Before discussing the results some points have to be clarified.

Since Gingival Index, Gingival Bleeding and Plaque Index scores were discrete values and Zn was a continuous variable its scores have been categorised. Thus, from the analysis of the distribution, three ranges of scores (1= 6,42-7,57; 2= 8,82-10,05; 3= 10,21-11,47) were identified. Another point to be explained is that to calculate the frequency for sextant of plaque index variable, it was necessary to have unique values. Consequently the approximations described in table 2 were done.

Plaque Index Scores	Approximations
0,25	0
0,50	1
1,25	1
1,50	2
1,75	2

Table 2. Approximations among Plaque Index scores.

Results

Study I

Questionnaire and oral hygiene habits

The comparison among the diabetic and the control groups about questionnaire items are displayed in table 3 and 4 . Regarding sugared foods and drinks several behaviours demonstrated a statistically significant difference in the examined groups (see table 1 for detail). Diabetic subjects had a statistically higher consumption of fresh-squeezed juice, bottle-juice, energy drink, and potato chip at school compared to control group. The comparison between diabetes with good metabolic control and diabetes with bad metabolic control showed statistically significant differences ($p<0.05$) for consumption at school of bottle-juices, diet beverage and potato chip while the consumption of soda beverage and milk outside school was higher in diabetics with bad metabolic control ($p<0.05$).

The correlation among diabetes in bad metabolic control and diabetes in good metabolic control observed a statistical significant difference for fresh-squeezed juice OS ($p<0.01$), juice in bottle S and OS ($p=0.03$ and $p=0.01$), diet beverage OS ($p<0.0001$), soda beverage OS ($p=0.02$), milk S and OS($p=0.02$ and $p<0.01$), potato chip S ($p=0.01$), ice cream OS ($p<0.001$), vegetable OS ($p<0.001$), fruits S and OS ($p<0.01$ and $p<0.001$).

The relation among the subject with diabetes in good metabolic and control group showed a significant statistical for potato S ($p=0.03$), vegetable OS ($p=0.04$) and fruits OS ($p<0.01$).

For other answer about frequency for drinking and snacking habits, no statistically differences were found when comparing any of the groups.

The table 4 illustrates oral hygiene habits in the examined groups. The use of fluoridated mouthwash was higher in control group respect to diabetic subjects (p=0.04). The comparison among diabetes in good metabolic control and diabetes in bad metabolic control highlighted a significan statistical difference for regular toothbrushing (“mostly every day” p=0.03) and for use of fluoridadated toothpaste (“yes” p=0.03 and “no” p=0.03).

The correlation between diabetes in bad metabolic control and control group showed a significant statistical for use of fluoridated toothpaste (“yes” p<0.01 and “no” p<0.01).

The relation among diabetes in good metabolic control and control group was demonstrated a significant statistical for toothbrushing regularly (“mostly every day” p. 0.02) and for use of fluoride supplements (“somentimes” p. <0.05).

*Table 3. Diet questionnaire regarding sugared food and drinks consumption (in school or outside school). The replies were treated as continuous ordinal variables. The table reports statistically significant differences (p<0.05) regarding the °comparison among diabetes group and non-diabetic group. *comparison among diabetes in good metabolic control (HbA1c ≤7.5) and diabetes in bad metabolic control (HbA1c >7.5). ◊comparison among diabetes in bad metabolic control and non-diabetic group ^comparison among diabetes in good metabolic control and non-diabetic group*

.

		Diabetic		Control	
Diet questionnaire	**Total group** *mean±SD*	**good-metabolic** *mean+SD*	**bad-metabolic** *mean+SD*	**Total group** *mean+SD*	**p Value**
Fresh-squeezed juice					
In school	1.10±1.66	0.83±1.27	1.23±1.81	0.87±1.24	
Outside school	2.06±2.06	1.45±1.86	2.33±2.12	1.49±1.44	°0.02 ◊<0.01
Juice in bottle					
In school	1.43±1.61	0.80±1.40	1.68±1.65	1.16±1.35	*0.04 ◊0.03
Outside school	2.18±1.64	2.00±1.90	2.26±1.54	1.65±1.39	°0.02 ◊0.01
Energy drink					
In school	0.08±0.37	0.08±0.29	0.09±0.42	0.24±0.55	°0.03
Outside school	0.07±0.37	0.18±0.60	0.00±0.00	0.27±0.59	°0.01
Diet beverage					
In school	0.76±1.39	0.64±1.29	0.82±1.47	0.89±1.14	
Outside school	2.00±1.81	1.08±1.24	2.44±1.89	1.30±1.61	°*<0.01 ◊<0.01
Soda Beverage					
In school	0.47±0.91	0.25±0.45	0.58±1.06	0.44±0.7	
Outside school	0.91±1.46	0.36±0.80	1.17±1.63	0.72±0.94	*0.04 ◊0.02
Milk					
In school	1.30±1.64	1.08±1.50	1.44±1.76	0.89±1.26	°0.04 ◊0.02
Outside school	2.37±2.18	1.54±2.14	2.78±2.12	1.89±1.89	*0.03 ◊<0.01
Coffee/Tea					
In school	0.18±0.53	0.08±0.28	0.25±0.63	0.27±0.53	
Outside school	0.18±0.72	0.42±1.16	0.04±0.21	0.20±0.56	*0.03
Potato Chip					
In school	0.94±1.35	0.40±0.70	1.67±1.49	1.08±1.39	*<0.01 ◊0.01 ^0.03
Outside school	1.11±1.45	0.61±0.65	1.32±1.64	1.26+1.53	
Salted biscuit					
In school	0.97±1.31	1.10±1.37	0.92±1.32	0.82±1.12	
Outside school	1.15±1.37	1.20±1.23	1.12±1.45	1.06±1.26	
Sweets					
In school	1.33±1.67	1.10±1.85	1.42±1.63	1.20±1.44	
Outside school	1.85±1.48	1.64±0.92	1.93±1.65	1.68±1.39	
Cake/Biscuits					
In school	1.08±1.44	1.25±1.60	1.00±1.38	1.06±1.29	
Outside school	1.09±1.31	0.87±0.83	1.16±1.43	0.93±1.25	
Dessert					
In school	0.90±1.58	0.89±1.45	0.90±1.66	1.00±1.53	
Outside school	1.48±1.52	1.20±1.13	1.59±1.65	1.15±1.45	
Ice cream					
In school	0.47±0.73	0.45±0.82	0.47±0.70	0.73±0.94	°<0.05
Outside school	1.61±1.39	1.25±0.75	1.76±1.57	1.06±1.12	°◊0<.01
Vegetable					
In school	1.94±1.88	1.83±1.85	2.00±1.94	1.45±1.68	
Outside school	/2.83±1.82	2.70±1.42	2.87±1.95	1.83±1.77	°◊<0.01 ^0.04
Fruits					
In school	2.49±2.08	2.00±1.81	2.72±2.18	1.78±1.95	°0.02 ◊<0.01
Outside school	3.60±1.54	3.64±1.29	3.59±1.64	2.42±1.89	°◊^<0.01

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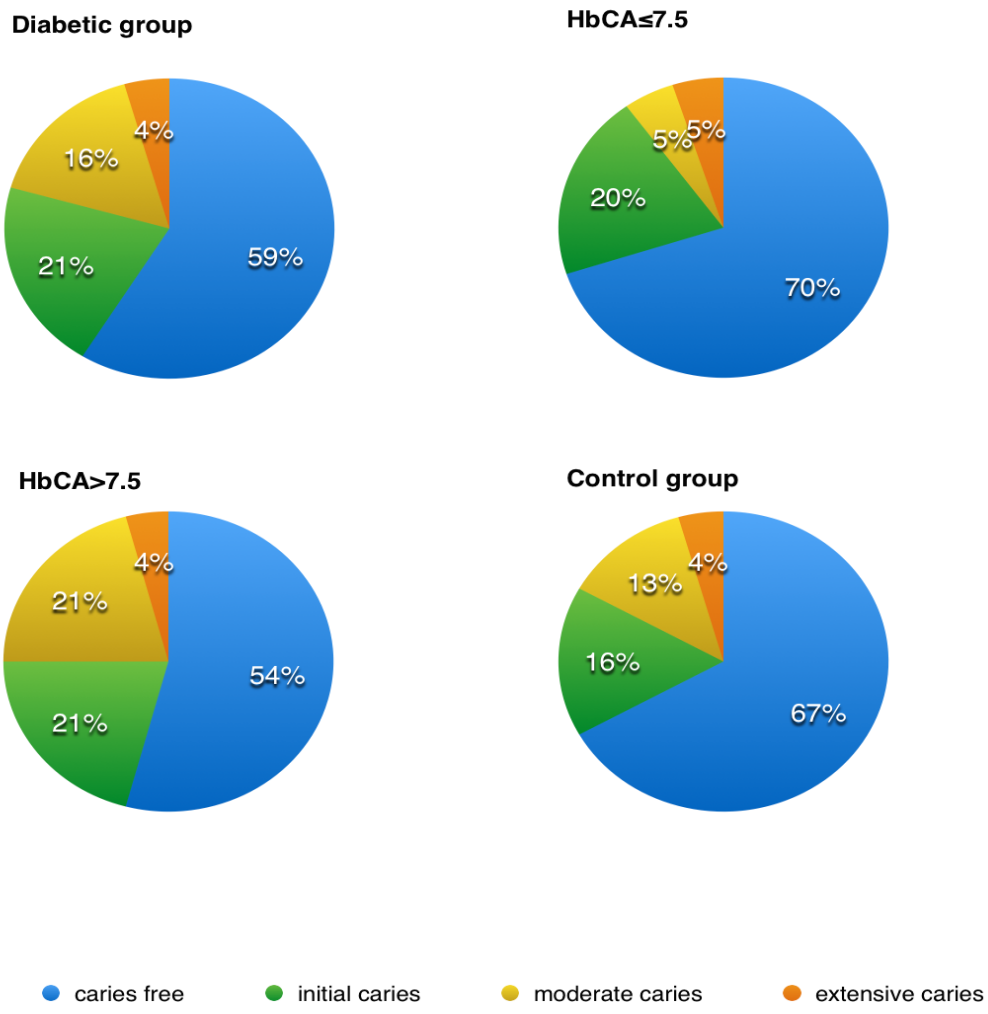
Table 4. *Oral hygiene habits in the examined groups. The replies were treated as continuous ordinal variables The table reports statistically significant differences (p<0.05) regarding the °comparison among diabetes group and non-diabetic group. *comparison among diabetes in good metabolic control (HbA1c ≤7.5) and diabetes in bad metabolic control (HbA1c >7.5). ◊comparison among diabetes in bad metabolic control and non-diabetic group ^comparison among diabetes in good metabolic control and non-diabetic group.*

		Diabetic		Control	
Oral hygiene Habits	**Total group** *% (n)*	**good-metabolic** *% (n)*	**bad-metabolic** *% (n)*	**Total group** *% (n)*	**p Value**
Do you brush your teeth regularly?					
No	72.1% (49)	65.0% (13)	75.0% (36)	72.1% (98)	
Sometimes	16.2% (11)	10.0% (2)	18.7% (9)	19.8% (27)	
Mostly every day	11.7% (8)	25.0% (5)	6.3% (3)	8.1% (11)	
Every day	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	*0.03 ^0.02
Toothbrusching frequency					
Once a day	16.2% (11)	10.0% (2)	18.7% (9)	19.9% (27)	
Twice a day	45.5% (31)	55.0% (11)	41.6% (20)	38.9% (53)	
Three time a day	32.4% (22)	25.0% (5)	35.4% (17)	35.3% (48)	
More than three times a day	5.9% (4)	10.0% (2)	4.1% (2)	5.9% (8)	
Fluoridated Toothpaste					
Yes	38.9% (21)	50.0% (10)	22.9% (11)	44.8% (61)	
No	69.1% (47)	50.0% (10)	77.1% (37)	55.2% (75)	*0.03 ◊<0.01
Fluoridated Mouthwash					
Yes	2.9% (2)	5.0% (1)	2.1% (1)	3.7% (5)	
Sometimes	17.7% (12)	10.0% (2)	20.8% (10)	30.8% (42)	
No	79.4% (54)	85.0% (17)	77.1% (37)	65.5% (89)	°0.04
Fluoride Supplements					
Yes	8.8% (6)	10.0% (2)	8.3% (4)	7.3% (10)	
Sometimes	16.2% (11)	25.0% (5)	12.5% (6)	9.6% (13)	
No	75.0% (51)	65.0% (13)	79.2% (38)	83.1% (113)	^<0.05
Dental check ups					
Only when in pain	22.1% (15)	10.0% (2)	27.1% (13)	27.9% (38)	
Each six months	26.5% (18)	20.0% (4)	29.2% (14)	31.6% (43)	
Once a year	41.2% (28)	55.0% (11)	35.4% (17)	33.9% (46)	
Every two years	10.2% (7)	15.0% (3)	8.3% (4)	6.6% (9)	

Clinical data

Caries data are presented in figure 2. Caries prevalence varies from 54% in diabetic subjects with good metabolic control to 70% in diabetic subjects in bad-metabolic control. Overall no statistically significant differences were observed between diabetic and control group, while the caries free subjects were statistically significant higher in diabetic subjects with good metabolic control compared diabetic subjects in bad-metabolic control. The other caries figures (Initial, Moderate and Extensive) were similar in all groups

Figure 2. Caries data



Microbiological analyses

The list of bacteria analysed and the association among diabetic subjects and control group is displayed in table 5. A significant association for *S. mutans, S. sobrinus, L. salivarius and L. fermentum* ($p < 0.05$) was found for the comparison among diabetic subjects and control group. The association between diabetic subjects with good metabolic control and bad metabolic control showed a significant association for the bacteria strain of S. Salivarius, L. Fermentum, L. Casei, S. Salivarium and S. Sobrinus ($p<0.05$).

A significant association was observed in relation to diabetic in bad control and control group for bacteria S. Sobrinus, S. Salivarius, L. Fermentum (p. < 0.01), L. Salivarius and L. Casei (p. 0.02), S. Mutans (p. <0.03). The relation between diabetes in good metabolic control and control group showed for S. Mutans (p. 0.05). For same bacteria, no statistically differences were found when comparing any of the groups.

Table 5. Microbiological analysis in the examined groups. The data were treated as continuous ordinal variables.

		Diabetic		Control	
Bacterial strain	**Total group**	**good-metabolic**	**bad-metabolic**	**Total group**	**p Value**
	mean±SD (range)	***mean±SD (range)***	***mean±SD (range)***	***mean±SD (range)***	
S. Mutans	3.38±1.15 (1-5)	3.35±1.19 (1-5)	3.35±1.15 (1-5)	2.83±1.07 (1-5)	°^0.04 ◊0.03
S. Sobrinus	1.88±0.70 (1-4)	1.70±0.47 (1-2)	1.96±0.77 (1-4)	1.27±0.96 (1-5)	°0.02 *0.04 ◊<0.01
S. Sanguinis	2.70±1.01 (1-5)	2.70±1.08 (1-5)	2.71±0.99 (1-5)	2.69±1.01 (1-5)	
S. Salivarius	2.81±1.07 (1-5)	2.60±0.94 (1-5)	2.89±1.11 (1-5)	2.46±0.95 (1-5)	°0.03 *0.04 ◊<0.01
S. Mitis	2.46±1.01 (1-5)	2.35±1,04 (1-5)	2.54±1.01 (1-5)	2.22±1.04 (1-5)	
S. Gordoni	2.23±0.98 (1-5)	2.10±0.85 (1-4)	2.29±1.03 (1-5)	2.21±0.87 (1-5)	
NSM	2.37±1.12 (1-5)	2.35±1.09 (1-5)	2.37±1.14 (1-5)	2.38±1.19 (1-5)	
L. Casei	2.03±0.90 (1-5)	1.85±0.67 (1-3)	2.10±0.97 (1-5)	1.98±1.03 (1-5)	*0.04 ◊0.02
L. Salivarius	2.48±0.95 (1-5)	2.45±1.10 (1-5)	2.50±0.90 (1-5)	2.27±1.15 (1-5)	°0.03 ◊0.02
L. Fermentum	2.48±0.97 (1-5)	2.25±0.85 (1-4)	2.58±1.01 (1-5)	2.15±1.21 (1-5)	°*0.03 ◊<0.01

Plaque pH measurements

The results from the plaque pH measurements are shown in table 6. The pH values recorded statistically significant differences for minimum pH, $AUC_{5.7}$ and $AUC_{6.2}$ between all diabetes subjects and the control group ($p<0.01$ or $p<0.05$) as well as between the subjects with bad metabolic control and the control group ($p<0.05$ or $p<0.01$). Comparison between the two

diabetes groups showed statistically significant differences for all four variables ($p<0.05$ or $p<0.01$). A statistically significant difference with more pronounced pH-falls were found during the whole 30-min period for the diabetes group vs the control group as well as for the subjects with good metabolic control compared to those with bad metabolic control.

Table 6. Plaque-pH (minimum pH, Maximum pH fall, $AUC_{6.2}$ $AUC_{5.7}$) evaluation.

	Diabetes Group *mean±SD*	HbA1c ≤ 7.5 *mean± SD*	HbA1c >7.5 *mean± SD*	Control group *mean± SD*	p-value
Minimum pH	5.35±0.45	5.69±0.24	5.21±0.45	6.02 ±0.30	°^*◊<0,01
Maximum pH fall	1.19±0.51	0.98±0.30	1.28±0.55	0.92±0.22	°*◊<0,01
$AUC_{6.2}$	5.79±3.2	5.54±3.82	6.25±3.41	3.06±1.48	°^*◊<0,01
$AUC_{5.7}$	2.22±1.27	1.97±2.28	2.38±1.38	1.27±1.82	°^*◊<0,01

°comparison among diabetes group and control group,
*comparison among diabetes in good metabolic control (HbA1c ≤7.5) and diabetes in bad metabolic control (HbA1c >7.5),
◊comparison among diabetes in bad metabolic control and control group
^ comparison among diabetes in good metabolic control and control group

Study II

Questionnaire

The results showed in table 7. At baseline the association among diabetes group and control group showed a significant association for fresh-squeezed juice no-school (p. 0.02), milk no school (p. <0.03), salted biscuit school (p. <0.04) , vegetable no-school (p. <0.04),

For other answer about frequency for drinking and snacking habits , no statistically differences were found when comparing any of the groups.

Table 7. The questionnaire study II.

Questionnaire	Diabetic Group1	Diabetic Group 2	p Value
Fresh-squeezed juice (S) Fresh-squeezed juice (NS)	0,90±1,55 1,44±1,75	1,35±1,80 2,59±2,19	NS 0,02
Juice in bottle (S) Juice in bottle (NS)	1,56±1,89 2,29±1,72	1,29±1,31 2,10±1,61	NS NS
Energy drink (S) Energy drink (NS)	0,15±0,50 0,00±0,00	0,00±0,00 0,13±0,50	NS NS
Diet beverage (S) Diet beverage (NS)	0,65±1,22 1,75±1,95	0,90±1,59 2,19±1,72	NS NS
Gas Beverage (S) Gas Beverage (NS)	0,44±0,70 0,63±1,15	0,50±1,10 1,16±1,68	NS NS
Milk (S) Milk (NS)	1,,20±1,61 1,67±2,23	1,40±1,72 2,80±2,08	NS 0,03
Coffee/The (S) Coffee/The (NS)	0,25±0,58 0,00±0,00	0,12±0,49 0,31±0,95	NS NS
Potato Chip (S) Potato Chip (NS)	1,12±1,45 0,89±0,96	0,76±1,25 1,27±1,71	NS NS
Salted biscuit (S) Salted biscuit (NS)	0,63±0,96 1,23±1,36	1,28±1,53 1,10±1,41	0,04 NS
Sweets (S) Sweets (NS)	1,44±1,72 2,11±1,18	1,22±1,66 1,62±1,69	NS NS
Cake/Biscuits (S) Cake/Biscuits (NS)	1,35±1,46 1,46±1,51	0,76±1,39 0,85±1,14	NS NS
Dessert (S) Dessert (NS)	0,67±1,18 1,33±1,40	1,13±1,89 1,59±1,62	NS NS
Ice cream (S) Ice cream (NS)	0,57±0,76 1,75±1,53	0,38±0,72 1,52±1,33	NS NS
Vegetable (S) Vegetable (NS)	1,90±1,89 2,29±1,69	2,00±1,94 3,20±1,85	NS 0,04
Fruits (S) Fruits (NS)	2,58±2,04 3,94±1,43	2,39±2,17 3,38±1,60	NS NS

Oral hygiene habit

The table 8 showed the results of oral hygiene habit .The association between diabetes group and control group showed a not statistically significant difference for all questions.

Table 8 Oral hygiene habit study II.

FACTOR	Diabetic 1	Diabetic 2	p Value (Chi-Quadro)
Oral Hygiene Habits			
Do you brush your teeth regularly? No Sometimes Mostly every day Every day	 70,6% (24) 17,6% (6) 11,8% (4) 0,0% (0)	 73,5 % (25) 14,7% (5) 11,8% (4) 0,0% (0)	 NS NS NS NS
Toothbrusching frequency Once a day Twice a day Three time a day More than three times a day	 11,8% (4) 50,0% (17) 29,4% (10) 8,8% (3)	 20,6% (7) 41,2% (14) 35,3% (12) 2,9% (1)	 NS NS NS NS
Fluoridated Toothpaste Yes No	 29,4% (10) 68,6% (24)	 32,4% (11) 67,6% (23)	 NS NS
Fluoridated Mouthwash Yes Sometimes No	 0,0% (0) 26,5% (9) 73,5% (25)	 5,9% (2) 8,8% (3) 85,3% (29)	 NS NS NS
Fluoride Supplements Yes Sometimes No	 5,9% (2) 20,6% (7) 73,5% (25)	 11,8% (4) 11,8% (4) 76,4% (26)	 NS NS NS
Dental check ups Only when in pain Each six months Once a year Every two years	 20,6% (7) 29,4% (10) 41,2% (14) 8,8% (3)	 23,5% (8) 23,5% (8) 41,2% (14) 11,8% (4)	 NS NS NS NS

Clinical data

The results are presented in table 9. Comparison between two groups showed no one different for status caries.

Table 9. Status caries Diabetic group 1 (control group) and Diabetic group 2 (test group)

Caries	Diabetic group 1	Diabetic group 2	p Value
Caries free	55,9% (19)	61,9% (21)	NS
Initial caries	23,5% (8)	17,6% (6)	NS
Moderate caries	14,7% (5)	17,6% (6)	NS
High caries	5,9% (2)	2,9% (1)	NS

Plaque pH measurements

The results from the plaque pH measurements are shown in table 10,11,12. The pH values recorded no statistically significant differences for maximun fall pH in each group after 90 days. No significant differences for control group when it valueted the minimal pH. A significant association for the test group with p. <0.001 after 90 days when it considered minimal pH. Comparison between the two diabetes groups showed no statistically significant differences when compared each group at baseline and after 90 days.

Table 10. Plaque pH measurement for control group at baseline, 30 days, 60 days, 90 days.

DIABETIC GROUP 1	TIME 0	TIME 1	TIME 2	TIME 3	P VALUE
FALL PH	1,18±0,56	1,13±0,51	1,06±0,45	1,07±0,51	NS
MINIMAL PH	5,34±0,50	5,39±0,44	5,48±0,40	5,53±0,43	NS

Table 11. Plaque pH measurement for test group at baseline, 30 days, 60 days, 90 days.

DIABETIC GROUP 2	TIME 0	TIME 1	TIME 2	TIME 3	P VALUE
FALL PH	1,20±0,46	1,10±0,42	1,02±0,35	0,98±0,29	NS
MINIMAL PH	5,37±0,41	5,46±0,37	5,59±0,29	5,69±0,24	<0,001

Table 12. Comparison test group and control group at baseline and after 90 days

	TIME 0 GROUP 1	TIME 0 GROUP 2	TIME 3 GROUP 1	TIME 3 GROUP 2	P VALUE
FALL PH	1,18±0,56	1,20±0,46	1,07±0,51	0,98±0,29	* NS ^NS
MINIMAL PH	5,34±0,50	5,37±0,41	5,53±0,43	5,69±0,24	* NS ^ NS

Microbiological analyses

The table 13 show the list of bacteria analysed and the association among diabetic test group and diabetic control group. A significant association for test group after intake of lozange of probiotic at 90 days (p.<0,001); for control group only *S. Sobribus* no one significant association.

The association between diabetic test group with diabetic control group showed a significant association at baseline only for the bacteria strain of *S. Salivarius* (p.<0.04) and *L. Casei* (p.<0.03);

after 90 days the comparison among diabetic test group and diabetic control group recorded a significant association for *S. Mutans, S. Sobrinus, L. Casei, L. Salivarius* (p. <0.01) and *S. Salivarius* (p.<0.02).

For other bacteria, no one statistically differences were found when comparing the groups in different time.

Table 13. Microbiological analyses (* control group, ° test group, ^ comparison among control group and test group baseline, ∞ comparison among control group and test group after 90 days)

Bacteria Strain	Diabetic Group 1				Diabetic Group 2				P Value
	Time 0	Time 1	Time 2	Time 3	Time 0	Time 1	Time 2	Time3	
S. Mutans	3,61±1,13	2,15±0,65	1,82±0,72	1,56±0,56	3,09±1,08	1,76±0,60	1,32±0,47	1,21±0,43	*<0,001 °<0,001 ^NS ∞<0,01
S. Sobrinus	1,76±0,74	1,50±0,70	1,47±0,66	1,44±0,66	2,00±0,65	1,20±0,41	1,11±0,32	1,06±0,24	*NS °<0,001 ^NS ∞ <0,01
S. Sanguinis	2,50±0,86	1,74±0,82	1,65±0,81	1,47±0,71	2,89±1,12	1,68±0,64	1,53±0,61	1,29±0,46	*<0,001 °<0,001 ^NS ∞ NS
S. Salivarius	2,14±1,01	1,64±0,69	1,59±0,61	1,47±0,56	2,61±0,92	1,56±0,50	1,32±0,47	1,20±0,41	<0,001 °<0,001 ^<0,04 ∞ <0,02
S. Mitis	2,26±1,08	1,50±0,75	1,38±0,65	1,32±0,59	2,56±1,11	1,56±0,66	1,41±0,56	1,12±0,33	*<0,001 °<0,001 ^NS ∞ NS
S. Gordoni	2,47±0,89	1,47±0,51	1,44±0,50	1,32±0,47	2,32±0,94	1,41±0,56	1,35±0,49	1,17±0,39	*<0,001 °<0,001 ^NS ∞ NS
L. Casei	3,00±1,81	1,76±0,70	1,65±0,60	1,47±0,51	2,24±1,05	1,38±0,55	1,32±0,47	1,18±0,39	*<0,01 °<0,001 ^<0,03 ∞ <0,01
L. Salivarius	2,09±0,62	1,65±0,65	1,68±0,72	1,53±0,66	2,50±1,05	1,50±0,62	1,35±0,48	1,15±0,36	*<0,01 °<0,001 ^NS ∞ <0,01
L. Fermentum	2,47±0,86	1,41±0,50	1,35±0,49	1,29±0,46	2,26±0,90	1,38±0,55	1,26±0,45	1,21±0,41	*<0,001 °<0,001 ^NS ∞ NS

Study III

Results

The majority of this cluster randomized sample is composed of individuals with no signs of gingival inflammation (72,63%), and 41,90% of the sample has a perfect oral hygiene (P.I. =0). In the remaining portion of the sample frequency for mild gingivitis is higher than for moderate ones (data not in table).

As regards the correlation among clinical records and Zn categorised values (Table 14), a significant P value shows how Zn Categorised Value 1 is related to healthy status of gums ($p<0.01$) in absence of plaque ($p<0.01$) or gingival bleeding ($p<0.01$). The same strong correlation ($p<0.01$) is confirmed as regards Zn categorised value 2 and mild gingivitis in absence of gingival bleeding. Zn Categorised at value 3 proved to be strictly related with moderate presence of dental plaque ($p<0.01$) and moderate gingivitis ($p<0.01$).

A strong correlation ($p<0.01$) is evidenced also among the absence of gingival inflammation, the absence of gingival bleeding the absence of dental plaque; similarly, mild gingival inflammation relates both with the presence of dental plaque and gingival bleeding. No correlation was found neither between the spectral values categorised at value 2 and the low presence of dental plaque nor between mild gingival inflammation and low presence of plaque on dental surfaces.

Table 14 Correlation matrix among spectral values and clinical variables..

	Absence of dental plaque	Low dental plaque	Moderate dental plaque	Absence of gingival bleeding	Presence of Gingival bleeding	Absence of gingival inflammation	Mild gingival inflammation	Moderate Gingival inflammation	Zn Cat. Value 1	Zn Cat. Value 2	Zn Cat Value 3
Absence of dental plaque	1.00										
Low dental plaque		1.00									
Moderate dental plaque			1.00								
Absence gingival bleeding	0.50 ***p<0.01***	0.12 *p>0.01*		1.00							
Presence of gingival bleeding			0.12 *p>0.01*		1.00						
Absence gingival inflammation	0.55 **p<0.01**			0.78 **p<0.01**		1.00					
Mild gingival inflammation		0.22 p>0.01		0.36 **p<0.01**			1.00				
Moderate gingival inflammation			0.48 **p<0.01**		0.42 **p<0.01**			1.00			
Zn Cat. Value 1	0.48 **p<0.01**			0.54 **p<0.01**		0.71 **p<0.01**			1.00		
Zn Cat. Value 2		0.10 p>0.01		0.50 **p<0.01**			0.55 **p<0.01**			1.00	
Zn Cat. Value 3			0.42 **p<0.01**		0.14 p>0.01			0.47 **p<0.01**			1.00

Discussion

Study I

The aim of this paper was to evaluate the different caries-related variables between diabetic and non-diabetic children aged 5-13 years old as well to compare the different caries-related variables of the diabetic children in relation to their metabolic control.

The main outcome of the study was that the risk for caries was higher in diabetic population when compared to the control group. The higher caries risk factor was due to the change of microflora bacteria, higher assumption for food/drink sweet, lower initial and final pH in diabetic group. These figures are even more pronounced when diabetic population was split in subjects bad metabolic control and subjects with good metabolic control. The result of plaque pH measurement after a sucrose rinse showed a trend towards a more important different pH drop in diabetic subjects with bad control metabolic and control group. One interesting observation was when we have been compared the subjects with metabolic control and group control because there is many difference for the value AUC pH 6.2 (3.06+4.48) / (6.25+7.41) and the value AUC pH5.7 (1.27+1.82) / (2.38+4.38). A statistically significant difference was recorded between the group with poor metabolic control and good metabolic control. In other

studies it was demonstrated that when the uncontrolled diabetics were compared with the non-diabetics had a decreased salivary pH; this can be explained to the changes in the metabolic process of the uncontrolled diabetic resulting in acidic pH and thus increased incidence of dental caries [30]. Yet, in this study the group with good metabolic control has the same status caries, different diet and the similar trend for the pH measurement when we was compared by the subjects with bad metabolic control To authors' knowledge no study on plaque acidogenecity was present. The strip-method used in this study is easy and appropriate for a chair-side clinical use [77]. The pH measurements in this study were performed in the interproximal space between the first and the second primary molars of the upper jaw; however, it is impossible to distinguish between plaque covering the adjacent tooth, *i.e.,* mesial and distal proximal surfaces.

The high prevalence of diagnosis diabetes type 1 in Sardegna doesn't permit to declare that the population is alike in all Italy. The highest incidence is seen in Finland. Sardegna in Italy together with Finland and Sweden are known to have the highest incidence of Type 1 diabetes in the world. The our sample can be compared with area such as Finland or Sweden. [3] [79] [80]

In other studies there are contradiction, it has been found that diabetic patients may have salivary dysfuction as well as different microbiologic salivary composition compared to non-diabetic . Yet, the salivary antimicrobial defence of diabetic patients may be better than that of non-diabetic individual, due to increased concentration of selected protective factor in saliva [16] [17] [18] [19], [20].

The relationship among diet and dental caries is often a question of particular interest when diabetes mellitus is concerned. Yet, possible variations in the dietary habits of diabetic patients compared to non- diabetic individuals might have an effect on caries increments over time, and change the analysis of the relation between dental caries and diabetes mellitus.

However, in the subjects found some differences between the diabetics and their controls with respect to frequency of meals or consumption of carbohydrates, as assessed by questionnaires. The factors of diet were not considered to contribute significantly to the effect of diabetes-related factors on the caries outcomes in this study.

The most important correlation was showed in the relationship amoug diabetic in bad metabolic control and control group, the bacteria S. Sobrinus, S. Salivarius, L. Fermentum ($p. < 0.01$) and L. Salivarius, L. Casei , S. Mutans ($p. <0.05$). Yet, in other comparisions have been recorded a significally correlation for the bacteria strain of L. Salivarius, L. Fermentum, L. Casei, S. Salivarium and S. Sobrinus ($p. <0.05$) between diabetic subjects with in good metabolic control and bad metabolic control; among diabetic subjects and control group was demonstrated a significant association for S. Mutans, S. Sobrinus, L. Salivarius and L. Fermentum ($p. < 0.05$).

The higher counts of bacteria like S. Mutans, S. Sobrinus, L. Salivarius and L. Fermentum in diabetic group may be correlated by glucose level in saliva in diabetic patients, in other study was found that salivary glucose values were higher among diabetic that in non-diabetic [81] and increased glucose concentrations in saliva of diabetics may origin as a result of no-correct neural regulation of the salivary gland function. [17].

The count of S. Mutans is similar when it was compared bad metabolic group and good metabolic group, this observation may be related only the variability diabetic type 1 because we were recorded different habit for drink/food.

Study II

The aim of this study was to evaluate the effect of probiotics (*Lactobacillus Brevis CD2*) on the oral microbiotic flora and biofilm acidogenicity in children diagnosed with diabetes since at least two years. Primary objective is the regular consumption of probiotic will lead to a significantly reduction of pathogenic bacteria compared to a placebo.

One of strengths of this study is that the study group consisted of children with diabetic type 1, with diagnoses diabetes at least 2 years, same oral hygiene habit and status caries for each group; for answer about frequency for drinking and snacking habits , no statistically differences were found when comparing any of the groups eccept for fresh-squeezed juice no-school (p. 0.02), milk no school (p. <0.03), salted biscuit school (p. <0.04) , vegetable no-school (p. <0.04) but we can say that in spite of these data the two groups can be considered similar.
One limitation in the present study is the number of children was quite small (n=34+34); this may influence the statistical comparison.
The our sample can be compared with area such as Finland or Sweden because the high prevalence of diagnosis diabetes type 1 is similar for these nations, they are known to have the highest incidence of Type 1 diabetes in the world.[79] [80]

In the subjects found some differences between the diabetics and their controls with respect to frequency of meals or consumption of carbohydrates, as assessed by questionnaires. The factors of diet were not considered to contribute significantly to the effect of diabetes-related factors on the caries outcomes in this study.

Oral hygiene habits don't demonstrate significant difference for the questions about toothbrushing frequency and dental check ups. Considering the caries status in each group we might suppose that the answers for oral hygiene habits of the all groups were not highlighted significant differences for our study.

The results from the plaque pH measurements are proved the pH values recorded no statistically significant differences for maximun fall pH in each group after 90 days. No one significant differences for control group when it valueted the minimal pH. A significant association for the test group with p. <0.001 after 90 days when it considered minimal pH. Comparison between the two diabetes groups showed no statistically significant differences when compared each group at baseline and after 90 days. After use of probiotics there was an improvement concerning the minimal ph in test group.

For bacteria analysed in diabetic test group and diabetic control group was found a significant association for test group after intake of lozange of probiotic at 90 days (p.<0,001); for control group only *S. Sobribus* no one significant association after 90 days.
The association between diabetic test group with diabetic control group showed a significant association at baseline only for the bacteria strain of *S. Salivarius* (p.<0.04) and *L. Casei* (p.<0.03);
after 90 days the comparison among diabetic test group and diabetic control group recorded a significant association for *S. Mutans, S. Sobrinus, L. Casei, L. Salivarius* (p. <0.01) and *S. Salivarius* (p.<0.02).
For other bacteria, no one statistically differences were found when comparing the groups in different time.

Study III

The hypotheses of this study was that reflectance spectrophotometry could be use even in cross sectional population studies. Here it has been proved that particularly spectra intensity ratio Zn (R 615/460) can be applied in that way to differentiate healthy and inflamed gums.

To the authors' knowledge, this was the first cross-sectional study carried out to evaluate the comparison between clinical indices about gingival inflammation and reflectance spectrophotometry. Generally, all the previous studied aimed to longitudinally assess the variance between spectral and clinical recordings in experimentally induced volunteers.

The most attractive aspect about reflectance spectrophotometry is that it is totally non-invasive, real-time, non-dangerous and it do not cause any discomfort to the patients during the procedure.

However, it is important to consider that since the dental arch morphology is not linear, as we go posteriorly towards the premolars and molar also gingival angulation changes. Thus any instrument, which is not dedicated to this aim, cannot detect with some certain precision teeth from fourth on. This leads obviously to a particular reflection.

On the base of the results it was possible to postulate that excluding the first molars from this assessment a statistic bias was generated.

On one hand it is in fact possible that registering first premolars scores in substitution of the molars' might be a bias, since they are nearest to the anterior sextant and thus generally assisted to be kept cleaned.

On the other hand, it is also important to consider that especially for the upper sextants the prevalence of the dental arch crowding is high and it mainly involves the third elements with a significant plaque retention in this region often affecting also the first premolars.

Nevertheless, since neither the variable rotation of the canine nor its alignment was considered in the present pilot study, this hypothesis could not be precisely disproved.

It is also important to consider that they have been enrolled only healthy adolescents declaring not to have any luxury habits. It would be interesting to assess if the results could change in case of a population deferring for ethnicity, average age or life habits.

However results could change, it is clear that reflectance spectrophotometry allows for permanent recording of changes in inflammation, helping operators in diagnosis as soon as the inflammation is taking place.

Despite the instrument chosen has been designed primarily to include the smile line elements and not actually for the purpose in which we approached with it, this pilot study demonstrated that its reliability could significantly contribute to guide the community health interventions to an increasingly preventive direction. Further studies should be performed to better understand if this system could be even able to predict gingival inflammation before clinical signs are manifest.

Conclusion

The conclusion after showed the resuls are:

Study I: Diabetes mellitus may have a direct effect on salivary pH reducing it from normal levels irrespective of diet.

Study II: The results of this study show that use of probiotic may improve the minimal pH in Diabetic children and may decrease the bacterial flora species as S. Mutans, S. Sobrinus, L. Casei, L. Salivarius decreasing the caries risk with good diet and oral hygiene.

Study III : Despite the shape of the mouthpiece of the instrument chosen, which has been design primarily to include the smile line elements and not for the manner in which we approached with it, this study demonstrated that its reliability could significantly contribute to guide the community health interventions.

References

1. American Diabetes, A., *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2014. **37 Suppl 1**: p. S81-90.
2. Singh-Husgen, P., et al., *Investigation of the oral status and microorganisms in children with phenylketonuria and type 1 diabetes.* Clin Oral Investig, 2016. **20**(4): p. 841-7.
3. Group, D.P., *Incidence and trends of childhood Type 1 diabetes worldwide 1990-1999.* Diabet Med, 2006. **23**(8): p. 857-66.
4. Kuzuya, T., et al., *Report of the Committee on the classification and diagnostic criteria of diabetes mellitus.* Diabetes Res Clin Pract, 2002. **55**(1): p. 65-85.
5. American Diabetes, A., *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2013. **36 Suppl 1**: p. S67-74.
6. Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes, M., et al., *Report of the committee on the classification and diagnostic criteria of diabetes mellitus.* J Diabetes Investig, 2010. **1**(5): p. 212-28.

7. *Variation and trends in incidence of childhood diabetes in Europe. EURODIAB ACE Study Group.* Lancet, 2000. **355**(9207): p. 873-6.
8. Vehik, K., et al., *Increasing incidence of type 1 diabetes in 0- to 17-year-old Colorado youth.* Diabetes Care, 2007. **30**(3): p. 503-9.
9. Hill, C., et al., *Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic.* Nature reviews Gastroenterology & hepatology, 2014. **11**(8): p. 506-14.
10. Devine, D.A. and P.D. Marsh, *Prospects for the development of probiotics and prebiotics for oral applications.* J Oral Microbiol, 2009. **1**.
11. Stamatova, I. and J.H. Meurman, *Probiotics: health benefits in the mouth.* Am J Dent, 2009. **22**(6): p. 329-38.
12. Koll-Klais, P., et al., *Oral lactobacilli in chronic periodontitis and periodontal health: species composition and antimicrobial activity.* Oral Microbiol Immunol, 2005. **20**(6): p. 354-61.
13. Goteiner, D., et al., *Periodontal and caries experience in children with insulin-dependent diabetes mellitus.* J Am Dent Assoc, 1986. **113**(2): p. 277-9.
14. Miko, S., et al., *Dental caries and adolescents with type 1 diabetes.* Br Dent J, 2010. **208**(6): p. E12.
15. Saes Busato, I.M., et al., *Association between metabolic control and oral health in adolescents with type 1 diabetes mellitus.* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2010. **109**(3): p. e51-6.
16. Siudikiene, J., et al., *Dental caries and salivary status in children with type 1 diabetes mellitus, related to the metabolic control of the disease.* Eur J Oral Sci, 2006. **114**(1): p. 8-14.
17. Mata, A.D., et al., *Effects of diabetes mellitus on salivary secretion and its composition in the human.* Mol Cell Biochem, 2004. **261**(1-2): p. 137-42.
18. Negrato, C.A. and O. Tarzia, *Buccal alterations in diabetes mellitus.* Diabetol Metab Syndr, 2010. **2**: p. 3.
19. Siudikiene, J., et al., *Dental caries increments and related factors in children with type 1 diabetes mellitus.* Caries Res, 2008. **42**(5): p. 354-62.
20. Lopez, M.E., et al., *Salivary characteristics of diabetic children.* Braz Dent J, 2003. **14**(1): p. 26-31.
21. Singh-Husgen, P., et al., *Investigation of the oral status and microorganisms in children with phenylketonuria and type 1 diabetes.* Clin Oral Investig, 2015.
22. Tagelsir, A., et al., *Dental caries and dental care level (restorative index) in children with diabetes mellitus type 1.* Int J Paediatr Dent, 2011. **21**(1): p. 13-22.
23. Chandki, R., P. Banthia, and R. Banthia, *Biofilms: A microbial home.* J Indian Soc Periodontol, 2011. **15**(2): p. 111-4.
24. Filoche, S., L. Wong, and C.H. Sissons, *Oral biofilms: emerging concepts in microbial ecology.* J Dent Res, 2010. **89**(1): p. 8-18.
25. Marsh, P.D., A. Moter, and D.A. Devine, *Dental plaque biofilms: communities, conflict and control.* Periodontol 2000, 2011. **55**(1): p. 16-35.
26. Beighton, D., *Can the ecology of the dental biofilm be beneficially altered?* Adv Dent Res, 2009. **21**(1): p. 69-73.

27. ten Cate, J.M., *Biofilms, a new approach to the microbiology of dental plaque.* Odontology, 2006. **94**(1): p. 1-9.
28. Pozhitkov, A.E., et al., *High-throughput methods for analysis of the human oral microbiome.* Periodontol 2000, 2011. **55**(1): p. 70-86.
29. Sakamoto, M., M. Umeda, and Y. Benno, *Molecular analysis of human oral microbiota.* J Periodontal Res, 2005. **40**(3): p. 277-85.
30. Nyvad, B., et al., *Dental caries from a molecular microbiological perspective.* Caries Res, 2013. **47**(2): p. 89-102.
31. Socransky, S.S., et al., *"Checkerboard" DNA-DNA hybridization.* Biotechniques, 1994. **17**(4): p. 788-92.
32. Wall-Manning, G.M., et al., *Checkerboard DNA-DNA hybridisation technology focused on the analysis of Gram-positive cariogenic bacteria.* J Microbiol Methods, 2002. **51**(3): p. 301-11.
33. Brito, L.C., et al., *Use of multiple-displacement amplification and checkerboard DNA-DNA hybridization to examine the microbiota of endodontic infections.* J Clin Microbiol, 2007. **45**(9): p. 3039-49.
34. Socransky, S.S. and A.D. Haffajee, *Periodontal microbial ecology.* Periodontol 2000, 2005. **38**: p. 135-87.
35. Mannaa, A., et al., *Intra-familial comparison of supragingival dental plaque microflora using the checkerboard DNA-DNA hybridisation technique.* Archives of oral biology, 2012. **57**(12): p. 1644-50.
36. Mannaa, A., et al., *Supragingival plaque microbial analysis in reflection to caries experience.* BMC Oral Health, 2013. **13**: p. 5.
37. Caglar, E., et al., *Oral colonization by Lactobacillus reuteri ATCC 55730 after exposure to probiotics.* Int J Paediatr Dent, 2009. **19**(5): p. 377-81.
38. Caglar, E., et al., *A probiotic lozenge administered medical device and its effect on salivary mutans streptococci and lactobacilli.* Int J Paediatr Dent, 2008. **18**(1): p. 35-9.
39. Caglar, E., et al., *Effect of chewing gums containing xylitol or probiotic bacteria on salivary mutans streptococci and lactobacilli.* Clin Oral Investig, 2007. **11**(4): p. 425-9.
40. Nase, L., et al., *Effect of long-term consumption of a probiotic bacterium, Lactobacillus rhamnosus GG, in milk on dental caries and caries risk in children.* Caries Res, 2001. **35**(6): p. 412-20.
41. Stecksen-Blicks, C., I. Sjostrom, and S. Twetman, *Effect of long-term consumption of milk supplemented with probiotic lactobacilli and fluoride on dental caries and general health in preschool children: a cluster-randomized study.* Caries Res, 2009. **43**(5): p. 374-81.
42. Petersson, L.G., et al., *Reversal of primary root caries lesions after daily intake of milk supplemented with fluoride and probiotic lactobacilli in older adults.* Acta Odontol Scand, 2011. **69**(6): p. 321-7.
43. Teughels, W., G. Loozen, and M. Quirynen, *Do probiotics offer opportunities to manipulate the periodontal oral microbiota?* J Clin Periodontol, 2011. **38 Suppl 11**: p. 159-77.

44. Lee, J.K., et al., *Modulation of the host response by probiotic Lactobacillus brevis CD2 in experimental gingivitis.* Oral Dis, 2015. **21**(6): p. 705-12.
45. Karuppaiah, R.M., et al., *Evaluation of the efficacy of probiotics in plaque reduction and gingival health maintenance among school children - A Randomized Control Trial.* Journal of international oral health : JIOH, 2013. **5**(5): p. 33-7.
46. Yanine, N., et al., *Effects of probiotics in periodontal diseases: a systematic review.* Clin Oral Investig, 2013. **17**(7): p. 1627-34.
47. Marttinen, A., et al., *Short-term consumption of probiotic lactobacilli has no effect on acid production of supragingival plaque.* Clinical oral investigations, 2012. **16**(3): p. 797-803.
48. Campus, G., et al., *Effect of a daily dose of Lactobacillus brevis CD2 lozenges in high caries risk schoolchildren.* Clin Oral Investig, 2014. **18**(2): p. 555-61.
49. Cagetti, M.G., et al., *The use of probiotic strains in caries prevention: a systematic review.* Nutrients, 2013. **5**(7): p. 2530-50.
50. Singh, R.P., S.G. Damle, and A. Chawla, *Salivary mutans streptococci and lactobacilli modulations in young children on consumption of probiotic ice-cream containing Bifidobacterium lactis Bb12 and Lactobacillus acidophilus La5.* Acta Odontol Scand, 2011. **69**(6): p. 389-94.
51. Chuang, L.C., et al., *Probiotic Lactobacillus paracasei effect on cariogenic bacterial flora.* Clin Oral Investig, 2011. **15**(4): p. 471-6.
52. Lexner, M.O., et al., *Microbiological profiles in saliva and supragingival plaque from caries-active adolescents before and after a short-term daily intake of milk supplemented with probiotic bacteria - a pilot study.* Oral health & preventive dentistry, 2010. **8**(4): p. 383-8.
53. Engelberger, T., et al., *Correlations among Papilla Bleeding Index, other clinical indices and histologically determined inflammation of gingival papilla.* J Clin Periodontol, 1983. **10**(6): p. 579-89.
54. Benamghar, L., et al., *Comparison of gingival index and sulcus bleeding index as indicators of periodontal status.* Bull World Health Organ, 1982. **60**(1): p. 147-51.
55. Abrams, K., J. Caton, and A. Polson, *Histologic comparisons of interproximal gingival tissues related to the presence or absence of bleeding.* J Periodontol, 1984. **55**(11): p. 629-32.
56. Greenstein, G., J. Caton, and A.M. Polson, *Histologic characteristics associated with bleeding after probing and visual signs of inflammation.* J Periodontol, 1981. **52**(8): p. 420-5.
57. Gleissner, C., et al., *Local gingival blood flow at healthy and inflamed sites measured by laser Doppler flowmetry.* J Periodontol, 2006. **77**(10): p. 1762-71.
58. Kerdvongbundit, V., et al., *Microcirculation of the healthy human gingiva.* Odontology, 2002. **90**(1): p. 48-51.
59. Hock, J., et al., *Clearance rates of xenon-113 in non-inflamed and inflamed gingiva of dogs.* Arch Oral Biol, 1980. **25**(7): p. 445-9.
60. Kinnen, E. and H.J. Goldberg, *The application of electrical impedance plethysmography to the study of gingival circulation.* J Periodontol, 1978. **49**(10): p. 528-33.

61. Vandersall, D.C. and H.A. Zander, *Experimental obstruction of the periodontal blood circulation.* Helv Odontol Acta, 1967. **11**(1): p. 74-9.
62. Baab, D.A., P.A. Oberg, and G.A. Holloway, *Gingival blood flow measured with a laser Doppler flowmeter.* J Periodontal Res, 1986. **21**(1): p. 73-85.
63. Matheny, J.L., et al., *Microcirculatory dynamics in experimental human gingivitis.* J Clin Periodontol, 1993. **20**(8): p. 578-83.
64. Hinrichs, J.E., L.L. LaBelle, and D. Aeppli, *An evaluation of laser Doppler readings obtained from human gingival sulci.* J Periodontol, 1995. **66**(3): p. 171-6.
65. Baab, D.A. and P.A. Oberg, *Laser Doppler measurement of gingival blood flow in dogs with increasing and decreasing inflammation.* Arch Oral Biol, 1987. **32**(8): p. 551-5.
66. Baab, D.A., A. Oberg, and A. Lundstrom, *Gingival blood flow and temperature changes in young humans with a history of periodontitis.* Arch Oral Biol, 1990. **35**(2): p. 95-101.
67. Liu, K.Z., et al., *In vivo determination of multiple indices of periodontal inflammation by optical spectroscopy.* J Periodontal Res, 2009. **44**(1): p. 117-24.
68. Ge, Z., et al., *Assessment of local hemodynamics in periodontal inflammation using optical spectroscopy.* J Periodontol, 2011. **82**(8): p. 1161-8.
69. Hanioka, T., et al., *Haemoglobin concentration and oxygen saturation in dog gingiva with experimentally induced periodontitis.* Arch Oral Biol, 1989. **34**(8): p. 657-63.
70. Hanioka, T., S. Shizukuishi, and A. Tsunemitsu, *Hemoglobin concentration and oxygen saturation of clinically healthy and inflamed gingiva in human subjects.* J Periodontal Res, 1990. **25**(2): p. 93-8.
71. Hanioka, T., S. Shizukuishi, and A. Tsunemitsu, *Changes in hemoglobin concentration and oxygen saturation in human gingiva with decreasing inflammation.* J Periodontol, 1991. **62**(6): p. 366-9.
72. Sekhar, P.C., et al., *Discrimination of periodontal diseases using diffuse reflectance spectral intensity ratios.* J Biomed Opt, 2012. **17**(2): p. 027001.
73. Zakian, C., et al., *In vivo quantification of gingival inflammation using spectral imaging.* J Biomed Opt, 2008. **13**(5): p. 054045.
74. Carta, G., et al., *Oral health inequalities in Italian schoolchildren--a cross-sectional evaluation.* Community dental health, 2014. **31**(2): p. 123-8.
75. Pitts, N.B., K.R. Ekstrand, and I. Foundation, *International Caries Detection and Assessment System (ICDAS) and its International Caries Classification and Management System (ICCMS) - methods for staging of the caries process and enabling dentists to manage caries.* Community Dent Oral Epidemiol, 2013. **41**(1): p. e41-52.
76. Campus, G., et al., *Effect of a sugar-free chewing gum containing magnolia bark extract on different variables related to caries and gingivitis: a randomized controlled intervention trial.* Caries Res, 2011. **45**(4): p. 393-9.
77. Carlen, A., H. Hassan, and P. Lingstrom, *The 'strip method': a simple method for plaque pH assessment.* Caries research, 2010. **44**(4): p. 341-4.
78. Viechtbauer, W., et al., *A simple formula for the calculation of sample size in pilot studies.* J Clin Epidemiol, 2015. **68**(11): p. 1375-9.

79. Patterson, C., et al., *Diabetes in the young - a global view and worldwide estimates of numbers of children with type 1 diabetes.* Diabetes Res Clin Pract, 2014. **103**(2): p. 161-75.
80. Tuomilehto, J., *The emerging global epidemic of type 1 diabetes.* Curr Diab Rep, 2013. **13**(6): p. 795-804.
81. Patel, B.J., et al., *Comparison and Correlation of Glucose Levels in Serum and Saliva of Both Diabetic and Non-diabetic Patients.* Journal of international oral health : JIOH, 2015. **7**(8): p. 70-6.

ORIGINAL PAPER

I

Submitted

Evaluation of the difference in caries experience in diabetic and non-diabetic children - A case control study

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ABSTRACT

Objective: The aim of this study was to evaluate the difference in caries experience and different caries-related variables between diabetic and non-diabetic children aged 5-13 years old. A further analysis was carried out on diabetic children after they were divided in two groups based on their metabolic control.

Material and Methods: We designed a case-control study on two categories of children: the first group consisted of 68 children diagnosed with type 1 diabetes and the second group consisted of 136 non-diabetic control children. The diabetic children were then divided into two subgroups: a) 20 children with adequate metabolic control (Hb1ac≤7.5) and b) 48 children with poor metabolic control (Hb1ac>7.5). Data on dietary and oral hygienic habits was obtained on all the subjects participating in the study. Collection of saliva was carried out after stimulating salivation by chewing on a piece of paraffin for 5 minutes. Microbial flora was analyzed using the checkerboard DNA-DNA hybridisation method. Plaque acidogenity was recorded using pH indicator strips up to 30 min after a sucrose rinse. Caries registration was performed using the ICDAS index.
Results: No statically significant difference in clinical data was found in the two study groups with similar caries status. No statistically significant difference was found for tooth brushing frequency, use of fluoridated toothpaste, mouthwash and other fluoride supplements and the pattern of dental check-ups between the groups examined. Statistically significant differences for plaque-pH when analyzed as minimum pH, $AUC_{6.2}$ and $AUC_{5.7}$ between all diabetic and the non-diabetic children ($p<0.01$ or $p<0.05$) were found. The bacterial counts differed significantly between diabetic and non-diabetic subjects regarding *S. Mutans, S. Sobrinus, L. Salivarius and L. Fermentum* ($p < 0.05$).
Conclusions: Type 1 Diabetes Mellitus patients showed a more cariogenic bacterial environment and a direct effect on plaque pH reducing it from normal levels was detected.

A 25-word summary of the abstract
Plaque acidogenicity and microbial flora were statistically different between diabetic and non-diabetic children and between subjects with a good-metabolic control and children with bad-metabolic control.

key phrases
Clinical studies; Epidemiology.

Introduction

Diabetes mellitus is a chronic disease resulting from a relative or absolute deficiency of insulin, which affects the metabolism of carbohydrate, protein, and fat. The most obvious finding is a high level of blood glucose especially following a meal. [1]

In Europe there is a north-south gradient in the disease incidence. The highest incidence is observed in Finland with 40.2 per 100,000 subjects and year, while the lowest incidence rates are reported by Balkan countries, particularly by Macedonia (3.2/100000/year). Finland, Sardinia (Italy) and Sweden are known to have the highest incidence of type 1 diabetes in the world. [2]

Diabetes mellitus can be divided into two main categories: insulin dependent (Type 1) and non insulin dependent (Type 2). The diagnosis of Type 1 diabetes mellitus in young patients is relatively easy, but in patients with adult-onset diabetes mellitus, the classification into either Type 1 or 2 can sometimes be difficult. [3] . Type 1 diabetes is mainly an autoimmunizing disease characterized by the specific destruction of pancreatic beta cells and the presence of specific autoantibodies. [4], [5]

The onset in children is on average under the age of fifteen years. Type 1 diabetes incidence increases as a child gets older and with an odd double at the age of 10-14 years old compared to the age of <5 years. This trend generally is not affected by gender. [2] [6] [7]

Various oral complications have been reported in diabetic patients including an increased presence of caries, even if no scientific evidence is described [10]. Changes in oral microflora of diabetics individuals with a poor glycaemic control may significantly influence the incidence of diseases caused by bacteria such as periodontal impairment and dental caries. [8] [9]

Furthermore, diabetes may cause changes in salivary glands, which may contribute to a slow flow rate, prolonged oral clearance and altered salivary composition. [10] [11]. Other factors of diabetes associated with the cariogenic changes in the oral environment of diabetic children and adolescents included less resting and stimulated whole saliva, lower saliva buffering capacity and acidic pH, higher salivary glucose, higher salivary albumin concentrations, high proportion of salivary Streptococcus mutans, and salivary yeast growth. [12]. [13], [14], However, contrary findings with lower prevalence have also been reported. [15], [16]

Aim of study

The aim of this study was to evaluate the difference in caries experience and different caries-related variables between diabetic and non-diabetic children aged 5-13 years old. A comparison was also made between the diabetic children in relation to their metabolic control. The null-hypothesis was that there was no difference regarding caries experience between diabetic and non-diabetic children and into diabetic group no difference regarding metabolic control.

Material and methods

Study design and population

A cross-sectional case control (rate 1:2 matched for age and gender) study was designed in which two categories of subjects participated; diabetic and non-diabetic children aged 5-13 years old. The study protocol was approved by the Ethical Committee of Sassari University of Medicine, Sassari, Italy [authorisation number 133/2014] and conducted according to the

principles of the Helsinki Declaration II. The number of people affects by diabetes in Sardinia is estimated in almost 95,000 individuals. In Sassari area 1013 children affected by Type 1 diabetes is reported (https://www.regione.sardegna.it/documenti/1_19_20120702174709.pdf in paediatric age in Sardinia). Inclusion criteria to be enrolled into the study were: 5-13 years old, diabetes diagnosed more than 2 years before the start of the study, living in Sassari and surrounding region (within a distance of 30-40 km from the city), good general health apart from diabetes, reporting to clean the teeth at least twice a day.

Power analysis was performed before the start of the study using the web-based openepi platform (http:/openinfo.com), taking into account a caries prevalence of about 50 % [17]. The number was increased by 20% taking account a modification in caries prevalence and a high number of non-responders. The number of diabetic children needs to enrolled was fixed in 64 with an actual power of 0.95. Information of the study was mailed to 225 parents/guardians (related to 75 diabetic children and 150 non-diabetic children) asking consent for their children to participated to the study. Finally, 72 diabetic children agree to participate and 68 were enrolled, so from the 150 bunch of non-diabetic children 136 subjects matched by gender and age were selected. The study was carried out at the Peadiatric diabetic children and in the Dental Clinic of the School of Medicine, University of Sassari, Sassari, Italy. The study was conducted from January 2015 to September 2015.

The diabetic children were, according to information in their medical charts, divided into two subgroups: a) 20 children with a good metabolic control (Hb1ac≤7.5) and b) 48 children with bad metabolic control (Hb1ac>7.5) [18], [19]. Subjects were instructed not to brush their teeth or to eat/drink anything during the last hour prior to examination.

Data collection

All subjects were examined in the Dental Clinic, of the University of Sassari. A plaque sample was collected for microbiological analyses, and acidogenicity of dental plaque was measured followed after a sucrose challenge. All parents/guardians fill in a questionnaire regarding oral hygiene and dietary habits of the children. For the diabetic children, data on their medical condition was also retrieved from their medical charts.

Questionnaire

A structured questionnaire with closed questions was used to elicit information of oral hygiene and fluoride habits as well as snacking and drinking habits. Each answer was assigned a code of intake frequency for drinking and snacking habits. The place of consumption of food and drink, at school (S) or outside school (OS), was registered. The answers were coded as never (Code 0), once a week (Code 1), two-four times a week (Code 2), five-six times a week (Code 3), once a day (Code 4), two-three times a day (Code 5) and over three times a day (Code 6). The answers of oral habits were obtained by questions focusing on regularity and frequency of toothbrushing, use of fluoridated toothpaste, the use of fluoride supplements and, frequency dental check-ups.

Clinical examination

The clinical examination was made under optimal lighting using a mirror and WHO-probe. For caries registration, the ICDAS II [22] index was used. The subjects were then merged and classified into the following four categories: caries-free (ICDAS 0), initial caries (ICDAS 1-2),

moderate caries (ICDAS 3-4), and severe caries (ICDAS 5-6). No radiographic caries registrations were made. [20]

Saliva samples and Microbiological analyses

Saliva sample collection was made after stimulated saliva by chewing on a piece of paraffin during 5 min with continuously spitting into test tube. The saliva samples were sent to Department of Microbiology, University of Bologna for evaluation of bacterial pathogens.

The analysis of the microbial flora was made using the checkerboard DNA-DNA hybridisation method [21]. Whole genomic probes were matched from 15 bacterial strains known to be associated with caries. An evaluation of the bacterial count in the samples was performed by matching the obtained signals with the ones generated by the pooled standard samples containing a count of 10^6 and 10^5 of each bacterial species, respectively. The signals were coded on a scale from 0 to 5: 0 = no signal; 1 = a signal density weaker than that of the low standard ($<10^5$ bacteria); 2 = a signal density equal to that of the low standard ($=10^5$ bacteria); 3 = a signal density higher than that of the low standard but lower than that of the high standard ($>10^5$ but $<10^6$ bacteria); 4=a signal density equal to that of the high standard ($=10^6$ bacteria) and 5 = a signal density higher than that of the high standard ($>10^6$ bacteria).

Plaque acidogenicity

The plaque acidogenity was assessed using the pH indicator strips. The strip measure a pH value in the range of 4.0–7.0 (Spezialindikator, pH range 4.0–7.0; Merck, Darmstadt, Germany) [22]. Each strip was cut into 4 pieces (approx. 2 mm in width) in order to get a strip that more easily could be inserted into the interproximal space. The strip was held into the interdental

space for 10 s after which it was removed and its colour compared to the colour index scheme supplied by the manufacturer. The pH was determined to one decimal of the value. For each subject, 3 measurements were carried out in 2 sites: 1) between the 2nd premolar and the 1st molar right or left upper jaw and 2) 2nd primary molar and 1st molar right or left upper jaw. In case the patient has not erupted 1st molar, the measurements were carried between 54/55 and 64/65. At each time point (t), measurements were performed before (0 min) and at 2, 5, 10, 15, 20 and 30 min after a mouth rinse with 10% sucrose.

Statistical analyses

The mean approximal plaque pH (±ES) for all participants at the different time points was calculated for the two interproximal sites. The maximum pH fall and minimum pH after the sucrose rinse were calculated for each individual curve. For each individual pH curve, the area below the critical pH of enamel ($AUC_{5.7}$) and of dentine ($AUC_{6.2}$) calculated using a software program [23]. The salivary bacterial concentrations were transformed to log10 values after which the mean and standard error (SE) was calculated for each group and time point. Comparisons of the different variables were made between the diabetic and non-diabetic subjects. The diabetics were also grouped into those in good metabolic control (HbA1c ≤7.5) and those in bad metabolic control (HbA1c >7.5). Statistical descriptive analyses were performed using STATA13® statistical software

RESULT

Questionnaire and oral hygiene habits

The comparison among the diabetic and the control groups about questionnaire items are displayed in Table 1 and 2. Regarding sugared foods and drinks several behaviours demonstrated a statistically significant difference in the examined groups (see table 1 for detail). Diabetic subjects had a statistically higher consumption of fresh-squeezed juice, bottle-juice, energy drink, and potato chip at school compared to control group. The comparison between diabetes with good metabolic control and diabetes with bad metabolic control showed statistically significant differences ($p<0.05$) for consumption at school of bottle-juices, diet beverage and potato chip while the consumption of soda beverage and milk outside school was higher in diabetics with bad metabolic control ($p<0.05$).

The correlation among diabetes in bad metabolic control and diabetes in good metabolic control observed a statistical significant difference for fresh-squeezed juice OS ($p<0.01$), juice in bottle S and OS ($p=0.03$ and $p=0.01$), diet beverage OS ($p<0.0001$), soda beverage OS ($p=0.02$), milk S and OS($p=0.02$ and $p<0.01$), potato chip S ($p=0.01$), ice cream OS ($p<0.001$), vegetable OS ($p<0.001$), fruits S and OS ($p<0.01$ and $p<0.001$).

The relation among the subject with diabetes in good metabolic and control group showed a significant statistical for potato S ($p=0.03$), vegetable OS ($p=0.04$) and fruits OS ($p<0.01$).

For other answer about frequency for drinking and snacking habits, no statistically differences were found when comparing any of the groups.

The table 2 illustrates oral hygiene habits in the examined groups. The use of fluoridated mouthwash was higher in control group respect to diabetic subjects (p=0.04). The comparison among diabetes in good metabolic control and diabetes in bad metabolic control highlighted a significan statistical differe ce for regular toothbrushing ("mostly every day" p=0.03) and for use of fluoridadated toothpaste ("yes" p=0.03 and "no" p=0.03).

The correlation between diabetes in bad metabolic control and control group showed a significant statistical for use of fluoridated toothpaste ("yes" p<0.01 and "no" p<0.01).

The relation among diabetes in good metabolic control and control group was demonstrated a significant statistical for toothbrushing regularly ("mostly every day" p. 0.02) and for use of fluoride supplements ("somentimes" p. <0.05).

Clinical data

Caries data are presented in figure 1. Caries prevalence varies from 54% in diabetic subjects with good metabolic control to 70% in diabetic subjects in bad-metabolic control. Overall no statistically significant differences were observed between diabetic and control group, while the caries free subjects were statistically significant higher in diabetic subjects with good metabolic control compared diabetic subjects in bad-metabolic control. The other caries figures (Initial, Moderate and Extensive) were similar in all groups.

Microbiological analyses

The list of bacteria analysed and the association among diabetic subjects and control group is displayed in table 3. A significant association for *S. mutans, S. sobrinus, L. salivarius and L. fermentum* ($p < 0.05$) was found for the comparison among diabetic subjects and control group. The association between diabetic subjects with good metabolic control and bad metabolic control showed a significant association for the bacteria strain of *S. Salivarius, L. Fermentum, L. Casei, S. Salivarium* and *S. Sobrinus* ($p<0.05$).

A significant association was observed in relation to diabetic in bad control and control group for bacteria *S. Sobrinus, S. Salivarius, L. Fermentum* (p. < 0.01), *L. Salivarius* and *L. Casei* (p. 0.02), S. Mutans (p. <0.03). The relation between diabetes in good metabolic control and control group showed for S. Mutans (p. 0.05). For same bacteria, no statistically differences were found when comparing any of the groups.

Plaque pH measurements

The pH values recorded statistically significant differences for minimum pH, $AUC_{5.7}$ and $AUC_{6.2}$ between all diabetes subjects and the control group ($p<0.05$) as well as between the subjects with bad metabolic control and the control group ($p<0.05$) (table 4). Comparison between the two diabetes groups showed statistically significant differences for all four variables ($p<0.05$). A statistically significant difference with more pronounced pH-falls were found during the whole 30-min period for the diabetes group vs the control group as well as for the subjects with good metabolic control compared to those with bad metabolic control.

DISCUSSION

The aim of this paper was to evaluate the different caries-related variables between diabetic and non-diabetic children aged 5-13 years old as well to compare the different caries-related variables of the diabetic children in relation to their metabolic control.

The main outcome of the study was that the risk for caries was higher in diabetic population respect to control group. The higher caries risk factor was due to the change of microflora bacteria, higher assumption for food/drink sweet, lower initial and final pH in diabetic group. These figures are even more pronounced when diabetic population was split in subjects bad metabolic control and subjects with good metabolic control. The result of plaque pH measurement after a sucrose rinse showed a trend towards a more important different pH drop in diabetic subjects with bad control metabolic and control group. One interesting observation was when we have been compared the subjects with metabolic control and group control because there is many difference for the value AUC pH6.2 (3.06+4.48) / (6.25+7.41) and the value AUC pH5.7 (1.27+1.82) / (2.38+4.38). A statistically significant was recorder between the group with bad metabolic control and good metabolic control. In other study demonstrated that when the uncontrolled diabetics were compared with the non-diabetics had a decreased salivary pH; this can be explained to the changes in the metabolic process of the uncontrolled diabetic resulting in acidic pH and thus increased incidence of dental caries [30]. Yet, in this study the group with good metabolic control has the same status caries, different diet and the similar trend for the pH measurement when we was compared by the subjects with bad metabolic control To authors' knowledge no study on plaque acidogenecity was present. The strip-method used in this study is easy and appropriate for a chair-side clinical use [24]. The pH measurements in this study were performed in the interproximal space between the first and the

second primary molars of the upper jaw; however, it is impossible to distinguish between plaque covering the adjacent tooth, *i.e.,* mesial and distal proximal surfaces.

The sample was not numerous but, the result with significant association (p.value <0,05) could be considered important success.

The high prevalence of diagnosis diabetes type 1 in Sardegna doesn't permit to declare that the population is alike in all Italy. The highest incidence is seen in Finland. Sardegna in Italy together with Finland and Sweden are known to have the highest incidence of Type 1 diabetes in the world. The our sample can be compared with area such as Finland or Sweden. [2] [25] [26]

In other studies there are contradiction, it has been found that diabetic patients may have salivary dysfuction as well as different microbiologic salivary composition compared to non-diabetic . Yet, the salivary antimicrobial defence of diabetic patients may be better than that of non-diabetic individual, due to increased concentration of selected protective factor in saliva [10] [11] [12] [13], [14].

The relationship among diet and dental caries is often a question of particular interest when diabetes mellitus is concerned. Yet, possible variations in the dietary habits of diabetic patients compared to non- diabetic individuals might have an effect on caries increments over time, and change the analysis of the relation between dental caries and diabetes mellitus.

However, in the subjects found some differences between the diabetics and their controls with respect to frequency of meals or consumption of carbohydrates, as assessed by questionnaires. The factors of diet were not considered to contribute significantly to the effect of diabetes-related factors on the caries outcomes in this study.

The most important correlation was showed in the relationship amoug diabetic in bad metabolic control and control group, the bacteria S. Sobrinus, S. Salivarius, L. Fermentum ($p. < 0.01$) and L. Salivarius, L. Casei , S. Mutans ($p. <0.05$). Yet, in other comparisions have been recorded a significally correlation for the bacteria strain of L. Salivarius, L. Fermentum, L. Casei, S. Salivarium and S. Sobrinus ($p. <0.05$) between diabetic subjects with in good metabolic control and bad metabolic control; among diabetic subjects and control group was demonstrated a significant association for S. Mutans, S. Sobrinus, L. Salivarius and L. Fermentum ($p. < 0.05$).

The higher counts of bacteria like S. Mutans, S. Sobrinus, L. Salivarius and L. Fermentum in diabetic group may be correlated by glucose level in saliva in diabetic patients, in other study was found that salivary glucose values were higher among diabetic that in non-diabetic [27] and increased glucose concentrations in saliva of diabetics may origin as a result of no-correct neural regulation of the salivary gland function. [11].

The count of S. Mutans is similar when it was compared bad metabolic group and good metabolic group, this observation may be related only the variability diabetic type 1 because we were recorded different habit for drink/food.

Conclusion

Diabetes mellitus may have a direct effect on salivary pH reducing it from normal levels irrespective of diet.

References

Dean AG, Sullivan KM, Soe MM. OpenEpi: Open Source Epidemiologic Statistics for Public Health, Versione. www.OpenEpi.com, access 2015/11/08.

1. American Diabetes, A., *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2014. **37 Suppl 1**: p. S81-90.
2. Group, D.P., *Incidence and trends of childhood Type 1 diabetes worldwide 1990-1999.* Diabet Med, 2006. **23**(8): p. 857-66.
3. Kuzuya, T., et al., *Report of the Committee on the classification and diagnostic criteria of diabetes mellitus.* Diabetes Res Clin Pract, 2002. **55**(1): p. 65-85.
4. American Diabetes, A., *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2013. **36 Suppl 1**: p. S67-74.
5. Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes, M., et al., *Report of the committee on the classification and diagnostic criteria of diabetes mellitus.* J Diabetes Investig, 2010. **1**(5): p. 212-28.
6. *Variation and trends in incidence of childhood diabetes in Europe. EURODIAB ACE Study Group.* Lancet, 2000. **355**(9207): p. 873-6.
7. Vehik, K., et al., *Increasing incidence of type 1 diabetes in 0- to 17-year-old Colorado youth.* Diabetes Care, 2007. **30**(3): p. 503-9.
8. Miko, S., et al., *Dental caries and adolescents with type 1 diabetes.* Br Dent J, 2010. **208**(6): p. E12.
9. Saes Busato, I.M., et al., *Association between metabolic control and oral health in adolescents with type 1 diabetes mellitus.* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2010. **109**(3): p. e51-6.
10. Siudikiene, J., et al., *Dental caries and salivary status in children with type 1 diabetes mellitus, related to the metabolic control of the disease.* Eur J Oral Sci, 2006. **114**(1): p. 8-14.
11. Mata, A.D., et al., *Effects of diabetes mellitus on salivary secretion and its composition in the human.* Mol Cell Biochem, 2004. **261**(1-2): p. 137-42.
12. Negrato, C.A. and O. Tarzia, *Buccal alterations in diabetes mellitus.* Diabetol Metab Syndr, 2010. **2**: p. 3.
13. Siudikiene, J., et al., *Dental caries increments and related factors in children with type 1 diabetes mellitus.* Caries Res, 2008. **42**(5): p. 354-62.
14. Lopez, M.E., et al., *Salivary characteristics of diabetic children.* Braz Dent J, 2003. **14**(1): p. 26-31.

15. Singh-Husgen, P., et al., *Investigation of the oral status and microorganisms in children with phenylketonuria and type 1 diabetes.* Clin Oral Investig, 2015.
16. Tagelsir, A., et al., *Dental caries and dental care level (restorative index) in children with diabetes mellitus type 1.* Int J Paediatr Dent, 2011. **21**(1): p. 13-22.
17. Carta, G., et al., *Oral health inequalities in Italian schoolchildren--a cross-sectional evaluation.* Community dental health, 2014. **31**(2): p. 123-8.
18. Chiang, J.L., et al., *Type 1 diabetes through the life span: a position statement of the American Diabetes Association.* Diabetes care, 2014. **37**(7): p. 2034-54.
19. Rewers, M.J., et al., *ISPAD Clinical Practice Consensus Guidelines 2014. Assessment and monitoring of glycemic control in children and adolescents with diabetes.* Pediatr Diabetes, 2014. **15 Suppl 20**: p. 102-14.
20. Pitts, N.B., K.R. Ekstrand, and I. Foundation, *International Caries Detection and Assessment System (ICDAS) and its International Caries Classification and Management System (ICCMS) - methods for staging of the caries process and enabling dentists to manage caries.* Community Dent Oral Epidemiol, 2013. **41**(1): p. e41-52.
21. Wall-Manning, G.M., et al., *Checkerboard DNA-DNA hybridisation technology focused on the analysis of Gram-positive cariogenic bacteria.* J Microbiol Methods, 2002. **51**(3): p. 301-11.
22. Campus, G., et al., *Effect of a sugar-free chewing gum containing magnolia bark extract on different variables related to caries and gingivitis: a randomized controlled intervention trial.* Caries Res, 2011. **45**(4): p. 393-9.
23. Larsen, M.J. and E.I. Pearce, *A computer program for correlating dental plaque pH values, cH+, plaque titration, critical pH, resting pH and the solubility of enamel apatite.* Arch Oral Biol, 1997. **42**(7): p. 475-80.
24. Carlen, A., H. Hassan, and P. Lingstrom, *The 'strip method': a simple method for plaque pH assessment.* Caries research, 2010. **44**(4): p. 341-4.
25. Patterson, C., et al., *Diabetes in the young - a global view and worldwide estimates of numbers of children with type 1 diabetes.* Diabetes Res Clin Pract, 2014. **103**(2): p. 161-75.
26. Tuomilehto, J., *The emerging global epidemic of type 1 diabetes.* Curr Diab Rep, 2013. **13**(6): p. 795-804.
27. Patel, B.J., et al., *Comparison and Correlation of Glucose Levels in Serum and Saliva of Both Diabetic and Non-diabetic Patients.* Journal of international oral health : JIOH, 2015. **7**(8): p. 70-6.

Figure 1

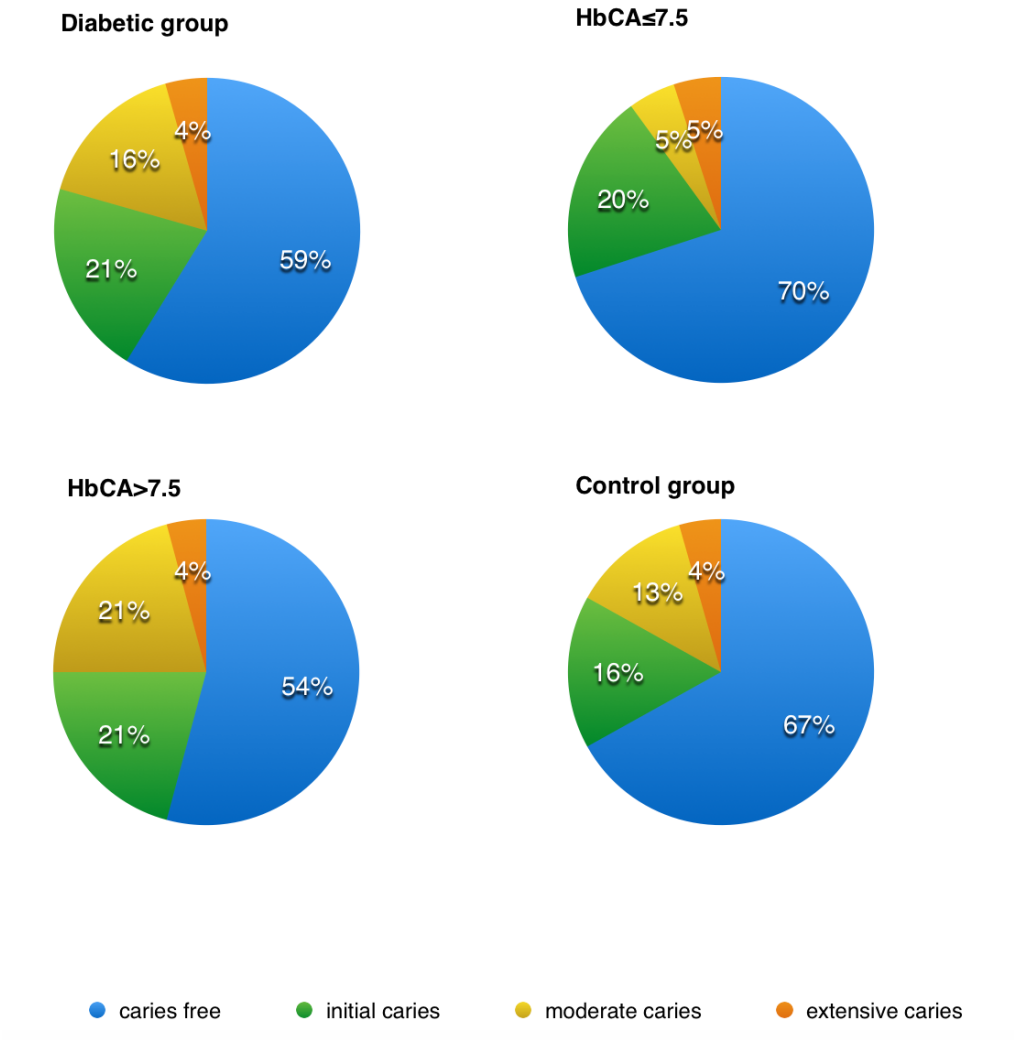
Diabetic group
4%
16%
21%
59%
HbCA≤7.5
5%
5%
20%
70%
HbCA>7.5
4%
21%
21%
54%
Control group
4%
13%
16%
67%
caries free
initial caries
moderate caries
extensive caries

Table 1. Diet questionnaire regarding sugared food and drinks consumption (in school or outside school). The replies were treated as continuous ordinal variables.

		Diabetic		Control	
Diet questionnaire	**Total group** *mean±SD*	**good-metabolic** *mean+SD*	**bad-metabolic** *mean+SD*	**Total group** *mean+SD*	**p Value**
Fresh-squeezed juice					
In school	1.10±1.66	0.83±1.27	1.23±1.81	0.87±1.24	
Outside school	2.06±2.06	1.45±1.86	2.33±2.12	1.49±1.44	°0.02 ◊<0.01
Juice in bottle					
In school	1.43±1.61	0.80±1.40	1.68±1.65	1.16±1.35	*0.04 ◊0.03
Outside school	2.18±1.64	2.00±1.90	2.26±1.54	1.65±1.39	°0.02 ◊0.01
Energy drink					
In school	0.08±0.37	0.08±0.29	0.09±0.42	0.24±0.55	°0.03
Outside school	0.07±0.37	0.18±0.60	0.00±0.00	0.27±0.59	°0.01
Diet beverage					
In school	0.76±1.39	0.64±1.29	0.82±1.47	0.89±1.14	
Outside school	2.00±1.81	1.08±1.24	2.44±1.89	1.30±1.61	°*<0.01 ◊<0.01
Soda Beverage					
In school	0.47±0.91	0.25±0.45	0.58±1.06	0.44±0.7	
Outside school	0.91±1.46	0.36±0.80	1.17±1.63	0.72±0.94	*0.04 ◊0.02
Milk					
In school	1.30±1.64	1.08±1.50	1.44±1.76	0.89±1.26	°0.04 ◊0.02
Outside school	2.37±2.18	1.54±2.14	2.78±2.12	1.89±1.89	*0.03 ◊<0.01
Coffee/Tea					
In school	0.18±0.53	0.08±0.28	0.25±0.63	0.27±0.53	
Outside school	0.18±0.72	0.42±1.16	0.04±0.21	0.20±0.56	*0.03
Potato Chip					
In school	0.94±1.35	0.40±0.70	1.67±1.49	1.08±1.39	*<0.01 ◊0.01 ^0.03
Outside school	1.11±1.45	0.61±0.65	1.32±1.64	1.26+1.53	
Salted biscuit					
In school	0.97±1.31	1.10±1.37	0.92±1.32	0.82±1.12	
Outside school	1.15±1.37	1.20±1.23	1.12±1.45	1.06±1.26	
Sweets					
In school	1.33±1.67	1.10±1.85	1.42±1.63	1.20±1.44	
Outside school	1.85±1.48	1.64±0.92	1.93±1.65	1.68±1.39	
Cake/Biscuits					
In school	1.08±1.44	1.25±1.60	1.00±1.38	1.06±1.29	
Outside school	1.09±1.31	0.87±0.83	1.16±1.43	0.93±1.25	
Dessert					
In school	0.90±1.58	0.89±1.45	0.90±1.66	1.00±1.53	
Outside school	1.48±1.52	1.20±1.13	1.59±1.65	1.15±1.45	
Ice cream					
In school	0.47±0.73	0.45±0.82	0.47±0.70	0.73±0.94	°<0.05
Outside school	1.61±1.39	1.25±0.75	1.76±1.57	1.06±1.12	°◊0<.01
Vegetable					
In school	1.94±1.88	1.83±1.85	2.00±1.94	1.45±1.68	
Outside school	/2.83±1.82	2.70±1.42	2.87±1.95	1.83±1.77	°◊<0.01 ^0.04
Fruits					
In school	2.49±2.08	2.00±1.81	2.72±2.18	1.78±1.95	°0.02 ◊<0.01
Outside school	3.60±1.54	3.64±1.29	3.59±1.64	2.42±1.89	°◊^<0.01

*The table reports statistically significant differences (p<0.05) regarding the °comparison among diabetes group and non-diabetic group. *comparison among diabetes in good metabolic control (HbA1c ≤7.5) and diabetes in bad metabolic control (HbA1c*

>7.5). ◊comparison among diabetes in bad metabolic control and non-diabetic group ^comparison among diabetes in good metabolic control and non-diabetic group.

Table 2. *Oral hygiene habits in the examined groups. The replies were treated as continuous ordinal variables.*

	Diabetic			Control	
Oral hygiene Habits	**Total group % (n)**	**good-metabolic % (n)**	**bad-metabolic % (n)**	**Total group % (n)**	**p Value**
Do you brush your teeth regularly?					
No	72.1% (49)	65.0% (13)	75.0% (36)	72.1% (98)	
Sometimes	16.2% (11)	10.0% (2)	18.7% (9)	19.8% (27)	
Mostly every day	11.7% (8)	25.0% (5)	6.3% (3)	8.1% (11)	
Every day	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	*0.03 ^0.02
Toothbrusching frequency					
Once a day	16.2% (11)	10.0% (2)	18.7% (9)	19.9% (27)	
Twice a day	45.5% (31)	55.0% (11)	41.6% (20)	38.9% (53)	
Three time a day	32.4% (22)	25.0% (5)	35.4% (17)	35.3% (48)	
More than three times a day	5.9% (4)	10.0% (2)	4.1% (2)	5.9% (8)	
Fluoridated Toothpaste					
Yes	38.9% (21)	50.0% (10)	22.9% (11)	44.8% (61)	
No	69.1% (47)	50.0% (10)	77.1% (37)	55.2% (75)	*0.03 ◊<0.01
Fluoridated Mouthwash					
Yes	2.9% (2)	5.0% (1)	2.1% (1)	3.7% (5)	
Sometimes	17.7% (12)	10.0% (2)	20.8% (10)	30.8% (42)	
No	79.4% (54)	85.0% (17)	77.1% (37)	65.5% (89)	°0.04
Fluoride Supplements					
Yes	8.8% (6)	10.0% (2)	8.3% (4)	7.3% (10)	
Sometimes	16.2% (11)	25.0% (5)	12.5% (6)	9.6% (13)	
No	75.0% (51)	65.0% (13)	79.2% (38)	83.1% (113)	^<0.05
Dental check ups					
Only when in pain	22.1% (15)	10.0% (2)	27.1% (13)	27.9% (38)	
Each six months	26.5% (18)	20.0% (4)	29.2% (14)	31.6% (43)	
Once a year	41.2% (28)	55.0% (11)	35.4% (17)	33.9% (46)	
Every two years	10.2% (7)	15.0% (3)	8.3% (4)	6.6% (9)	

*The table reports statistically significant differences (p<0.05) regarding the °comparison among diabetes group and non-diabetic group. *comparison among diabetes in good metabolic control (HbA1c ≤7.5) and diabetes in bad metabolic control (HbA1c >7.5). ◊comparison among diabetes in bad metabolic control and non-diabetic group ^comparison among diabetes in good metabolic control and non-diabetic group.*

Table 3. Microbiological analysis in the examined groups. The data were treated as continuous ordinal variables.

	Diabetic			Control	
Bacterial strain	**Total group** *mean±SD (range)*	**good-metabolic** *mean±SD (range)*	**bad-metabolic** *mean±SD (range)*	**Total group** *mean±SD (range)*	**p Value**
S. Mutans	3.38±1.15 (1-5)	3.35±1.19 (1-5)	3.35±1.15 (1-5)	2.83±1.07 (1-5)	°^0.04 ◊0.03
S. Sobrinus	1.88±0.70 (1-4)	1.70±0.47 (1-2)	1.96±0.77 (1-4)	1.27±0.96 (1-5)	°0.02 *0.04 ◊<0.01
S. Sanguinis	2.70±1.01 (1-5)	2.70±1.08 (1-5)	2.71±0.99 (1-5)	2.69±1.01 (1-5)	
S. Salivarius	2.81±1.07 (1-5)	2.60±0.94 (1-5)	2.89±1.11 (1-5)	2.46±0.95 (1-5)	°0.03 *0.04 ◊<0.01
S. Mitis	2.46±1.01 (1-5)	2.35±1,04 (1-5)	2.54±1.01 (1-5)	2.22±1.04 (1-5)	
S. Gordoni	2.23±0.98 (1-5)	2.10±0.85 (1-4)	2.29±1.03 (1-5)	2.21±0.87 (1-5)	
NSM	2.37±1.12 (1-5)	2.35±1.09 (1-5)	2.37±1.14 (1-5)	2.38±1.19 (1-5)	
L. Casei	2.03±0.90 (1-5)	1.85±0.67 (1-3)	2.10±0.97 (1-5)	1.98±1.03 (1-5)	*0.04 ◊0.02
L. Salivarius	2.48±0.95 (1-5)	2.45±1.10 (1-5)	2.50±0.90 (1-5)	2.27±1.15 (1-5)	°0.03 ◊0.02
L. Fermentum	2.48±0.97 (1-5)	2.25±0.85 (1-4)	2.58±1.01 (1-5)	2.15±1.21 (1-5)	°*0.03 ◊<0.01

Table 4. Plaque-pH (minimum pH, Maximum pH fall, $AUC_{6.2}$ $AUC_{5.7}$) evaluation.

	Diabetes Group *mean±SD*	HbA1c ≤ 7.5 *mean± SD*	HbA1c >7.5 *mean± SD*	Control group *mean± SD*	p-value
Minimum pH	5.35±0.45	5.69±0.24	5.21±0.45	6.02 ±0.30	°^*◊<0,01
Maximum pH fall	1.19±0.51	0.98±0.30	1.28±0.55	0.92±0.22	°*◊<0,01
$AUC_{6.2}$	5.79±3.2	5.54±3.82	6.25±3.41	3.06±1.48	°^*◊<0,01
$AUC_{5.7}$	2.22±1.27	1.97±2.28	2.38±1.38	1.27±1.82	°^*◊<0,01

°comparison among diabetes group and control group,

*comparison among diabetes in good metabolic control (HbA1c ≤7.5) and diabetes in bad metabolic control (HbA1c >7.5),

◊comparison among diabetes in bad metabolic control and control group

^ comparison among diabetes in good metabolic control and control group

ORIGINAL PAPER

II

Submitted

Clinical and microbilogical evaluation of the effect of a probiotic lozenge (Inersan®) on caries-related variables in diabetics children

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ABSTRACT

Objective: The aim of this study was to evaluate the effect of probiotics on the oral microbiotic flora and biofilm acidogenicity in children diagnosed with diabetes since at least two years. The null hypothesis is that the probiotic lozenge containing Lb CD2 would not reduce the pathogenic bacteria and modify the plaque-pH. Primary objective is the regular consumption of probiotic will lead to a significantly reduction of pathogenic bacteria compared to a placebo. Regular oral hygiene is permitted.

Material and Methods: A double-blind, longitudinal study was performed including 68 diabetic subjects shared into two group test control and control group each were 34 subjects. The dosage in the treatment phase subjects will use 2 oral tablets a day for 56 days. The inclusion criteria for the diabetic children were: 1) 5-13 years old, 2) diabetes diagnosed >2 years ago, 3) living in Sassari and surrounding region (rural area within a distance of 30-40 km from the city), 4) good general health, and 5) average oral hygiene (cleaning the teeth at least twice a day)

Result: The pH values recorded no statistically significant differences for maximun fall pH in each group after 90 days. No significant differences for control group when it valueted the minimal pH. A significant association for the test group with p. <0.001 after 90 days when it considered minimal pH. Comparison between the two diabetes groups showed no statistically significant differences when compared each group at baseline and after 90 days A significant association for test group after intake of lozange of probiotic at 90 days (p.<0,001); for control group only *S. Sobribus* no one significant association.

The association between diabetic test group with diabetic control group showed a significant association at baseline only for the bacteria strain of *S. Salivarius* (p.<0.04) and *L. Casei* (p.<0.03);

after 90 days the comparison among diabetic test group and diabetic control group recorded a significant association for *S. Mutans, S. Sobrinus, L. Casei, L. Salivarius* (p. <0.01) and *S. Salivarius* (p.<0.02).

For other bacteria, no one statistically differences were found when comparing the groups in different time.

Conclusion: The results of this study show that use of probiotic may improve the minimal pH

in Diabetic children and may decrease the bacterial flora species as S. Mutans, S. Sobrinus, L. Casei, L. Salivarius decreasing the caries risk with good diet and oral hygiene.

INTRODUCTION

Diabetes mellitus is a chronic disease in children with a relative or absolute deficiency of insulin, which affects the metabolism of carbohydrate and protein. [1] Commonly occuring complications are nephropathy, dyslipidemia, neuropathy and retinopathy. [2] The highest incidence is seen in Finland and the lowest incidence rates in the area Balkan countries, particularly in Macedonia. However, both Sardegna in Italy together with Finland and Sweden are known to have the highest incidence of Type 1 diabetes in the world. [3] [4] [5] Diabetes mellitus can be divided into two main broad categories: insuline dependent (Type 1) occuring in children and non insulin dependent (Type 2) affecting adults and elderly. [6] [1]

Diabetics patients do often show high incidence of gingivitis, parodontal disease and xerostomia, and these complication can be correlated with disease duration and degree of glycemic control. [7] The correlation between diabetes and dental caries is vague. However, with reduced salivary flow there is an increased risk for enamel hypomineralization and caries formation. [8]

In previous studies were conducted for counting Streptococcus mutans and Lactobacillus from the saliva and compered with dental caries in childrean with diagnosis of diabetes type 1. It is showed that with same diet the dental caries would be more prevalent in diabetic children. In other study demonstrated that diet with carbodrhydrate and simple sugar in diabetic patient comparison non-diabetic was lower and in diabetic patient was found dental caries location prevalent on the buccal and labial cervical area.

Differences in the oral microflora of diabetic and non-diabetic individuals may significantly influence the incidence of bacteria-related diseases such as periodontal impairment and dental caries. [9] [10] [11]

Different strategies have been suggested in order to prevent oral diseases. The effect of probiotics (Lb CD2), distributed in association with antibiotic on gingivitis, has been tested. In different fields of oral healthcare probiotics a clinical effect has been demonstrated on

different oral conditions such as halitosis, oral candidiasis and dental caries. [12] [13] [14] [15] In order to prevent dental caries the effect of probiotics have been evaluated in different studies using various types of Lactobacilli strains. They have been proven to obtain a reduction in caries incidence, reduced number of mutans streptococci and lactobacilli, decrease plaque acidogenicity and a reversal of root caries lesions. [16] [17] [18] [19] [20] [21]

The aim of this study was to evaluate the effect of probiotics on the oral microbiotic flora and biofilm acidogenicity in children diagnosed with diabetes since at least two years. The null hypothesis is that the probiotic lozenge containing Lb CD2 would not reduce the pathogenic bacteria and modify the plaque-pH. Primary objective is the regular consumption of probiotic will lead to a significantly reduction of pathogenic bacteria compared to a placebo. Regular oral hygiene is permitted.

MATERIAL AND METHODS

Study design and study centre

A double-blind, longitudinal study was performed including 68 diabetic subjects shared into two group test control and control group each were 34 subjects. The dosage in the treatment phase subjects will use 2 oral tablets a day for 56 days. The inclusion criteria for the diabetic children were: 1) 5-13 years old, 2) diabetes diagnosed >2 years ago, 3) living in Sassari and surrounding region (rural area within a distance of 30-40 km from the city), 4) good general health, and 5) average oral hygiene (cleaning the teeth at least twice a day). Exclusion criteria were: 1) ongoing oral/dental treatment except for emergency treatment, 2) known allergic reactions to an oral hygiene product and/or medication and/or dental material previously used in the mouth or pharynx, 3) pathological changes of the oral mucosa, 4) use of fluoride-containing products (pastes, mouthrinses) within the 14 days prior to the introduction of the intraoral appliances, 5) antibiotic therapy within the past six months, 6) any non-permitted therapy. The study was carried out from in two centra: the Clinic of Pediatric at the University of Sassari, School of Medicine, the University of Sassari, Sassari and in the Clinic

of Dentistry at the University of Sassari, School of Medicine, the University of Sassari, Sassari, Italy. The study was carraied out from May 2014 to May 2015. Proposed start of the recruitment of test subjects was June 2014. The study protocol was approved by the Ethical Committee of Sassari University of Medicine, Sassari, Italy [authorisation number 133/2014] and conducted according to the principles of the Helsinki Declaration II. The study was conducted according to the principles of the Helsinki Declaration II. It was obtained from all study participants and their parents before to start.

Collection of data

All subjects arrived the Clinic of Dentistry, University of Sassari for a clinical examination. A plaque sample was collected for microbiological analyses, saliva test for microbiological analyses and acidogenicity of dental plaque was made after a sucrose challenge at baseline and then after 30, 60, 90 days. At baseline the subjects compiled with answer a questionnaire. For the diabetic children, data on their medical condition was also from found their medical journals.

Clinical examination

At baseline the clinical examination was made in the Clinic Pediatric and Clinic Dentistry, University of Sassari under optimal lighting using a mirror and WHO-probe. The ICDAS index was used for caries registration. The subjects were classified into the following four categories: caries-free (ICDAS 0), initial caries (ICDAS 1-2), moderate caries (ICDAS 3-4), and severe caries (ICDAS 5-6). No radiographic caries registration was made. [22]

Saliva samples

The collection the samples of saliva was performed after stimulated saliva by chewing on a piece of paraffin during 5 min and continuously spitting the obtained saliva into test tube. The samples of saliva were sent to Department of Microbiology, University of Bologna for

evaluation with DNA Checkerboard Method for Bacterial Pathogen Identification and count bacterial strain of oral microflora.

Microbiological analyses

The analysis of the microbial flora was obtained by the checkerboard DNA-DNA hybridisation method [23]. Whole genomic probes were matched from 9 bacterial strains known to be associated to caries (Table 1). And measurement of the bacterial count in the samples was performed by matching the obtained signals with the ones generated by the pooled standard samples containing a count of 10^6 and 10^5 of each bacterial species, respectively. The signals were coded on a scale from 0 to 5: 0 = no signal; 1 = a signal density weaker than that of the low standard ($<10^5$ bacteria); 2 = a signal density equal to that of the low standard ($=10^5$ bacteria); 3 = a signal density higher than that of the low standard but lower than that of the high standard ($>10^5$ but $<10^6$ bacteria); 4=a signal density equal to that of the high standard ($=10^6$ bacteria) and 5 = a signal density higher than that of the high standard ($>10^6$ bacteria). Other information regarding the technique are retrived in the article "Intra-familial comparison of supragingival dental plaque microflora using the checkerboard DNA–DNA hybridisation technique". [24] [25]

Plaque acidogenicity

The plaque acidogenity was followed with the method of pH indicator strips. The strips measuring a pH value in the range of 4.0–7.0 (Spezialindikator, pH range 4.0–7.0; Merck, Darmstadt, Germany) were used [26]. This method allows to determine changes in plaque pH, discriminating differences at the level of 0.2–0.5 pH units following a sugar challenge to the same extent as the microtouch method (correlation coefficient 0.99). The use of strips is easily and performing for a chair-side clinical use [27]. Each strip was cut into 4 pieces (approx. 2 mm in width) in order to get a strip that could be more simple inserted into the interproximal space. The strip was held into the interdental space for 10 s, after which it was removed and its color compared to the color index scheme supplied by the manufacturer. The pH was determined to one decimal of the value. For each subject, 3 measurements were carried out on 2 sites, between the 2nd premolar and the 1st molar right and left of the upper jaw or 2nd primary molar and 1st molar right and left of the upper jaw; in the case the patient has not

erupted 1st molar the measurements were carried between 54/55 and 64/65. At each time point (t), measurements were performed before 0 min and at 2, 5, 10, 15, 20 and 30 min after a mouth rinse with 10% sucrose.

Questionnaire

The questionnaire was organized in the Italian language with closed questions was used to elicit information of oral hygiene and fluoride habits and their snaking and drinking habits. The informations was collected from answers to questions. Every answer was corresponded a code of intake frequency for drinking and snacking habits. They were registered the place of consumption of food and drink if they occurred at school or no-school. The answers were: never (Code 0), once a week (Code 1), two-four times a week (Code 2), five-six times a week (Code 3), once a day (Code 4), two-three times a day (Code 5) and over three times a day (Code 6). The answers of oral habits was obtained by questions like regularity and frequency toothbrushing, if the children used the mouthrinse and toothpaste fluoridate, frequency dental check ups and to intake supplement fluoride.

Statistical analyses

The mean approximal plaque pH (±ES) for all participants at the different time points was calculated for the two interproximal sites. The maximum pH fall and minimum pH after a 10% sucrose rinse were also calculated at baseline and after 30, 60, 90 days. The salivary ms concentrations were transformed to log10 values to normalise the data, and the mean and standard error (SE) was calculated for each group and time point.

RESULTS

Questionnaire

The results showed in table 1. At baseline the association among diabetes group and control group showed a significant association for fresh-squeezed juice no-school (p. 0.02), milk no school (p. <0.03), salted biscuit school (p. <0.04) , vegetable no-school (p. <0.04),

For other answer about frequency for drinking and snacking habits , no statistically differences were found when comparing any of the groups.

Table 1. The questionnaire.

Questionnaire	Diabetic Group1	Diabetic Group 2	p Value
Fresh-squeezed juice (S) Fresh-squeezed juice (NS)	0,90±1,55 1,44±1,75	1,35±1,80 2,59±2,19	NS 0,02
Juice in bottle (S) Juice in bottle (NS)	1,56±1,89 2,29±1,72	1,29±1,31 2,10±1,61	NS NS
Energy drink (S) Energy drink (NS)	0,15±0,50 0,00±0,00	0,00±0,00 0,13±0,50	NS NS
Diet beverage (S) Diet beverage (NS)	0,65±1,22 1,75±1,95	0,90±1,59 2,19±1,72	NS NS
Gas Beverage (S) Gas Beverage (NS)	0,44±0,70 0,63±1,15	0,50±1,10 1,16±1,68	NS NS
Milk (S) Milk (NS)	1,,20±1,61 1,67±2,23	1,40±1,72 2,80±2,08	NS 0,03
Coffee/The (S) Coffee/The (NS)	0,25±0,58 0,00±0,00	0,12±0,49 0,31±0,95	NS NS
Potato Chip (S) Potato Chip (NS)	1,12±1,45 0,89±0,96	0,76±1,25 1,27±1,71	NS NS
Salted biscuit (S) Salted biscuit (NS)	0,63±0,96 1,23±1,36	1,28±1,53 1,10±1,41	0,04 NS
Sweets (S) Sweets (NS)	1,44±1,72 2,11±1,18	1,22±1,66 1,62±1,69	NS NS
Cake/Biscuits (S) Cake/Biscuits (NS)	1,35±1,46 1,46±1,51	0,76±1,39 0,85±1,14	NS NS
Dessert (S) Dessert (NS)	0,67±1,18 1,33±1,40	1,13±1,89 1,59±1,62	NS NS
Ice cream (S) Ice cream (NS)	0,57±0,76 1,75±1,53	0,38±0,72 1,52±1,33	NS NS
Vegetable (S) Vegetable (NS)	1,90±1,89 2,29±1,69	2,00±1,94 3,20±1,85	NS 0,04
Fruits (S) Fruits (NS)	2,58±2,04 3,94±1,43	2,39±2,17 3,38±1,60	NS NS

Oral hygiene habit

The table 2 showed the results of oral hygiene habit .The association between diabetes group and control group showed a no significant statistical for all questions.

Table 2 Oral hygiene habit.

FACTOR	Diabetic 1	Diabetic 2	p Value (Chi-Quadro)
Oral Hygiene Habits			
Do you brush your teeth regularly?			
No	70,6% (24)	73,5 % (25)	NS
Sometimes	17,6% (6)	14,7% (5)	NS
Mostly every day	11,8% (4)	11,8% (4)	NS
Every day	0,0% (0)	0,0% (0)	NS
Toothbrusching frequency			
Once a day	11,8% (4)	20,6% (7)	NS
Twice a day	50,0% (17)	41,2% (14)	NS
Three time a day	29,4% (10)	35,3% (12)	NS
More than three times a day	8,8% (3)	2,9% (1)	NS
Fluoridated Toothpaste			
Yes	29,4% (10)	32,4% (11)	NS
No	68,6% (24)	67,6% (23)	NS
Fluoridated Mouthwash			
Yes	0,0% (0)	5,9% (2)	NS
Sometimes	26,5% (9)	8,8% (3)	NS
No	73,5% (25)	85,3% (29)	NS
Fluoride Supplements			
Yes	5,9% (2)	11,8% (4)	NS
Sometimes	20,6% (7)	11,8% (4)	NS
No	73,5% (25)	76,4% (26)	NS

Dental check ups			
Only when in pain	20,6% (7)	23,5% (8)	NS
Each six months	29,4% (10)	23,5% (8)	NS
Once a year	41,2% (14)	41,2% (14)	NS
Every two years	8,8% (3)	11,8% (4)	NS

Clinical data

The results are presented in Table 3. Comparison between two groups showed no one different for status caries.

Table 3. Status caries Diabetic group 1 (control group) and Diabetic group 2 (test group)

Caries	Diabetic group 1	Diabetic group 2	p Value
Caries free	55,9% (19)	61,9% (21)	NS
Initial caries	23,5% (8)	17,6% (6)	NS
Moderate caries	14,7% (5)	17,6% (6)	NS
High caries	5,9% (2)	2,9% (1)	NS

Plaque pH measurements

The results from the plaque pH measurements are shown in Table 4,5,6. The pH values recorded no statistically significant differences for maximun fall pH in each group after 90 days. No significant differences for control group when it valueted the minimal pH. A significant association for the test group with p. <0.001 after 90 days when it considered minimal pH. Comparison between the two diabetes groups showed no statistically significant differences when compared each group at baseline and after 90 days.

Table 4. Plaque pH measurement for control group at baseline, 30 days, 60 days, 90 days.

DIABETIC GROUP 1	TIME 0	TIME 1	TIME 2	TIME 3	P VALUE
FALL PH	1,18±0,56	1,13±0,51	1,06±0,45	1,07±0,51	NS
MINIMAL PH	5,34±0,50	5,39±0,44	5,48±0,40	5,53±0,43	NS

Table 5. Plaque pH measurement for test group at baseline, 30 days, 60 days, 90 days.

DIABETIC GROUP 2	TIME 0	TIME 1	TIME 2	TIME 3	P VALUE
FALL PH	1,20±0,46	1,10±0,42	1,02±0,35	0,98±0,29	NS
MINIMAL PH	5,37±0,41	5,46±0,37	5,59±0,29	5,69±0,24	<0,001

Table 6. Comparison test group and control group at baseline and after 90 days.

	TIME 0 GROUP 1	TIME 0 GROUP 2	TIME 3 GROUP 1	TIME 3 GROUP 2	P VALUE
FALL PH	1,18±0,56	1,20±0,46	1,07±0,51	0,98±0,29	* NS ^NS
MINIMAL PH	5,34±0,50	5,37±0,41	5,53±0,43	5,69±0,24	* NS ^ NS

Microbiological analyses

The table 7 show the list of bacteria analysed and the association among diabetic test group

and diabetic control group. A significant association for test group after intake of lozange of probiotic at 90 days (p.<0,001); for control group only *S. Sobribus* no one significant association.

The association between diabetic test group with diabetic control group showed a significant association at baseline only for the bacteria strain of *S. Salivarius* (p.<0.04) and *L. Casei* (p.<0.03);

after 90 days the comparison among diabetic test group and diabetic control group recorded a significant association for *S. Mutans, S. Sobrinus, L. Casei, L. Salivarius* (p. <0.01) and *S. Salivarius* (p.<0.02).

For other bacteria, no one statistically differences were found when comparing the groups in different time.

Bacteria Strain	Diabetic Group 1				Diabetic Group 2				P Value
	Time 0	Time 1	Time 2	Time 3	Time 0	Time 1	Time 2	Time3	
S. Mutans	3,61±1,13	2,15±0,65	1,82±0,72	1,56±0,56	3,09±1,08	1,76±0,60	1,32±0,47	1,21±0,43	*<0,001 °<0,001 ^NS ∞<0,01
S. Sobrinus	1,76±0,74	1,50±0,70	1,47±0,66	1,44±0,66	2,00±0,65	1,20±0,41	1,11±0,32	1,06±0,24	*NS °<0,001 ^NS ∞ <0,01
S. Sanguinis	2,50±0,86	1,74±0,82	1,65±0,81	1,47±0,71	2,89±1,12	1,68±0,64	1,53±0,61	1,29±0,46	*<0,001 °<0,001 ^NS ∞ NS
S. Salivarius	2,14±1,01	1,64±0,69	1,59±0,61	1,47±0,56	2,61±0,92	1,56±0,50	1,32±0,47	1,20±0,41	<0,001 °<0,001 ^<0,04 ∞ <0,02
S. Mitis	2,26±1,08	1,50±0,75	1,38±0,65	1,32±0,59	2,56±1,11	1,56±0,66	1,41±0,56	1,12±0,33	*<0,001 °<0,001 ^NS ∞ NS
S. Gordoni	2,47±0,89	1,47±0,51	1,44±0,50	1,32±0,47	2,32±0,94	1,41±0,56	1,35±0,49	1,17±0,39	*<0,001 °<0,001 ^NS ∞ NS
L. Casei	3,00±1,81	1,76±0,70	1,65±0,60	1,47±0,51	2,24±1,05	1,38±0,55	1,32±0,47	1,18±0,39	*<0,01 °<0,001 ^<0,03 ∞ <0,01
L. Salivarius	2,09±0,62	1,65±0,65	1,68±0,72	1,53±0,66	2,50±1,05	1,50±0,62	1,35±0,48	1,15±0,36	*<0,01 °<0,001 ^NS ∞ <0,01
L. Fermentum	2,47±0,86	1,41±0,50	1,35±0,49	1,29±0,46	2,26±0,90	1,38±0,55	1,26±0,45	1,21±0,41	*<0,001 °<0,001 ^NS ∞ NS

Table 7. Microbiological analyses (* control group, ° test group, ^ comparison among control group and test group baseline, ∞ comparison among control group and test group after 90 days)

DISCUSSION

The aim of this study was to evaluate the effect of probiotics (*Lactobacillus Brevis CD2*) on the oral microbiotic flora and biofilm acidogenicity in children diagnosed with diabetes since at least two years. Primary objective is the regular consumption of probiotic will lead to a significantly reduction of pathogenic bacteria compared to a placebo.

One of strengths of this study is that the study group consisted of children with diabetic type 1, with diagnoses diabetes at least 2 years, same oral hygiene habit and status caries for each group; for answer about frequency for drinking and snacking habits , no statistically differences

were found when comparing any of the groups eccept for fresh-squeezed juice no-school (p. 0.02), milk no school (p. <0.03), salted biscuit school (p. <0.04) , vegetable no-school (p. <0.04) but we can say that in spite of these data the two groups can be considered similar.
One limitation in the present study is the number of children was quite small (n=34+34); this may influence the statistical comparison.

The method for evaluating changes in plaque pH, the strip method used is easy and fast. The use of strips is easily and appropriate for a chair-side clinical use [27].

The our sample can be compared with area such as Finland or Sweden because the high prevalence of diagnosis diabetes type 1 is similar for these nations, they are known to have the highest incidence of Type 1 diabetes in the world.[4] [5]

In the subjects found some differences between the diabetics and their controls with respect to frequency of meals or consumption of carbohydrates, as assessed by questionnaires. The factors of diet were not considered to contribute significantly to the effect of diabetes-related factors on the caries outcomes in this study.

Oral hygiene habits don't demonstrate significant difference for the questions about toothbrushing frequency and dental check ups. Considering the caries status in each group we might suppose that the answers for oral hygiene habits of the all groups were not highlighted significant differences for our study.

The results from the plaque pH measurements are proved the pH values recorded no statistically significant differences for maximun fall pH in each group after 90 days. No one significant differences for control group when it valueted the minimal pH. A significant association for the test group with p. <0.001 after 90 days when it considered minimal pH. Comparison between the two diabetes groups showed no statistically significant differences when compared each group at baseline and after 90 days. After use of probiotics there was an improvement concerning the minimal ph in test group.

For bacteria analysed in diabetic test group and diabetic control group was found a significant association for test group after intake of lozange of probiotic at 90 days (p.<0,001); for control group only *S. Sobribus* no one significant association after 90 days.

The association between diabetic test group with diabetic control group showed a significant association at baseline only for the bacteria strain of *S. Salivarius* (p.<0.04) and *L. Casei* (p.<0.03);

after 90 days the comparison among diabetic test group and diabetic control group recorded a significant association for *S. Mutans, S. Sobrinus, L. Casei, L. Salivarius* (p. <0.01) and *S. Salivarius* (p.<0.02).

For other bacteria, no one statistically differences were found when comparing the groups in different time.

CONCLUSION

The results of this study show that use of probiotic may improve the minimal pH in Diabetic children and may decrease the bacterial flora species as S. Mutans, S. Sobrinus, L. Casei, L. Salivarius decreasing the caries risk with good diet and oral hygiene.

REFERENCES

1. American Diabetes, A., *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2013. **36 Suppl 1**: p. S67-74.
2. Silverstein, J., et al., *Care of children and adolescents with type 1 diabetes: a statement of the American Diabetes Association.* Diabetes care, 2005. **28**(1): p. 186-212.
3. Group, D.P., *Incidence and trends of childhood Type 1 diabetes worldwide 1990-1999.* Diabet Med, 2006. **23**(8): p. 857-66.
4. Patterson, C., et al., *Diabetes in the young - a global view and worldwide estimates of numbers of children with type 1 diabetes.* Diabetes Res Clin Pract, 2014. **103**(2): p. 161-75.
5. Tuomilehto, J., *The emerging global epidemic of type 1 diabetes.* Curr Diab Rep, 2013. **13**(6): p. 795-804.
6. Kuzuya, T., et al., *Report of the Committee on the classification and diagnostic criteria of diabetes mellitus.* Diabetes Res Clin Pract, 2002. **55**(1): p. 65-85.
7. Moore, P.A., et al., *Type 1 diabetes mellitus, xerostomia, and salivary flow rates.* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2001. **92**(3): p. 281-91.
8. Rayman, S., E. Dincer, and K. Almas, *Xerostomia: diagnosis and management in dental practice.* Todays FDA, 2011. **23**(6): p. 56-61.
9. Negrato, C.A. and O. Tarzia, *Buccal alterations in diabetes mellitus.* Diabetol Metab Syndr, 2010. **2**: p. 3.
10. Siudikiene, J., et al., *Dental caries increments and related factors in children with type 1 diabetes mellitus.* Caries Res, 2008. **42**(5): p. 354-62.
11. Lopez, M.E., et al., *Salivary characteristics of diabetic children.* Braz Dent J, 2003. **14**(1): p. 26-31.
12. Teughels, W., G. Loozen, and M. Quirynen, *Do probiotics offer opportunities to manipulate the periodontal oral microbiota?* J Clin Periodontol, 2011. **38 Suppl 11**: p. 159-77.
13. Lee, J.K., et al., *Modulation of the host response by probiotic Lactobacillus brevis CD2 in experimental gingivitis.* Oral Dis, 2015. **21**(6): p. 705-12.
14. Karuppaiah, R.M., et al., *Evaluation of the efficacy of probiotics in plaque reduction and gingival health maintenance among school children - A Randomized Control Trial.* Journal of international oral health : JIOH, 2013. **5**(5): p. 33-7.
15. Yanine, N., et al., *Effects of probiotics in periodontal diseases: a systematic review.* Clin Oral Investig, 2013. **17**(7): p. 1627-34.
16. Marttinen, A., et al., *Short-term consumption of probiotic lactobacilli has no effect on acid production of supragingival plaque.* Clinical oral investigations, 2012. **16**(3): p. 797-803.
17. Campus, G., et al., *Effect of a daily dose of Lactobacillus brevis CD2 lozenges in high caries risk schoolchildren.* Clin Oral Investig, 2014. **18**(2): p. 555-61.
18. Cagetti, M.G., et al., *The use of probiotic strains in caries prevention: a systematic review.* Nutrients, 2013. **5**(7): p. 2530-50.

19. Singh, R.P., S.G. Damle, and A. Chawla, *Salivary mutans streptococci and lactobacilli modulations in young children on consumption of probiotic ice-cream containing Bifidobacterium lactis Bb12 and Lactobacillus acidophilus La5.* Acta Odontol Scand, 2011. **69**(6): p. 389-94.
20. Chuang, L.C., et al., *Probiotic Lactobacillus paracasei effect on cariogenic bacterial flora.* Clin Oral Investig, 2011. **15**(4): p. 471-6.
21. Lexner, M.O., et al., *Microbiological profiles in saliva and supragingival plaque from caries-active adolescents before and after a short-term daily intake of milk supplemented with probiotic bacteria - a pilot study.* Oral health & preventive dentistry, 2010. **8**(4): p. 383-8.
22. Pitts, N.B., K.R. Ekstrand, and I. Foundation, *International Caries Detection and Assessment System (ICDAS) and its International Caries Classification and Management System (ICCMS) - methods for staging of the caries process and enabling dentists to manage caries.* Community Dent Oral Epidemiol, 2013. **41**(1): p. e41-52.
23. Wall-Manning, G.M., et al., *Checkerboard DNA-DNA hybridisation technology focused on the analysis of Gram-positive cariogenic bacteria.* J Microbiol Methods, 2002. **51**(3): p. 301-11.
24. Mannaa, A., et al., *Intra-familial comparison of supragingival dental plaque microflora using the checkerboard DNA-DNA hybridisation technique.* Archives of oral biology, 2012. **57**(12): p. 1644-50.
25. Mannaa, A., et al., *Supragingival plaque microbial analysis in reflection to caries experience.* BMC Oral Health, 2013. **13**: p. 5.
26. Campus, G., et al., *Effect of a sugar-free chewing gum containing magnolia bark extract on different variables related to caries and gingivitis: a randomized controlled intervention trial.* Caries Res, 2011. **45**(4): p. 393-9.
27. Carlen, A., H. Hassan, and P. Lingstrom, *The 'strip method': a simple method for plaque pH assessment.* Caries research, 2010. **44**(4): p. 341-4.

ORIGINAL PAPER

III
Submitted

A comparative assessment of gingivitis through reflectance spectrophotometry among Italian adolescents. A pilot study.

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Abstract

Objective. To assess gingival inflammation objectively through reflectance spectrophotometry in cross-sectional population studies. **Materials and methods**. This pilot study was carried out during academic year 2014 among 88 cluster sampling chosen Italian schoolchildren, first assessing the prevalence of gingival inflammation and then comparing sextants clinical records to spectral images using a portable MTH SpectroshadeTM Micro. Spectral ratio at 615 and 460 nm was identified as a method to detect properly the earliest periodontal breakdown. **Results**. Although over the 70% of the sample did not show any sign of gingival inflammation, concerning the portion of subjects affected of gingivitis the correlation among the spectral values categorized during data elaboration phase and the clinical records was always strict and stable ($p<0.01$ among all the variables), proving that reflectance spectrophotometry could be a powerful tool to fulfil the initial purpose. **Conclusions**. Despite the shape of the mouthpiece of the instrument chosen, which has been design primarily to include the smile line elements and not for the manner in which we approached with it, this study demonstrated that its reliability could significantly contribute to guide the community health interventions.

Introduction

Advances in research over recent years have led to a fundamental change in the understanding of the periodontal diseases process and in the assessment of the risk among adolescents or adults.

Gingival inflammation has been studied and assessed with several approaches. A variety of indices or schemes for evaluating the severity and extent on gingivitis have been formulated in the past. All are to some degree subjective in nature, depending on individual examiner skills, perceptions and judgments of gingival appearance. Among these scoring systems, the Gingival Index (GI), developed by Löe and Silness has gained the most widespread acceptance and use. Presence or absence of gingival bleeding has been associated with the visual clinical signs of gingivitis and with histologically detection of inflammation on gums (Engelberger et al., 1983; Benamghar, 1982; Abras et al., 1984; Greenstein et al., 1981; Barnett et al., 1980).

Several investigations demonstrated also that gingival microcirculation exhibits a dramatic, dynamic change in response to the development and progression of gingivitis. In particular, they proved the growth of blood flow in inflamed gingiva in comparison with healthy gingiva both in human and in animal models (Gleissner et al., 2006; Kedvongbundit et al., 2002; Wilder Smith et al., 1988; Kerdvongbundt et al., 2002; Forsslund, 1958). Thus, all these morphologic alterations occurring in gingival inflammation and currently scored subjectively using the indices reported above may be related to functional changes in the gingival microcirculation, which can on the contrary, be mapped objectively.

Therefore, gingival microvascular function has been examined with a variety of techniques even if sometimes their invasive or toxic nature prevented their application for in vivo

assessment in human volunteers. This is the case of infusion of labelled and unlabelled microspheres, electrical impendance plethysmography, high-speed cinematography, thermal clearance and radioisotope clearance (Kaplan et al., 1973; Vandersall and Zander, 1967; Kinnen and Goldberg, 1978; Hock et al., 1980).

As concerning non-invasive techniques, several authors have employed laser Doppler flowmetry as a method of studying gingival microvascular dynamic (Baab et al., 1986; Childress et al., 1991).

In human subjects, it has been reported laser Doppler flowmetrically was able to measure gingival blood flow in experimentally induced human gingivitis. Patients showed a reduction in gingival blood flow in labial marginal gingiva as compared with baseline measurements of healthy gingiva, indicating that human blood flow slows in the presence of inflammation (Matheny et al., 1993).

Laser Doppler Flowmetry has been successfully used to determine gingival blood flow in normal human gingiva, dog gingiva with increasing and decreasing inflammation and in young humans with histories of various periodontal diseases (Hinrichs et al., 1995; Baab et Oberg, 1987: Baab et al., 1990).

Optical spectrophotometry proves itself to be able to simultaneously determine multiple inflammatory indices related to periodontal disease directly in gingival tissues in vivo (Liu et al., 2009; Ge et al; 2011).

Reflectance spectrophotometry has been used as a noninvasive measure of microvascular function too, in particular to estimate the haemoglobin (Hb) concentration and Hb oxygen saturation and then to quantify severity and extent of gingival inflammation.

Many surveys showed that reflectance spectrophotometry can continuously measure the

gingival Hb concentration and Hb oxygen saturation in situ and discovered an increase in Hb concentration and a decrease in Hb oxygen saturation with increasing inflammation both in dog and human gums, suggesting that the increase in blood supply may not be enough to meet the oxygen demand in inflamed gingiva (Hanioka et al., 1989; Hanioka et al., 1990; Hanioka et al 1991; Sekhar et al., 2012; Sekhar et al., 2013).

In addition, spectral ratios at specific wavelengths have been used to extract relevant information from the absorption haemoglobin spectra (Sekjar et al., 2012).

It is mostly useful to distinguish two wavelength bands (A from 453 to 500 nm and B from 685 to 805 nm) in order to understand absorption coefficient for the oxygenated blood is higher than deoxygenated blood within Band A, and the opposite happens within Band B. Spectral ratio at wavelengths 460 and 615 from MHT Spectroshade™ Micro multispectral imaging system has been thus demonstrated to fall within bands A and B. It was especially assumed that comparing reflectance at these waveleghts would have provided accurate details about the blood content in the tissue (Zakian et al., 2008)

Considering properly the variety of risk factors that can start occurring during the particular stage of adolescence, it seemed significant to estimate the feasibility of a method possibly able to: (1) help in measuring the smallest changes in periodontal inflammation, (2) monitor the progression of the disease longitudinally (3) detect as much preventively as possible the earliest conditions in population cross sectional studies.

Based on these previous considerations a clinical and experimental cross sectional pilot study was carried out as presented below.

Material and methods

Sampling selection

A cross sectional pilot study was designed and approved by the Ethics Committee of the University of Sassari (2014_23_0598) and performed in Sassari (Italy) during academic year 2014-2015.

Sample size for this pilot study was performed through the online sample size calculator for pilot studies setting confidence at 0.99 and probability at 0.05. Prevalence of gingival disease among 13-15 year adolescences derived from a previous epidemiological study not yet published.

The estimated sample size was of 89.8 patients, but since two of the patients missed the appointment several times and no extra agreements were obtained, our final sample was 88 patients, which was considered quite enough to perform our cross sectional pilot study as well (Viechtbauer et al., 2015).

The project was first discussed with the adolescents in a way to get their interest and a better collaboration, then a letter explaining the aim and purpose of the study was sent to their parents associated with a consent form to be signed in order to attest their understanding of the contents and to authorize to enrol their child in the study.

Children with systemic medicals problems or those undergoing orthodontic treatment with fixed appliances were excluded.

A cluster sampling was performed using the class as a cluster; the number of school classes was inserted into the list and then randomly selected. The design and procedure of the study is displayed in figure 1.

Materials and measures

One single prior trained and calibrated examiner carried out spectral assessments assisted by a scribe across the whole study. All clinical examinations were performed under standard conditions in the infirmary of the high schools using natural daylight as a source of illumination. No radiographic examination was made.

To acquire spectra of gingival erythema a portable MHT Spectroshade™ Micro device was employed. This is a precision dental spectrophotometer largely used to evaluate the tooth colour and for this reason able also to be applied also on soft tissues. Its illumination system consists in light emitting from 410 to 620 nm. It acquires calibrated images from 400 to 720 nm. A black and white charge-couple device (CCD) fitted with the system was used to capture the reflectance image.

Prior to the clinical assessment it was decided to consider the oral cavity as divided into sextants and to investigate just a teeth for each of them. Since the shape and the dimensions of the device enabled to detect gingival tissues from elements five to seven, a comparative assessment was completed including teeth 14, 11, 24, 34, 31, 44. That way each sextant was investigated anyway and two scans could be easily obtained.

Measurements of reflectance spectra were always performed with a special mouthpiece before clinical assessment of disease stage, as clinical examinations may disturb the sites causing bleeding, thereby interfering with the spectral measurements. Each data capture session was preceded by the calibration of the device through a green and white ceramic tile specific to the unit chosen. Once acquired each image captured was processed by a second examiner not involved in the clinical session. Edge detection was applied to find the line contour corresponding to the gingival margin. To normalize the results, for each portion of gingival

tissue selected spectral ratio (named below as Zn) was calculated considering the average with the two evaluations corresponding to the scans. The inflammation at the spectral reflectance ratio R(615)/(R460) was then calculated.

All patients were clinically assessed by recording the Gingival Index, the Plaque index and the Gingival bleeding after plaque removal scores. Superficial plaque was removed through a WHO ball ended probe. In view of the average age of the population, in order to avoid assessing false pockets associated with tooth eruption, no probe was inserted into the sulcus (World Health Organization, 2013).

Data analysis

All data, first collected in tables with Excel® software, were then moved and elaborated with Stata ® statistical software version 12. Before discussing the results some points have to be clarified.

Since Gingival Index, Gingival Bleeding and Plaque Index scores were discrete values and Zn was a continuous variable its scores have been categorised. Thus, from the analysis of the distribution, three ranges of scores (1= 6,42-7,57; 2= 8,82-10,05; 3= 10,21-11,47) were identified. Another point to be explained is that to calculate the frequency for sextant of plaque index variable, it was necessary to have unique values. Consequently the approximations described in table 1 were done.

Results

The majority of this cluster randomized sample is composed of individuals with no signs of gingival inflammation (72,63%), and 41,90% of the sample has a perfect oral hygiene (P.I.

=0). In the remaining portion of the sample frequency for mild gingivitis is higher than for moderate ones (data not in table).

As regards the correlation among clinical records and Zn categorised values (Table 2), a significant P value shows how Zn Categorised Value 1 is related to healthy status of gums ($p<0.01$) in absence of plaque ($p<0.01$) or gingival bleeding ($p<0.01$). The same strong correlation ($p<0.01$) is confirmed as regards Zn categorised value 2 and mild gingivitis in absence of gingival bleeding. Zn Categorised at value 3 proved to be strictly related with moderate presence of dental plaque ($p<0.01$) and moderate gingivitis ($p<0.01$).

A strong correlation ($p<0.01$) is evidenced also among the absence of gingival inflammation, the absence of gingival bleeding the absence of dental plaque; similarly, mild gingival inflammation relates both with the presence of dental plaque and gingival bleeding.

No correlation was found neither between the spectral values categorised at value 2 and the low presence of dental plaque nor between mild gingival inflammation and low presence of plaque on dental surfaces.

Discussion

The hypotheses of this study was that reflectance spectrophotometry could be use even in cross sectional population studies. Here it has been proved that particularly spectra intensity ratio Zn (R 615/460) can be applied in that way to differentiate healthy and inflamed gums.

To the authors' knowledge, this was the first cross-sectional study carried out to evaluate the comparison between clinical indices about gingival inflammation and reflectance spectrophotometry. Generally, all the previous studied aimed to longitudinally assess the variance between spectral and clinical recordings in experimentally induced volunteers.

The most attractive aspect about reflectance spectrophotometry is that it is totally non-invasive, real-time, non-dangerous and it do not cause any discomfort to the patients during the procedure.

However, it is important to consider that since the dental arch morphology is not linear, as we go posteriorly towards the premolars and molar also gingival angulation changes. Thus any instrument, which is not dedicated to this aim, cannot detect with some certain precision teeth from fourth on. This leads obviously to a particular reflection.

On the base of the results it was possible to postulate that excluding the first molars from this assessment a statistic bias was generated.

On one hand it is in fact possible that registering first premolars scores in substitution of the molars' might be a bias, since they are nearest to the anterior sextant and thus generally assisted to be kept cleaned.

On the other hand, it is also important to consider that especially for the upper sextants the prevalence of the dental arch crowding is high and it mainly involves the third elements with a significant plaque retention in this region often affecting also the first premolars.

Nevertheless, since neither the variable rotation of the canine nor its alignment was considered in the present pilot study, this hypothesis could not be precisely disproved.

It is also important to consider that they have been enrolled only healthy adolescents declaring not to have any luxury habits. It would be interesting to assess if the results could change in case of a population deferring for ethnicity, average age or life habits.

However results could change, it is clear that reflectance spectrophotometry allows for permanent recording of changes in inflammation, helping operators in diagnosis as soon as the inflammation is taking place.

Despite the instrument chosen has been designed primarily to include the smile line elements and not actually for the purpose in which we approached with it, this pilot study demonstrated that its reliability could significantly contribute to guide the community health interventions to an increasingly preventive direction. Further studies should be performed to better understand if this system could be even able to predict gingival inflammation before clinical signs are manifest.

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Figure 1. Flow chart of the study.

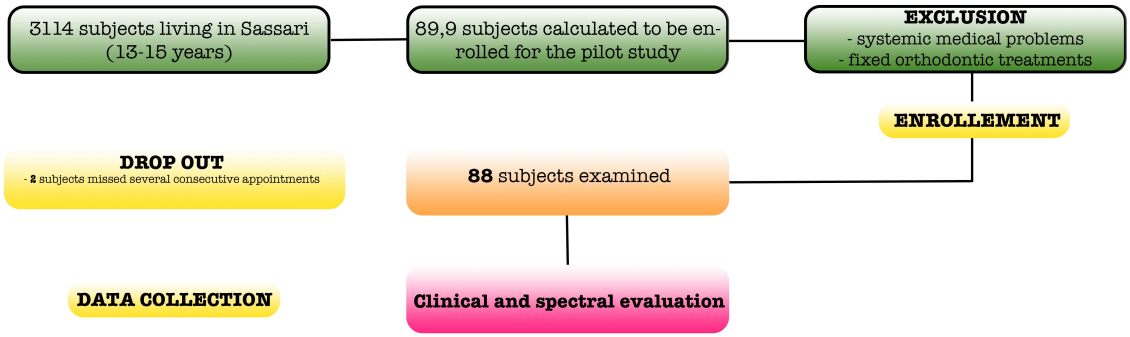

Table 1. Approximations among Plaque Index scores.

Plaque Index Scores	Approximations
0,25	0
0,50	1
1,25	1
1,50	2
1,75	2

Table 2. Correlation matrix among spectral values and clinical variables.

	Absence of dental plaque	Low dental plaque	Moderate dental plaque	Absence of gingival bleeding	Presence of Gingival bleeding	Absence of gingival inflammation	Mild gingival inflammation	Moderate Gingival inflammation	Zn Cat. Value 1	Zn Cat. Value 2	Zn Cat Value 3
Absence of dental plaque	1.00										
Low dental plaque		1.00									
Moderate dental plaque			1.00								
Absence gingival bleeding	0.50 **p<0.01**	0.12 *p>0.01*		1.00							
Presence of gingival bleeding			0.12 *p>0.01*		1.00						
Absence gingival inflammation	0.55 **p<0.01**			0.78 **p<0.01**		1.00					
Mild gingival inflammation		0.22 p>0.01		0.36 **p<0.01**			1.00				
Moderate gingival inflammation			0.48 **p<0.01**		0.42 **p<0.01**			1.00			
Zn Cat. Value 1	0.48 **p<0.01**			0.54 **p<0.01**		0.71 **p<0.01**			1.00		
Zn Cat. Value 2		0.10 p>0.01		0.50 **p<0.01**			0.55 **p<0.01**			1.00	
Zn Cat. Value 3			0.42 **p<0.01**		0.14 p>0.01			0.47 **p<0.01**			1.00

boldface indicates statistical significance (p<0.01)

References

1. Abrams K, Caton J, Polson A (1984). Histological comparison of interproximal gingival tissues related to presence or absence of bleeding index as indicators of periodontal status. *J Periodontol* **55**: 629-632
2. Baab DA, Oberg A, Lundstrom A (1990). Gingival blood flow and temperature changes in young humans with a history of periodontitis. *Arch Oral Biol* **35**: 95–101.
3. Baab DA, Oberg PA, Holloway GA (1986). Gingival blood flow measured with a laser Doppler flowmeter. *J Periodontol* **21**: 73–85.
4. Baab DA, Oberg PA (1987). Laser Doppler measurement of gingival blood flow in dogs with increasing and decreasing inflammation. *Arch Oral Biol* **32**: 551–555.
5. Barnett ML, Ciancio SG, Mather ML (1980). The modified papillary bleeding index: comparison with gingival index during the resolution of gingivitis. *J Prev Dent* **6**: 135-138
6. Benamghar L (1982). Comparison of gingival index and bleeding index as indicators of periodontal status. *Bull World Health Org* **60**: 147-151
7. Childress K, Abrams H, Johnson D, Toth G, Matheny J (1991). Gingivitis and human gingival circulation: videomicroscopy and laser Doppler evaluation. *J Dent Res* 70-590

8. Engelberger T, Hefti A, Kallenberger A, Rateitschak KH (1983). Correlations among papilla bleeding index, other clinical indices and histologically determined inflammation of gingival papilla. *J Clin Periodontol* **10**: 579-589
9. Forsslund G (1958). The structure and function of the capillary system in the gingiva in man. *Acta Odontol Scand* **17**: 1-44
10. Ge Z., Liu KZ, Xiang X, Yang Q, Jianhua H, Elicia K, Sowa MG (2011). Assessment of local Hemodynamics in Periodontal inflammation using optical spectroscopy. *J. Periodontol* **82**: 1161-1168
11. Gleissner C, Kempki O, Peylo S, Glabel JH, Willershausen B (2006). Local gingival blood flow at healty and inflamed sites measured by Laser Doppler flowmetry. *J Periodontol* **77**: 1762-1771
12. Greenstein G, Caton J and Polson AM (1981). Histological characteristics associated with bleeding after probing and visual signs of inflammation. *J Periodontol* **52**: 420-425
13. Hanioka T, Shizuhuishi S, Tsunemitsu A (1991). Changes in Hemoglobin concentration and oxygen saturation in human gingiva with decreasing inflammation. *J Periodontol* **62**(6): 366-369
14. Hanioka T, Shizukuishi S, Tsunemitsu A, Joh S, Sugiyama K, Sato N (1989). Haemoglobin concentration and oxygen saturation in dog gingiva with experimentally induced periodontitis. *Archs Oral Biol* **34**: 657-663
15. Hanioka T, Shizukuishi S, Tsunemitsu A (1990). Hemoglobin concentration and oxygen saturation of clinically healthy and inflamed gingiva in human subjects. *J Periodont Res* **25**:93-988

16. Hinrichs JE, LaBelle LL, Aeppli D (1995). An evaluation of laser Doppler readings obtained from human gingival sulci. *J Periodontol* **66**: 171-176
17. Hock J, Nuki K, Schlenker R, Hawks A (1980). Clearance rates of xenon-133 in non-inflamed gingiva of dogs. *Arch Oral Biol* **25**: 445-9
18. Hock J, Nuki K (1976). Erythrocyte velocity in vascular networks of young non inflamed dog gingiva. *J Dent Res* **55**: 1058-60
19. Kaplan ML, Davis MA, Glodhaber P (1973). Blood flow measurements in selected oral tissues in dog usig radiolabelled microspheres and rubidium-85. *Arch Oral Biol* **23**: 281-284
20. Kerdvongbundlt V, Vongsavan N, Soo-Ampon S, Phankosol P, Hasegawa A (2002). Microcirculation of healthy human gingiva. *Odontology* **90**: 48-51
21. Kerdvongbundt V, Siriat M, Sirikulsathean A, Kasetsuwan J, Hasegawa A (2002). Blood flow and human periodontal status. *Odontology* **90**: 52-56
22. Kinnen E, Golberg HJ (1978). The application of electrical impendance plethysmography to the study of gingival circulation. *J Periodontol* **49**: 528-533
23. Liu KZ, Xiang XM, Man A, Sowa MG, Cholakis N, Ghiabi E, Singer DL, Scott DA (2009). In vivo determination of multiple indices of periodontal inflammation by optical spectroscopy. *J Perio Res* **44**: 117–124
24. Matheny JL, Abrams H, Johnson DT, Roth GI (1993). Microcirculatory dynamics in experimental human gingivitis. *J Clin Periodontol* **20**: 578-583
25. Sekhar PC, Betsy J, Jayanthi J, Nisha UG, Presanthila J, Subhash N (2013). In vivo inflammation mapping of periodontal disease based on diffuse reflectance spectral imaging: a clinical study. *J Biomed Opt* **18**(2): 026019

26. Sekhar PC, Betsy J, Presanthila J, Subhash N, Jayanthi JL, Presanthila J (2012). Non – invasive detection of periodontal disease using diffuse reflectance spectroscopy: a clinical study. Biomedical Applications of Light Scattering VI, edited by Adam P. Wax, Vadim Backman, Proc. of SPIE Vol. 8230, 82301
27. Sekhar PC, Betsy J, Presanthila J, Subhash N (2012). Discrimination of periodontal diseases using diffuse reflectance spectral intensity ratios. *J Biomed Opt* **17**(2): 027001-1-027001-9
28. Vandersall DC and Zander HA (1967). Experimental obstruction of the periodontal blood circulation. *Helv Odontol Acta* **11**: 74-79
29. Viechtbauer W, Smits L, Kotz D, Budé L, Spigt M, Serroyen J, Crutzen R (2015). A simple formula for the calculation of sample size in pilot studies. *J Clin Epidemiol* (In Press)
30. Wilder Smith P and Frosh P (1988). Laser Doppler flowmetry: a method for the determination of the blood flow. *Dtsch Zahanarztl Z* **43**: 994-997
31. World Health Organization (2013). Oral Health Surveys. Basic Methods. Introduction. 5th edition. World Health Organization: Geneva, p. 7
32. Zakian C, Pretty I, Ellwood R, Hamlin D (2008). In vivo quantification of gingival inflammation using spectral im