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Scuola di dottorato in medicina clinica e sperimentale

Dipartimento di Scienze Odontostomatologiche e
Maxillo –Facciali

DOTTORATO DI RICERCA IN:
SCIENZE ODONTOSTOMATOLOGICHE

XXII CICLO

TESI

“A BIOLOGICAL WAY IN CARIES PREVENTION”.

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Anni Accademici 2006-2009

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INTRODUCTION

Oral health is a part of general health and hence affects the total well-being of individuals. Dental and oral diseases affect various aspects of quality of life. Dental caries has been associated with numerous adverse effects on children's health including pain, restricted dietary intake, impaired growth, and reduced body weight [Hamissi et al., 2008].

Even though the onset of dental caries has decreased through the use of preventive systems (improved oral hygiene, usage of fluoride-containing toothpaste, fluoride content in drinking water, sealing), tooth decay is still one of the most prevalent chronic worldwide diseases [Al-Malik and Rehbini, 2006; de Almeida et al., 2003; Ferrazzano et al., 2006; Ferro et al., 2007; Jacobsen and Young, 2003; Selwitz et al., 2007; Simonsen, 2002].

In fact, a decline in DMFT (decayed, missing, filled teeth) indices from 5.0 to 0.9 in the period from 1980 to 2002 was observed among 12-year-olds in Denmark; in Sweden from 3.1 to 0.9 during 1985–2001. A decline in the DMFT index from 5.1 to 3.8 was found in Poland during 1991–2000. National epidemiological surveys conducted by the Brazilian Ministry of Health in 1996 and 2003 showed a DMFT reduction of 9.15% (3.06 to 2.78) in 12-year-olds [Cypriano et al., 2008; Milciuviene et al., 2009].

Furthermore, significant gains in oral health levels have been observed over the last few years in countries like Germany, England, USA, Scandinavia, Scotland, Norway and Australia [Cypriano et al., 2008].

However, with the decline of dental caries in several countries the chance of extending oral health care to other groups of the population has increased significantly [Petersen, 2005].

In fact, there are profound disparities in oral health. The indigenous poor populations, immigrants, racial and ethnic minority groups, and medically compromised patients are those who suffer the worst oral health conditions [Skeie et al., 2006].

Recent evidence indicates that immigrants and minority ethnic groups should be regarded as "whole populations at risk" on the verge of oral health deterioration. People crossing national and cultural frontiers originally are characterized by disease patterns, health behaviours and health care modalities different from those at their destinations [Vered et al., 2008].

Recent reports from developed countries indicate inequalities in caries prevalence among preschoolers, with a higher caries prevalence and severity in certain ethnic and immigrant groups [Hallett and Rourke, 2006; Wyne, 2008].

For example, in both Denmark [Sundby and Petersen, 2003] and the UK [Gray et al., 2000], children from different ethnic groups present higher levels of caries than is seen in the native population. In The Hague (Netherlands) [Truin et al., 2005], a study has described higher caries prevalence among low-social-class children of Turkish and Moroccan origin when compared with Dutch children from the same social class.

This indicates that there are still shortcomings in the areas of both preventive and curative dental care. More oral health education programs must be deployed in an attempt to control oral diseases and school-based approaches should be combined with family- and community-directed preventive programs. Also, specific strategies should be developed for the different situations concerning oral health status [Hamissi et al., 2008].

There is a need to identify areas with high caries levels, even if the DMFT =3 goal had already been achieved, and to develop oral health strategies for the groups with the highest caries prevalence. Finally, individualized approaches should be applied, promoting reasonable, effective and lasting health actions. In this view, because caries affects individuals disproportionately, it is essential that those at the highest risk are identified early so that preventive therapies can be targeted toward those who are most likely to benefit.

A significant improvement in dental caries worldwide levels might be reached by implementation of caries preventive strategies, paying special attention to high-risk groups, represented by people with low economic-social status in developed countries and people who lives in less developed countries.

For this reason our research is focused on the elaboration of a new preventive methodology against dental caries, based on the identification and the characterization of natural active principles (derived from animals) [Walker et al., 2006], having anti-caries activity.

In this way, we should reduce the severity of this illness, first of all, only by changing the dietary behaviours of the population, then, by adding these natural

active principles to food, toothpaste, mouthwash, tablets. These anti-cariogenic agents would be particularly useful, being non toxic and relatively cheap.

The food group most recognised as exhibiting anti-caries activity is dairy products [Addeo et al., 1994; Aimutis, 2004; Ferranti et al., 1997].

Using in vitro, animal and human in situ models, the anti-cariogenic activity of milk and milk products was attributed to the direct chemical effects of casein phosphopeptides, calcium and phosphate [McDougall, 1977; Reynolds, 1998].

SCIENTIFIC BACKGROUND

Casein phosphopeptides

Casein phosphopeptides (CPPs) are phosphorylated casein-derived peptides produced by proteolytic digestion of α_{s1} -, α_{s2} -, and β -casein during the natural digestive process in vivo, by action of proteolytic enzymes in vitro, and by proteolytic starter cultures during manufacturing of dairy products as fermented milk, yogurt and cheese [Bouhallab and Bouglé, 2004; Cai et al., 2003; Ramalingam et al., 2005; Walker et al., 2006].

CPPs, containing the sequence Ser(*P*)-Ser(*P*)-Ser-(*P*)-Glu-Glu, stabilize nanoclusters of amorphous calcium phosphate (ACP) in metastable solution. These multiple phosphoseryl residues of the CPPs bind to forming nanoclusters of ACP in supersaturated solutions, preventing growth to the critical size required for phase transformations.

CPPs-ACP localize ACP in dental plaque, which buffers the free calcium and phosphate ion activities, helping to maintain a state of supersaturation with respect to tooth enamel, depressing demineralisation and enhancing remineralisation [Cross et al., 2004; Cross et al., 2005].

In particular, CPPs stabilize calcium and phosphate ions under neutral and alkaline conditions forming metastable solutions that are supersaturated with respect to the basic calcium phosphate phases. Under these conditions, the CPPs bind their equivalent weights of calcium and phosphate .

The preventive action of CPPs, in vivo, takes place when there are demineralising agents (acid pH) that can enhance the release of calcium from the CPP-ACP

complex, thus increasing the Ca cation concentration and promoting a supersaturation condition that will prevent demineralisation and enhance the remineralisation of early enamel caries [Reynolds et al., 2003].

On the basis of the generally accepted molecular formula for ACP [$\text{Ca}_3(\text{PO}_4)_2 \cdot n\text{H}_2\text{O}$], ACP also may be considered a tricalcium phosphate. There is no conclusive evidence that ACP is an integral mineral component in hard tissues. It likely plays a special role as a precursor to bioapatite and as a transient phase in biomineralization [Azarpazhooh and Limeback, 2008].

In summary, CPP-ACP complexes have a multiple action mechanism: on one hand, providing an oversaturation of calcium and phosphate ions in the dental biofilm and saliva, conferring the potential to be biological delivery vehicles for calcium and phosphate; on the other hand, inhibiting adhesion of cariogenic bacteria to the hydroxyapatite making it possible to modulate the activity of plaque bacteria and favour colonization by less cariogenic bacteria. This can help to reduce acid formation in the biofilm and reduce enamel demineralisation. The final effect is that saliva and plaque are kept in calcium and phosphate supersaturation with respect to the enamel which allows a reduction in demineralisation and favours remineralisation [Azarpazhooh and Limeback, 2008; Llana et al., 2009].

Several laboratory, animal and human *in situ* experiments have demonstrated that both synthetic casein phosphopeptide-amorphous calcium phosphate (CPPs-ACP) nanocomplexes contained in mouthrinses and sugar-free chewing gum, and natural CPPs contained in dairy products (such yogurt) are anticariogenic [Ferrazzano et al., 2008; Iijima et al., 2004; Manton et al., 2008; Morgan et al., 2008; Shen et al., 2001].

Furthermore, Guggenheim et al. found that CPP-ACP taken with a cariogenic diet in rats significantly reduced the numbers of streptococcus sobrinus by interfering with bacterial adherence and therefore colonization [Guggenheim et al., 1995].

In addition, a commercial paste containing CPP-ACP has shown to remineralise initial enamel lesions [Kumar et al., 2008].

The application of a CPPs toothpaste and sodium fluoride (Colgate Neutrafluor 9000 ppm) (NaF) can provide significant additional prevention of enamel demineralisation when resin-modified glass ionomer cement (RMGIC) is used for bonding molar tubes for orthodontic patient as preventive actions [Sudjalim et al., 2007].

An in vitro study to evaluate the remineralisation of incipient enamel lesions by the topical application of Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) using laser fluorescence and scanning electron microscope showed high scores of remineralisation [Pai et al., 2008].

However, as suggested by Walker, in liquid milk the majority of the casein and calcium and phosphate ions are bound in micelles and then upon consumption would not necessarily be available to promote enamel remineralisation [Walker et al., 2006].

In fact, bovine milk contains around 30 mM-calcium, where only approximately 10 mM is not bound in casein micelles and of that only approximately 2 mM is free calcium ions [Neville et al., 1994].

The calcium in casein micelles is unlikely to be available to diffuse across a relatively intact enamel surface layer into a subsurface lesion and therefore milk may have limited ability to remineralise enamel lesions in situ.

Therefore, since milk couldn't be used as a natural product against dental caries, because it needs the addition of 2.0–5.0 g CPP-ACP to substantially increase its ability to remineralise enamel subsurface lesions [Walker et al., 2006], the remineralisation effect of other dairy products (yogurt, cheese etc..) should be investigated.

Having milk and yogurt similar components, it can be supposed that the mechanisms by which yogurt may have a protective effect against dental caries are the same as milk [Ferrazzano et al., 2007; Ferrazzano et al., 2008].

The protein content of yogurt is generally higher than that of milk because of the addition of non-fat dry milk during processing and concentration, which increases the protein content of the final product.

Yogurt is, then, very rich in calcium and phosphorus. In fact, fermentation has little effect on the mineral content of milk and therefore the total mineral content remains unaltered in the yogurt. Moreover, because of the lower pH of yogurt compared with that of milk, calcium is present in yogurt mostly in ionic form [Adolfsson et al., 2004]. Additionally, CPP content in yogurt is higher than that in milk due to the proteolytic activity of microorganisms contained in the yogurt [Rasić and Kurmann, 1983].

The effect of yogurt on cariogenic bacteria

Dental caries has a multifactorial aetiology [Nishikawara et al., 2007]. It has been suggested to be triggered by three main factors: carbohydrate (diet), bacteria (dental plaque), and susceptible teeth (the host) [Keyes and Jordan, 1963].

Fundamentally, caries is a bacterial infectious disease. The critical bacterial virulence factors are carbohydrate-derived organic acids.

The caries process may be described as reflecting the imbalance between the effect of saliva, the dominant protector of the tooth surface, on one hand, and the combined impact of the factors dietary carbohydrate and the plaque flora on the other. Since saliva cannot always control the latter factors completely, a critical focus on the bacterial composition of plaque as a decisive cariogenic determinant is justified [van Ruyven et al., 2000].

At present, particularly, mutans streptococci (MS) and lactobacilli are well known as cariogenic oral bacteria [Hamada and Slade, 1980; Loesche, 1986]. MS, including streptococcus mutans and streptococcus sobrinus, are well known as a group of oral micro-organisms which have virulence factors and which are harbored on the tooth surface within the oral bio-film. The key virulence factors are synthesized water insoluble glucan from sucrose, acidogenicity and acid tolerance [Hamada et al., 1984].

Meanwhile, lactobacilli do not avidly colonize teeth surfaces, but they may be transiently found in the oral cavity even before teeth erupt.

They preferentially colonize the dorsum of the tongue and are carried into saliva by sloughing of the tongue epithelium [Van Houte et al., 1972].

Their numbers in saliva appear to reflect the consumption of simple carbohydrates by the host [Staat et al., 1975].

Lactobacilli are acidogenic in the presence of carbohydrates, they are acid tolerant [Crossner et al., 1989], and they are often isolated from established carious lesions [Loesche and Syed, 1973].

It is also known that large amounts of mutans streptococci and lactobacilli inhabit caries lesions [Arneberg et al., 1984].

Among the three factors responsible for caries development (carbohydrate, bacteria and susceptible teeth), bacteria have been suggested to have the strongest effect on the prevalence or incidence of dental caries [Hardie et al., 1977]. Therefore, it is necessary to use antibacterial substances able to reduce the pathogenicity of these bacteria.

Yogurt has traditionally been considered a probiotic-carrier food with health-promoting effects [García-Albiach et al., 2008].

It is defined by the Codex Alimentarius of 1992 as a coagulated milk product that results from fermentation of lactic acid in milk by *Lactobacillus bulgaricus* and *Streptococcus thermophilus*.

In fresh yoghurt the amount of these micro-organisms together are in a concentration of 10^8 cells/ml. Other lactic acid bacteria (LAB) species can be combined with *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. In the finished product, the LAB must be alive and in substantial amounts. LAB have been used for thousands of years to produce fermented food and milk products. Fermented products contain a variety of fermenting micro-organisms belonging to various genera and species, all of which produce lactic acid [Bourlioux and Pochart, 1988].

With few exceptions, milk and yogurt have similar vitamin and mineral compositions. During fermentation, vitamins B-12 and C are consumed and folic acid is produced. The differences in other vitamins between milk and yogurt are small and depend on the strain of bacteria used for fermentation. Although milk and yogurt have similar mineral compositions, some minerals, eg, calcium, are more

bioavailable from yogurt than from milk. In general, yogurt also has less lactose and more lactic acid, galactose, peptides, free amino acids, and free fatty acid than milk has [Shahani and Chandan, 1979].

In many modern societies, fermented dairy products make up a substantial proportion of the total daily food consumption.

Furthermore, it has long been believed that consuming yogurt and other fermented milk products provides various health benefits [Saint-Eve et al., 2006].

In recent years, many investigators have studied the therapeutic effects of yogurt and the bacterial cultures used in the production of yogurt [Elli et al., 2006]. The benefits of yogurt and LAB on health have been investigated in animal models and, occasionally, in human subjects. Some studies using yogurt, individual LAB species, or both showed promising health benefits for diseases such as cancer, infection, gastrointestinal disorders (lactose intolerance, constipation, diarrheal diseases, inflammatory bowel disease, helicobacter pylori infection), asthma and allergies [Adolfsson et al., 2004].

Abundant evidence implies that specific bacterial species used for the fermentation of dairy products such as yogurt have powerful anti-pathogenic and anti-inflammatory properties [Meydani and Ha, 2000].

Since there is clear evidence that yogurt can restore the physiological microbial equilibrium in several areas of the digestive tract [Parvez et al., 2006], it is possible to hypothesise a similar activity in restoring microbial homeostasis of dental plaque, disrupted by factors such as high-frequency intake of fermentable carbohydrates. However, the possible impact on oral health is less explored.

It has been suggested that bacteria present in yogurt may have a positive effect on the ecology of dental plaque, re-establishing the microbial homeostasis, broken down by the bacterial metabolism. This hypothesis could represent a favourable outcome in terms of prevention of dental caries.

Recent studies reported that yogurt and probiotic-containing milk and cheese consumption has been associated with a decrease in mutans streptococci in dental plaque and/or saliva [Ahola et al., 2002; Caglar et al., 2005; Nikawa et al., 2004].

Petti et al. in an *in vitro* study investigated the differences in susceptibility to yogurt between several strains of viridans streptococci. Two strains of each of the following streptococcus species were tested: mutans, sobrinus, gordonii, oralis, parasanguinis and sanguinis. Survival rates after incubation with fat-free plain yogurt containing streptococcus thermophilus and lactobacillus bulgaricus were 8% (*S. mutans* 6519T), 12% (*S. mutans* 31738), 35% (*S. oralis* 25671) and >50% (all other species tested) after 15 min, and 0.01% (*S. mutans*) and >10% (all other species tested) after 30 min. The authors concluded that *in vitro* yogurt had bactericidal activity against *s. mutans*, and suggested that the decrease in the oral level of mutans streptococci *in vivo* as a result of daily yogurt intake was partly attributable to direct and selective bactericidal activity of yogurt against *s. mutans* [Petti et al., 2008].

The protective role of saliva

During dental caries, enamel and dentine are in contact with an acid H⁺ releasing system. This may be plaque during an *in situ* or *in vivo* experiment or an acidic solution or acidic gel *in vitro*. The H⁺ ions cause local dissolution of the tissue, and

the resulting dissolution products (calcium and phosphate ions) diffuse out of the tooth [Arends et al., 1997].

Over the course of human life, enamel and dentin undergo unlimited cycles of demineralisation and remineralisation. The crux of this argument lies in the balance of these two competing phenomena. A tip in the balance one way or the other will either lead to stronger healthier teeth or greater susceptibility to dental caries and other oral complications. This mineral loss compromises the mechanical structure of the tooth and could lead to cavitation over a long period of time. Saliva alone has the capability to increase plaque pH with bicarbonates although typically this process may take up to 2 hours. The susceptibility of apatite in enamel surface layers makes it critical to control the acidity of the plaque fluid and the calcium and phosphate ion concentrations in saliva [Featherstone, 2000]. The subsequent remineralisation process is nearly the reverse. When oral pH returns to near neutral, calcium and phosphate ions in saliva incorporate themselves into the depleted mineral layers of enamel as new apatite. The demineralised zones in the crystal lattice act as nucleation sites for new mineral deposition. Essentially, the sudden drop in pH following meals produces an under-saturation of those essential ions in the plaque fluid with respect to tooth mineral. This promotes the dissolution of the enamel. Instead, at elevated pH, the ionic supersaturation of plaque shifts the equilibrium the other way, causing a mineral deposition in the tooth.

Saliva plays multiple roles in these oral processes. Aside from providing a constant rinse, the value of saliva as a reservoir for calcium, phosphate and fluoride has been well established [Larsen and Pearce, 2003]. Saliva offers a myriad of other benefits,

although many are not widely known but contribute significantly to enamel remineralisation [Amaechi and Higham, 2001].

The buffering capacity of human saliva plays a major role in countering fluctuations in pH. In particular, bicarbonates in saliva played a major role in elevating low oral pH after meals [Aiuchi et al., 2008]. Other buffers present in saliva include urea proteins. Urease enzyme in plaque fluid metabolizes urea producing ammonia and an increase in plaque pH. Arginine rich proteins in saliva can also metabolize into alkaline substances such as arginine and ammonia. Phosphate has also been found to contribute to buffering capabilities [Larsen and Richards, 2001]. Various salivary components also demonstrate antibacterial capability. Iron binding protein lactoferrin has been shown to inhibit aerobic and facultative anaerobic bacteria (such as streptococcus mutans) which require iron to metabolize. Lysozyme also exhibits direct antibacterial function [Flink et al., 2007].

Transverse microradiography (TMR)

In caries research, the level of mineral content in enamel and dentin is a good indication of the demineralisation and remineralisation process. It is important for small changes in tooth mineral content to be measurable [White et al., 1992]. Mineral content, as measured by most methods, refers to the concentrations of the major components of dental hard tissues, calcium and phosphate. Currently, transverse microradiography (TMR) is the most accepted tool for the above purpose [Damen et al., 1997].

Microradiography has been used in more than 2000 studies during the last four decades [Ngo et al., 1997]. Several reviews in which mineral quantification

techniques are compared show that three different "generations" of microradiography have been developed:

- contact microradiography or transverse microradiography (TMR), in which thin sections (slices ~ 90 µm thick) are analyzed;
- longitudinal microradiography (LMR), in which thick tooth slices (~ 400 µm) are used and analyzed;
- wavelength-independent microradiography (WIM), suitable for mineral quantification of whole teeth [Arends and ten Bosch, 1992; ten Bosch and Angmar-Månsson, 1991].

Transverse microradiography (TMR) of thin sections is the principal method for determining mineral changes in experimental lesions [Damen et al., 1997].

For the determination of the mineral loss from experimental incipient lesions, transverse microradiography (TMR) is the analytical method which yields the most detailed quantitative information to date [Arends and ten Bosch, 1992]. It is not a new method, but since its first application in dental research [Angmar et al., 1963], the technique has been refined constantly, and especially the developments in computer-aided video-image analysis of microradiographs have made TMR an appropriate tool for determining small changes in mineral density profiles in time.

The TMR results are expressed in terms of the vol% of mineral as a function of depth from the surface [Ngo et al., 1997].

Arends et al. were the first to quantify the changes in mineral content in dental hard tissues, after a sample was exposed to de-/re-mineralisation cycles [Arends et al., 1983].

For TMR analysis, a thin piano-parallel slice (approximately 80 µm thick) was sectioned transversally from each dental block. Contact microradiograms, by means of monochromatic x-rays, were made for each slice, together with a calibration aluminum step-wedge. After development, the x-ray adsorption was reflected directly in the optical density of the film. Using microdensitometry, it is possible to calculate the mineral content in volume percentage using Angmar's formula [Angmar et al., 1963].

AIMS

The intent of this research program was to determine a new way in caries prevention. The score was the valuation of anti-caries effects of bio-active molecules of natural origin (derived from animals), in order to elaborate a new preventive methodology. The under discussion molecules were casein phosphopeptides (CPPs) of dairy origin.

For this aim, this research project has been divided in 5 part.

Part 1: in vitro evaluation of the effectiveness of synthetic CPPs in dental caries prevention;

Part 2: in vitro evaluation of the effectiveness of natural CPPs in dental caries prevention;

Part 3: in vivo evaluation of the effectiveness of synthetic and natural CPPs in dental caries prevention;

Part 4: in vivo evaluation of the antibacterial power of commercial yogurt against oral cariogenic microflora;

Part 5: in vitro effectiveness of TMR (transverse micro-radiography) in assessing dental enamel demineralisation and remineralisation.

The final score will be the creation of a new preventive methodology against dental caries. In fact, on the basis of the results obtained from this research, the protection against tooth decay could be done in different ways. First of all, people could protect their self simply by eating food containing CPPs , then these active principles could be exploited by nutritional and pharmacological industries by adding these bio-molecules both in food and in commercial products used for oral hygiene (mouth-rinse, toothpaste).

Part 1

In vitro evaluation of the effectiveness of synthetic CPPs in dental caries prevention.

ABSTRACT

Aim Objective of this study is in vitro testing of the capability of synthetic CPPs to prevent demineralisation and promote remineralisation of early enamel lesions.

Materials and Methods 159 samples of dental enamel were divided into 3 groups, which subsequently underwent 3 different chemical treatments: the samples from group I (control group) were preserved in distilled water; the samples from group II were treated with a demineralising solution for producing artificial caries; the samples from group III underwent the same treatment as group II, but with the addition of synthetic CPPs. The effects of these procedures were evaluated by quantitative analysis (change in weight and calcium titration) and qualitative analysis (SEM). **Statistics** Statistical analysis of the results was performed using ANOVA.

Results In presence of CPPs, acid dissolution of human enamel is reduced by over 50% in vitro. **Discussion** Our results demonstrate that CPPs could be a valid preventive system against demineralisation of early enamel lesions.

MATERIALS AND METHODS

In the present study, 53 human molars, extracted for orthodontic reasons or impaction, were cleaned with sterile gauze imbibed in distilled water and stored at 4°C in sterile containers with 5% formalin solution. The roots were then cut and the crowns polished with pumice dust and non-fluoride toothpaste, using a circular brush with nylon bristles mounted on a dental hand-piece; they were then rinsed in distilled water. Subsequently, the crowns were sectioned, through microtome, in 3 fragments getting a total of 159 samples. On anyone, through red acid-resistant nail varnish, an enamel area of 5x2 mm was delimited. These samples were divided again into three groups so that, in each of them, there was a representative fragment of each dental element.

The three groups were submitted to a different chemical experimental treatment (Tab. 1). The samples from the group I (control group) were preserved in distilled water at T° 4°C. Each sample, belonging to group II, was immersed for 4 days (with one change of solution after 48 hours) in 50 ml of demineralising solution, containing lactic acid (0.1M/l), 0.02% of carboxymethylcellulose (CMC Az -ko, Italy), at PH 4.8 and T° 37°C, for producing, on its surface, artificial caries. The samples belonging to group III underwent the same treatment as group II, but with addition of a remineralising agent (CPPs 1%).

All the samples were submitted to a quantitative and qualitative analysis with the aim of evaluating and quantifying preventive effectiveness of CPPs.

Quantitative analysis measures the samples' weight changes and the calcium concentration of the solutions after the chemical treatments. Weight changes were

calculated by a digital analytical scale with a sensitivity of 0.01 mg (Gibertini Electronics, Novate (MI)- Italy). All the samples were weighed before (t0) and after (t96) the treatments. The amount of calcium released by the teeth in solution was determined by compleximetric titration with EDTA-Na2 (disodium ethylenediamine tetraacetate) using Eriochrome Black T as an indicator. The calcium concentration in solution was calculated before (t0) and after the treatments (t96). The results obtained were submitted to statistic elaboration through the use of the ANOVA system, program SPSS 10.0.

The micro-morphological alterations presented on the samples following the chemical treatments were valued through observation to Scanning Electron Microscopy (SEM) (Stereoscan 250 MK3s, Cambridge, UK).

SAMPLES	CHEMICAL TREATMENS
GROUP I	distilled water at T° 4°C
GROUP II	acid solution at PH 4.8 (lactic acid 0.1 M/l; CMC 0.02%)
GROUP III	acid solution at PH 4.8 containing CPPs (lactic acid 0.1 M/l; CMC 0.02%; CPPs 1%)

Table 1: summary of the used solutions.

RESULTS

The samples belonging to the control group (group I) were not submitted to any chemical treatment and quantitative analysis gave as a result a mean weight change and a mean quantity of the calcium released in solution I of ± 0 mg.

The mean weight change, related to the samples treated with the demineralising solution (group II), is 1.43 ± 0.49 mg (Fig. 1); the mean quantity of the calcium released in solution II, following the dissolutive action on the enamel, is 0.86 ± 0.28 mg (Fig. 2).

The samples belonging to group III, in which the demineralising action has been effected in presence of CPPs, show a mean weight change of 0.76 ± 0.27 mg (Fig. 3); whereas the mean quantity of the calcium released in solution III is 0.49 ± 0.18 mg (Fig. 4).

The samples belonging to group I (control group) show, to the SEM, a healthy, smooth and regular enamel surface (Fig. 5). In SEM observation of the samples belonging to group II a lesion with an average depth of around $200 \mu\text{m}$ with a superficial micro-morphology “etched-like” for the presence of excavations that are 4 to $20 \mu\text{m}$ deep can be noticed (Fig. 6). On the samples belonging to group III there aren't deep lesions, but it is possible to appreciate a slightly irregular surface with a rough look, due to the acid pH (Fig. 7).

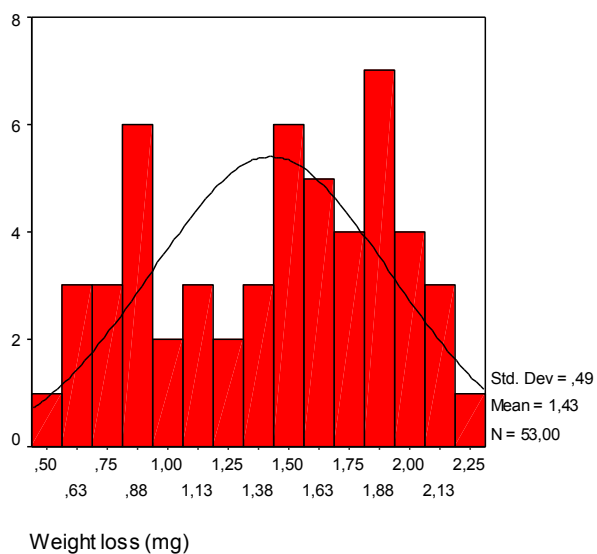


Fig. 1 : distribution of samples weight changes (group II).

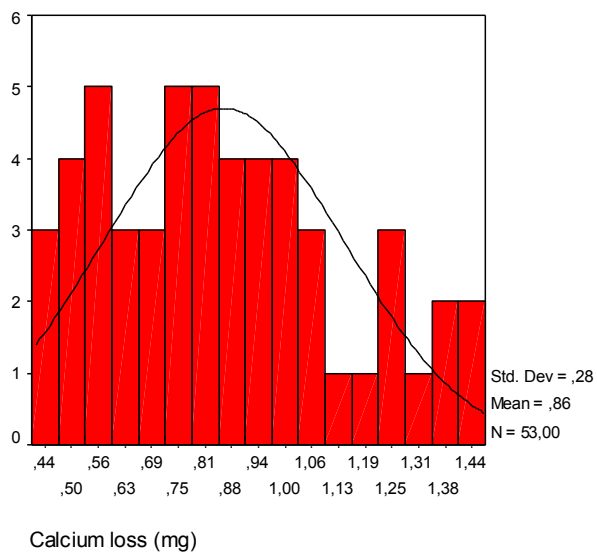


Fig. 2: distribution of calcium released in solution (group II).

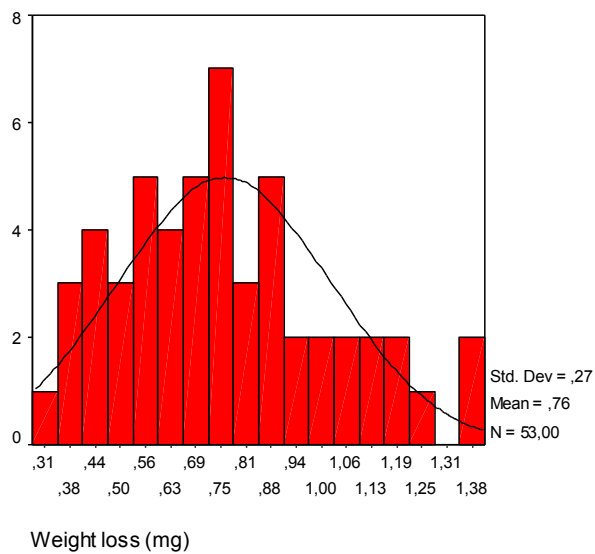


Fig. 3: distribution of samples weight changes (group III).

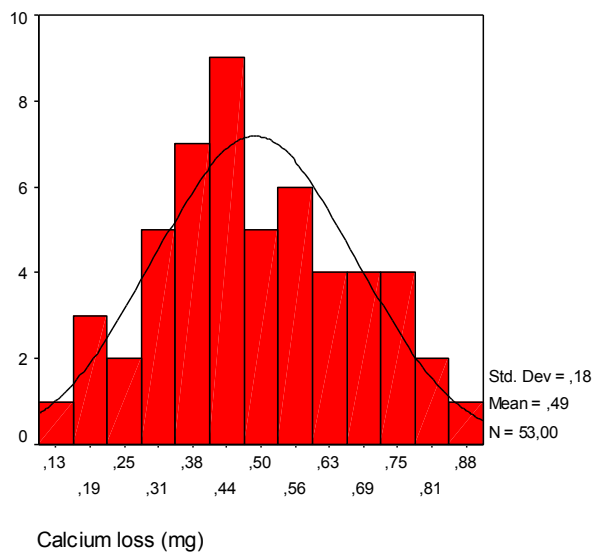


Fig. 4: distribution of calcium released in solution (group III).

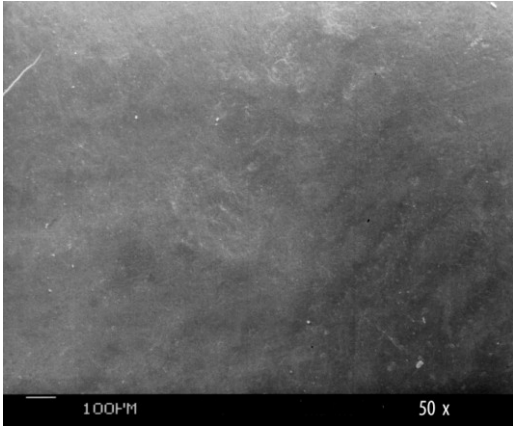


Fig. 5: healthy enamel surface (group I) (50x).

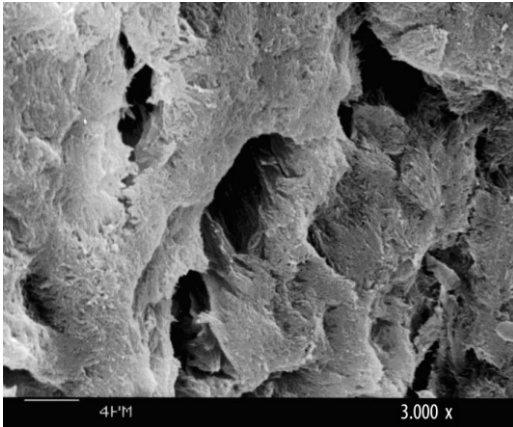


Fig. 6: demineralised enamel surface at pH 4.8 (group II) (3000x).

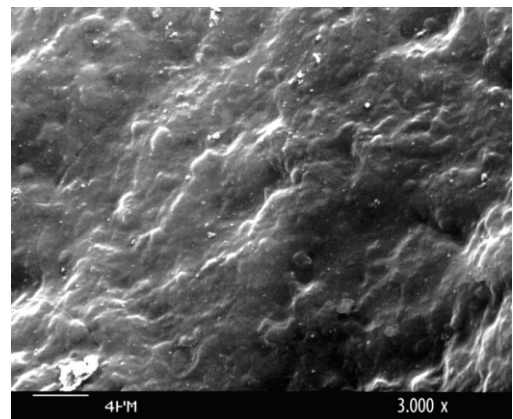


Fig.7: remineralised enamel surface at pH 4.8 and CPPs (group III) (3000x).

DISCUSSION

The present study shows that CPPs, if used in acid solutions, are able to inhibit the process of enamel demineralisation, in vitro.

Analysis of the results of samples mean weight change leads us to affirm that enamel demineralisation in presence of CPPs is appreciably reduced in vitro.

In fact, the samples of group II undergo, on average, a loss double than group III samples. The difference between mean weight change and mean quantity of calcium released in solution for group II and III, respectively, is, also, statistically significant ($P < 0.01$).

This result is amply confirmed by group III SEM images where the samples show the fewest superficial alterations and with no evidence of lesions which can be seen in the samples of group II.

These results are consistent with the proposed mechanism of the anticariogenicity for CPPs, which is the localization of ACP on the tooth surface, buffering plaque pH, depressing enamel demineralisation and enhancing remineralisation [Reynolds, 1998].

Furthermore, CPPs are able to influence many biological activities, such as bone mineralization, gastrointestinal and digestive functions, analgesic properties, hormonal, immunological and neurological responses. [Clare and Swaisgood, 2000].

Tooth decay is still a critical health problem. For this reason, future researches should be focused on in vivo and epidemiological effects of bioactive consumption in reducing or eliminating dental caries.

Dairy products and CPPs could, then, represent a system able to prevent the onset of tooth decay in its first phases and strengthening the physiological mechanisms of protection.

Therefore, CPPs, being natural derivative of milk, could be added to sugar-containing foods and dental professional products without adverse organoleptic effects.

Part 2

In vitro evaluation of the effectiveness of natural CPPs (extracted from yogurt) in dental caries prevention.

ABSTRACT

Aim In vitro testing of the ability of natural CPPs (contained in the yogurt) to prevent demineralisation and promote remineralisation of dental enamel.

Methods Eighty human molars were used. After standardizing an in vitro demineralisation protocol for producing artificial caries (Group 1: pH 4.8; Group 2: pH 3.97), this procedure was used on teeth, but with the addition of natural CPPs (Group 3: pH 4.8; Group 4: pH 3.97). The effects of these procedures were evaluated by quantitative analysis (change in weight and calcium titration) and qualitative analysis (SEM). Statistical analysis of the results was performed using ANOVA.

Results Statistical analysis shows significant differences in weight changes between the groups with and without natural CPPs. The SEM observation shows the protective effects of natural CPPs.

Discussion The results demonstrate that CPPs contained in yogurt have an inhibitory effect on demineralisation and promote the remineralisation of dental enamel.

MATERIALS AND METHODS

First of all, we set up an experimental protocol to standardize a demineralisation procedure of dental enamel [White et al., 1992] at two different pH levels. This step was necessary in order to obtain reproducible dental enamel caries (Groups 1 and 2 as controls). These standardized procedures were later performed in the presence of remineralising agents naturally present in the yogurt supernatant (Groups 3 and 4).

Preparation of enamel specimens

Eighty human teeth, extracted for orthodontic reasons or impaction, were cleaned with sterile gauze imbibed in distilled water and stored at 4°C in sterile containers with 5 per cent formalin solution. The roots were then cut by mean of a diamond disk assembled on a laboratory hand-piece (NSK IS-65, A3311968, Japan) and the crowns polished with pumice dust and non-fluoride toothpaste, using a circular brush with nylon bristles mounted on a dental hand-piece (KAVO INTRAMATIC LUX 3 20 LH, D481631, Germany); they were then rinsed in distilled water. From each crown, three enamel areas (6X4mm² each) were isolated using an acid-resistant varnish (Fig. 8). The 80 specimens were divided into four groups, which subsequently underwent four different chemical treatments (Tab. 2).

Origin of solution	Solution	pH	Remineralizanting agent	Demineralizanting agent
Synthetic	1	4,8		Lactic acid
	2	3,97		Lactic acid
Natural	3	4,8	Yogurt supernatant	Lactic acid
	4	3,97	Yogurt supernatant	Lactic acid

Tab. 2: summary of the used solutions.



Fig. 8: isolated specimen.

Demineralising systems

The 20 specimens from Group 1 were immersed for 96 hours (with one change of solution after 48 hours) in a demineralising solution containing: 50ml of 0.1M lactic acid, 0.02 per cent carboxymethylcellulose (Akzo Nobel, Netherlands) (pH 4.8, T° 37°C) [Iijima et al., 1999].

The 20 specimens from Group 2 underwent the same treatment as Group 1, but at a pH value of 3.97.

Preparation of CPP additives

The specimens from Groups 3 and 4 were treated with a remineralising solution containing a probiotic-enriched yogurt (Bifidobacterium Bb-12, L. acidophilus), chosen for its high CPP content) [Chianese et al., 2002].

A fraction enriched in CPPs was prepared as whey resulting after the centrifugation (CL40R, Thermo Electron, Waltham - United States) of yogurt at 4000g at 25°C for 10min. After three times the supernatant was collected.

The yogurt was separated into two fractions by centrifugation; with this procedure the insoluble fraction precipitates at the bottom of the test tube, whereas the soluble fraction containing the CPPs remains in suspension (supernatant).

All the Group 3 specimens underwent the same treatment as Group 1 (50ml of 0.1M lactic acid, 0.02 per cent carboxymethylcellulose), but with addition of 50ml of yogurt supernatant, for four days at 37°C, with change of solution after two days.

The pH of the solution was adjusted to 4.8.

The 20 specimens from Group 4 underwent the same treatment as Group 3, but at a pH value of 3.97 (the pH of the yogurt).

Assessment methods

After four days, all specimens were rinsed in distilled water and dried with a jet of warm air for 3seconds. The effects of these procedures were evaluated by quantitative and qualitative analysis.

The aim of the quantitative analysis was to assess the specimens' weight changes and the calcium concentration of the solutions after the chemical treatments. Weight

changes were measured by a digital analytical scale with a sensitivity of 0.01 mg (Gibertini Electronics, Novate (MI) – Italy). All the specimens were weighed before (t_0) and after (t_{96}) the treatments to assess hydroxyapatite weight content.

The amount of calcium released into the solution was determined by compleximetric titration with EDTA- Na_2 (disodium ethylenediamine tetraacetate) using Eriochrome Black T as an indicator. The calcium concentration in solution was calculated before (t_0) and after the treatments (t_{96}). The results obtained were submitted to statistic elaboration through the use of the ANOVA system, program SPSS 10.0.

Furthermore, five specimens from each group were dehydrated in a graded series of ethanol, critical point dried (SPC-900/EX, The Bomar Co., Tacoma, Washington), mounted in stubs, sputter-coated with gold (E 306, Edwards,UK) and observed by SEM (Stereoscan 250 MK3, Cambridge UK) (qualitative analysis).

RESULTS

Statistical analysis showed that the mean weight change of the specimens from Group 1 was $-3.28 \pm (\text{SD}) 2.20\text{mg}$. The mean quantity of the calcium released in solution 1 was $0.63 \pm (\text{SD}) 0.37\text{mg}/50\text{ml}$. Figure 9 shows a SEM image of the surface micro-morphology of the artificial lesions created in the Group 1 specimens.

The mean weight change in the specimens from Group 2 was $-16.50 \pm (\text{SD}) 5.88\text{mg}$. The mean quantity of the calcium released in solution 2 was $2.31 \pm (\text{SD}) 0.57\text{mg}/50\text{ml}$. Figure 10 shows a SEM image of the surface micro-morphology of a specimen from Group 2.

The mean weight change in Group 3 specimens was $2.73 \pm (\text{SD}) 1.95\text{mg}$. The mean quantity of the calcium released in solution 3 was $-0.69 \pm (\text{SD}) 3.06\text{mg}/50\text{ml}$. Figure 11 shows a SEM image of the surface micro-morphology of a specimen from Group 3.

The mean weight change of Group 4 was $-1.27 \pm (\text{SD}) 2.59\text{mg}$. The mean quantity of the calcium released in solution 4 was $1.69 \pm (\text{SD}) 2.38\text{mg}/50\text{ml}$. Figure 12 shows a SEM image of the surface micro-morphology of a specimen from Group 4.

Analysis of the results obtained after chemical treatment shows that artificial demineralization in presence of natural “protective” factors (yogurt supernatant) provides significantly lower weight and calcium loss.

The mean difference in weight changes between Groups 1 and 3 is statistically significant ($F = 83.76$; $p < 0.001$) (Fig. 13).

Furthermore, SEM observation of the specimens from Group 1 shows substantial lesions, where the surface micromorphology looks like “etched” (Fig. 9). The SEM images of the specimens from Group 3 do not show any macroscopic erosive lesions (Fig. 11). Comparing these images with the ones from Group 1, it is clear that the CPPs contained in yogurt play a positive role.

When Group 2 is compared against Group 4, it is clear that the difference between the means of the weight changes is statistically significant ($F = 112.25$; $p < 0.001$) (Fig. 14). In Group 2 the conspicuous weight loss is due to the greater acidity of the solution, which causes a considerable loss of substance. By contrast, the Group 4 specimens lost less weight, despite the fact that they were exposed to the same pH as Group 2.

SEM images of the specimens from Group 2 show deep and irregular erosions, and the surface appears disarranged and heterogeneous (Fig. 10).

SEM images of the Group 4 specimens show slightly irregular surfaces characterized by microporosities, due to the acid pH (Fig. 12). These results are consistent with CPPs' ability to inhibit enamel demineralization.

Analysis of the results of released calcium in solution confirms what has been reported to date. A comparison between the concentration of the calcium released in solution 1 and in solution 2 clearly reveals that the difference between the means of the calcium content is statistically significant ($F = 123.25$; $p < 0.001$) (Fig. 15).

A comparison between the concentration of the calcium released in solution 3 and in solution 4 shows that the difference between the means of the calcium content is not statistically significant ($F = 7.57$; $p < 0.001$) (Fig. 16).

These results are consistent with the previous data concerning weight loss. Therefore, it can be stated that the calcium increase in solution is directly proportional to the decrease in pH; the protective effect of natural CPPs is valid at a low pH value; the remineralising treatment can determine the transport of calcium from the solution to the tooth.

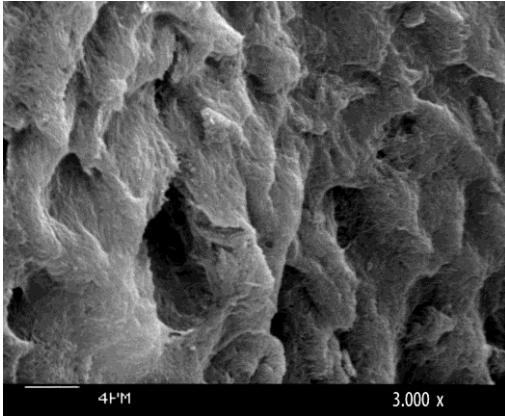


Fig. 9: demineralised enamel surface at pH 4.8 (3000x).

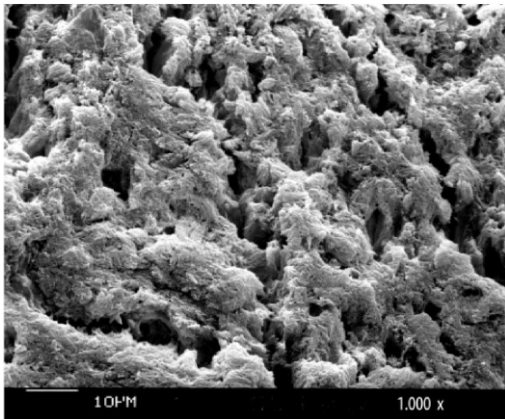


Fig.10: demineralised enamel surface at pH 3.97 (1000x).

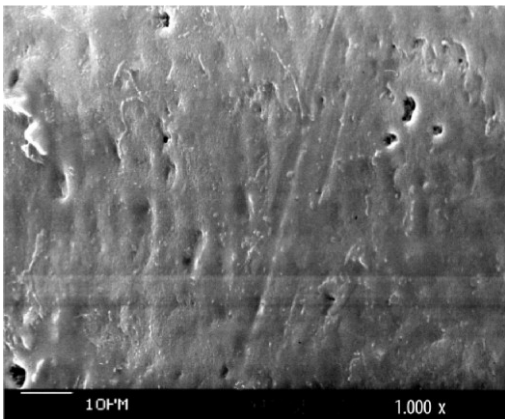


Fig. 11: remineralised enamel surface at pH 4.8 and supernatant of yogurt (1000x).

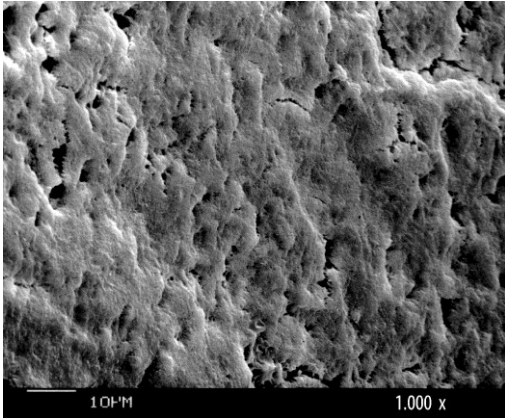


Fig. 12: remineralised enamel surface at pH 3.97 and supernatant of yogurt (1000x).

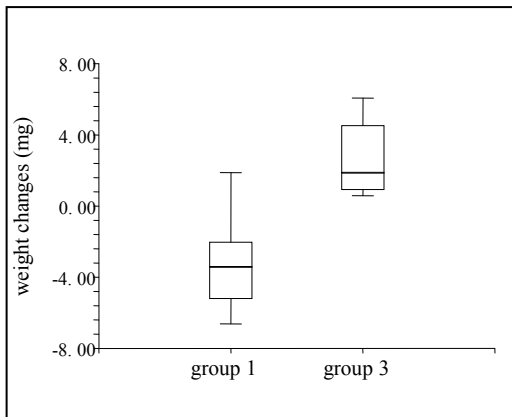


Fig. 13: significance of weight changes for Groups 1 and 3.

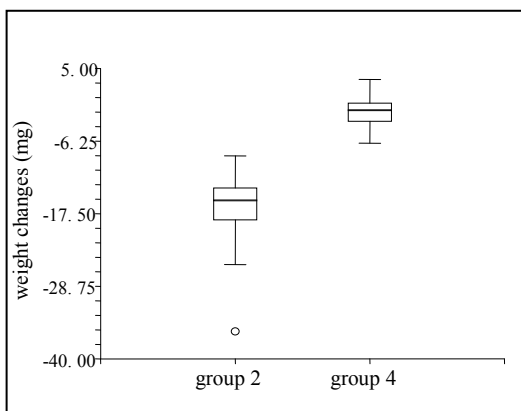


Fig. 14: significance of weight changes for Groups 2 and 4.

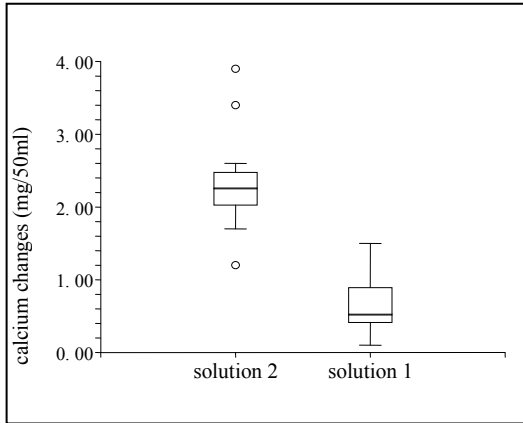


Fig. 15: significance of released calcium concentration for Solutions 1 and 2.

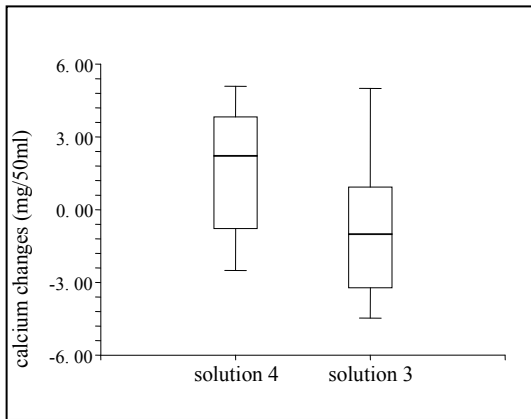


Fig.16: significance of released calcium concentration for Solutions 3 and 4.

DISCUSSION

The obtained data show that yogurt extract is protective against enamel demineralisation in vitro; this result reflected what was found in literature, where the association between dairy products consumption and reduction of dental caries emerged [Merritt et al., 2006].

International scientific literature was analysed to understand the mechanisms by which yogurt may have anti-caries effect.

In particular, an epidemiological study indicated adolescents with low incidence of dental caries drank more milk than those with high caries incidence [Petridou et al., 1996].

Furthermore, Silva et al., using a human intra-oral caries model, showed that a water extract of cheddar cheese significantly reduced enamel demineralisation [Silva et al., 1987].

As observed from literature review, several mechanisms by which dairy products may reduce enamel demineralisation have been proposed.

Levine supposed that milk proteins are adsorbed onto the enamel surface and may impede enamel demineralisation; milk fat is adsorbed onto the enamel surface and may have a protective role; milk enzymes may have a role in reducing the growth of acidogenic plaque bacteria [Levine, 2001].

Herod, also, reported that milk and cheese could reduce the effects of metabolic acids, and could help restore the enamel that is lost during eating. Postulated mechanisms involve buffering, salivary stimulation, reduction of bacterial adhesion, reduction of enamel demineralisation, and/or promotion of remineralisation by casein and ionizable Ca and P [Herod, 1991].

Several constituents in dairy products could exert a direct effect on the tooth surface: calcium, phosphorus and CPPs.

In particular, milk and milk products release calcium and phosphorus and, increasing their concentrations in the dental plaque, inhibit demineralisation and favour remineralisation by a common-ion effect [Silva et al., 1987].

Furthermore, CPPs, containing the sequence Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu, stabilize nanoclusters of amorphous calcium phosphate (ACP) in metastable solution. These multiple phosphoseryl residues of the CPPs bind to forming nanoclusters of ACP in supersaturated solutions, preventing growth to the critical size required for phase transformations. CPPs-ACP localize ACP in dental plaque, which buffers the free calcium and phosphate ion activities, helping to maintain a state of supersaturation with respect to tooth enamel, depressing demineralisation and enhancing remineralisation [Cross et al., 2005].

Having milk and yogurt similar components, it can be supposed that the mechanisms by which yogurt may have protective effect against dental caries are the same as milk.

In fact, regarding the composition of yogurt, it is based on the nutrient composition of the milk from which it is derived. Other variables that play a part during processing of milk are temperature, duration of heat exposure, exposure to light, and storage conditions. The final composition of yogurt is, also, influenced by the species and strains of bacteria used in the fermentation, the source and type of milk solids that may be added before fermentation, and the temperature and duration of the fermentation process.

The protein content of yogurt is generally higher than that of milk because of the addition of non-fat dry milk during processing and concentration, which increases the protein content of the final product. In addition, yogurt is an excellent source of calcium and phosphorus. In fact, fermentation has little effect on the mineral content of milk and therefore the total mineral content remains unaltered in the yogurt.

Furthermore, because of the lower pH of yogurt compared with that of milk, calcium is present in yogurt mostly in ionic form [Adolfsson et al. 2004].

Additionally, CPPs content in yogurt is higher than that in milk due to proteolytic activity of microorganisms contained in the yogurt [Rasić and Kurmann, 1983].

Furthermore, consumption of probiotic products, for example yogurt containing live microorganisms, improves the oral health status.

In fact, recent experimental studies and results from randomized controlled trials have shown that yogurt, containing *L. reuteri* and *Bifidobacterium* DN-173 010, may reduce the levels of selected caries-associated microorganisms in saliva [Caglar et al., 2005; Nikawa et al., 2004].

To conclude, in light of the results obtained by this study it can be stated that CPPs contained in the yogurt may have a remineralising action in vitro when associated with a demineralising agent. This preventive effectiveness is not due to the fact that dental enamel is strengthened, but to the inhibition of demineralisation.

Therefore, though CPPs do not represent a treatment method, they nevertheless provide valid prevention against early demineralisation of enamel when protective physiological mechanisms are insufficient.

It should be emphasized that our experimental procedure has demonstrated its validity as far as the original aim of the study is concerned, and it is sufficiently sensitive in quantifying the changes of weight and calcium concentration after demineralisation and remineralisation of dental enamel.

Future researches should, then, be focused on in vivo and epidemiological effects of yogurt consumption in reducing or eliminating dental caries.

Part 3

An in vivo model for the remineralisation of enamel lesions by natural and synthetic casein phosphopeptides: SEM analysis.

ABSTRACT

Aim To test in vivo the effectiveness of casein phosphopeptides (CPPs) naturally present in dairy products (yogurt) and synthetic CPPs present in a commercial product to inhibit demineralisation and promote remineralisation of early enamel caries.

Materials and methods Forty volunteers (age range 10-18 years) were recruited and divided in 2 groups (Group A and B). Forty extracted sound molars were divided in 4 pieces each and some of them were artificially demineralised. To twenty subjects (Group A) were placed, through adhesive method, 2 enamel specimens on buccal surfaces of first molars (1 sound and 1 demineralised). The subjects were instructed to eat natural CPPs (yogurt) daily for a month. To twenty subjects (Group B) were placed similarly two demineralised enamel specimens on the buccal surface of the first molars. The subjects were required to apply 3 times daily for a month a commercial product containing 10% of CPPs (GC Tooth Mousse) only on right specimen and a placebo mousse on the left one. The remaining 2 enamel sections of

4 obtained from each sectioned tooth were used as control outside the oral environment. The effects of these procedures were evaluated by SEM.

Results SEM analysis revealed a diffused and homogeneous mineral coating that reduced the surface alterations in the decayed artificially specimens treated with natural and synthetic CPPs for 30 days into the mouth.

Discussion Results demonstrate that CPPs could be a valid preventive system against demineralisation of early enamel lesions.

MATERIALS AND METHODS

Subjects and study design

The study was carried out in the Department of Paediatric Dentistry of the University of Naples “Federico II”, Italy.

For this study 200 subjects were recruited but only 40 healthy patients (18 males and 22 females) with an age range from 10 to 18 years were selected with the following criteria. Inclusion criteria were: good general health (ASA I-II) and agreement to comply with study procedures. An intra-oral examination confirmed that each member had no periodontal disease or other oral pathology.

Exclusion criteria were: milk protein allergies and conditions that interfered with the examination procedure (not cooperating subjects).

Participation was voluntary. After parents and patients had been given verbal and written explanations of the experimental protocol and the study aims, written informed consent was signed by them prior to the start of the study. Permission was received from the appropriate authorities.

The subjects were divided into two groups of 20 (Groups A and B) but 3 subjects from Group A and 2 subjects from Group B withdrew during the treatment period. In addition, 1 specimens placed to a subject from Group A was broken during the experimental procedures and the matched specimens were withdrawn from analyses. Consequently, at the end of the experiment, 16 volunteers from Group A and 18 from Group B completed the study.

Preparation of enamel specimens

40 human teeth, extracted for orthodontic reasons or impaction were obtained from Department of Paediatric Dentistry of the University of Naples “Federico II”, Italy.

Teeth were cleaned with sterile gauze imbibed in distilled water and stored at 4°C in sterile containers with 5 per cent formalin solution. The roots were cut by mean of a diamond disk assembled on a laboratory hand-piece and the crowns polished with pumice dust and non-fluoride toothpaste, using a circular brush with nylon bristles mounted on a dental hand-piece and, then, rinsed in distilled water. From each crown 4 longitudinal sections of enamel of about 3x2x2 mm were obtained by cutting with a diamond disk.

Experiment design -Group A

Eighty specimens were used for this group. Two of the 4 specimens obtained from each tooth were submitted to a demineralisation procedure by immersion for 96 hours (with one change of solution after 48 hours) in a demineralising solution containing: 50ml of 0.1M lactic acid, 0.02 per cent carboxymethylcellulose (Akzo Nobel, Netherlands) (pH 4.8, T° 37°C) [Iijima et al., 1999]. The other 2 specimens from each tooth were left intact and stored in distilled water (with one change of solution after 48 hours) .

After 4 days, all specimens were rinsed in distilled water, dried with a jet of warm air for 3', packaged in individual paper autoclavable bags and autoclaved at 121 degrees C at 15 lbs psi for 30 min [Kumar et al., 2005].

Two enamel sections (1 sound and 1 demineralised, respectively) of 4 obtained from each tooth were stored in distilled water (with one change of solution every 48 hours) and used as control outside of the oral environment (Subgroup α_1 and α_2) (Tab. 3).

To twenty subjects belonging to the Group A the remaining 2 enamel specimens (1 sound and 1 demineralised, respectively)(Subgroup α_3 and α_4) (Tab. 3) were placed, with the dentinal face towards the tooth, through adhesive cementing systems, on buccal surfaces of first or second upper molars for the remineralisation protocol with natural protective factors; therefore all the subjects were instructed to eat 125 gr of yogurt (corresponding to a pot of yogurt in order to have a standard value that might be easy to find in every supermarket) daily for a month and were asked to complete a diary documenting their eating yogurt habits.

No alterations were made to the subjects diet and oral hygiene procedures during the study.

20 sound specimens	Subgroup α_1	Outside of oral environment
20 demineralised specimens	Subgroup α_2	Outside of oral environment
20 sound specimens	Subgroup α_3	Intra-oral environment and yogurt
20 demineralised specimens	Subgroup α_4	Intra-oral environment and yogurt

Tab. 3: Group A : remineralisation protocol with yogurt.

Experiment design-Group B

Eighty specimens were used for this group. Three specimens of the four obtained from each tooth were submitted to a demineralisation procedure by immersion for 96 hours (with one change of solution after 48 hours) in a demineralising solution containing: 50ml of 0.1M lactic acid, 0.02 per cent carboxymethylcellulose (Akzo Nobel, Netherlands) (pH 4.8, T° 37°C) [Iijima et al., 1999]. The other 1 specimen from each tooth was left intact and stored in distilled water (with one change of solution after 48 hours).

After four days, all specimens were rinsed in distilled water and dried with a jet of warm air for 3', packaged in individual paper autoclavable bags and autoclaved at 121 degrees C at 15 lbs psi for 30 min [Kumar et al., 2005].

Two enamel sections (one sound and one demineralised, respectively) of 4 obtained from each tooth were stored in distilled water (with one change of solution every 48 hours) and used as control outside the oral environment (Subgroup β_1 and β_2) (Tab. 4).

To 20 subjects belonging to the Group B the remaining two enamel specimens (both demineralised) were placed, with the dentinal face towards the tooth, through adhesive cementing systems, on buccal surfaces of the right and the left first or second upper molars for the remineralisation protocol with synthetic casein phosphopeptide. Therefore, each subject was required to apply a commercial product for professional use containing 10% of casein phosphopeptide (Tooth Mousse GC Corp. Tokyo, Japan) only on right slab (Subgroup β_3) (Tab.4) and a glycerine based (without remineralising substances) placebo gel on the left slab (Subgroup β_4) (Tab.4), on a daily basis, 3 times daily for a month at the following times 7.00-14.00-

21.00. In addition, subjects were asked to complete a diary documenting their Tooth Mousse application habits.

The GC Tooth Mousse were provided by GC industry as coded products and stored in a secured area at room temperature.

No alterations were made to the subjects diet and oral hygiene procedures during the study.

20 sound specimens	Subgroup β_1	Outside of oral environment
20 demineralized specimens	Subgroup β_2	Outside of oral environment
20 demineralized specimens	Subgroup β_3	Intra-oral environment and GC Tooth Mousse
20 demineralized specimens	Subgroup β_4	Intra-oral environment and placebo

Tab. 4 : Group B : remineralisation protocol with GC Tooth Mousse.

SEM

After completion of each treatment period, the enamel specimens in the mouth were removed by orthodontic pliers and were polished with pumice dust and non-fluoride toothpaste, using a circular brush with nylon bristles mounted on a dental hand-piece. Then, they were rinsed in distilled water and compared with the control slabs to assess the morphological changes by SEM.

For the ultra-structural examination of the tooth surfaces, the specimens were dehydrated in a graded series of ethanol, critical point dried (SPC-900/EX, The Bomar Co., Tacoma, Washington), mounted in stubs, sputter-coated with gold

(E 306, Edwards, UK) and observed by SEM (Stereoscan 250 MK3, Cambridge UK) at 50x, 100x, 600x, 3000x, 6000x magnifications.

RESULTS

In succession are reported some images that are representative for each group.

Specimens from Group A

Subgroup α_1

SEM evaluation of the enamel specimens from subgroup α_1 revealed that they were like a typical sound enamel surface (Fig. 17).

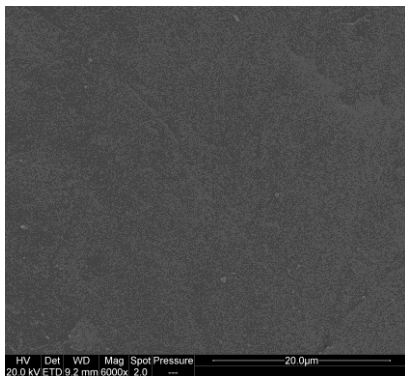


Fig. 17: Sound enamel surface subgroup α_1 (6000x).

Subgroup α_2

The sections from subgroup α_2 presented roughening surfaces, in which tissue loss gave the enamel a porous appearance. The entire prism core was dissolved. In all the

samples, the characteristic honeycomb structure of demineralised enamel was clearly evident with relatively deep, but tapered etched pits varied in size from 4-20 μm in diameter (Fig. 18, 19).

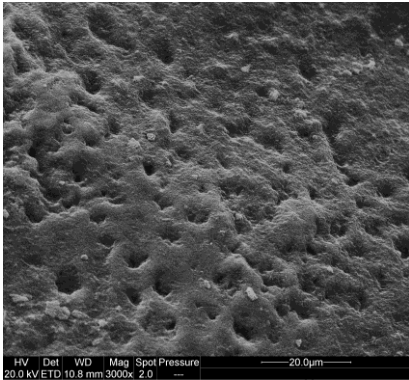


Fig.18: Demineralised enamel surface subgroup α_2 (3000x).

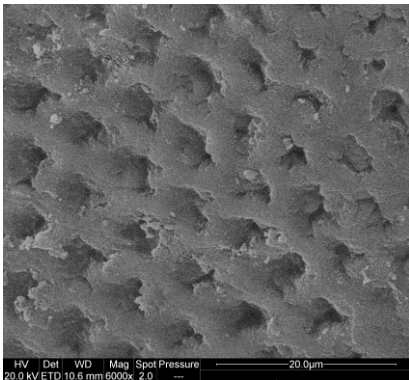


Fig.19: Demineralised enamel surface subgroup α_2 (6000x).

Subgroup α_3

The images of the enamel specimens from subgroup α_3 did not show demineralised areas nor a smooth and regular surface as the enamel sections of the control group. In

fact, some specimens slightly changed their morphological features, showing the presence of uneven mineral sediment (Fig. 20,21).

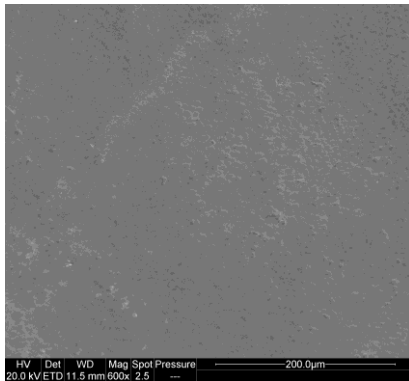


Fig. 20: Sound enamel surface and yogurt subgroup α_3 (600x).

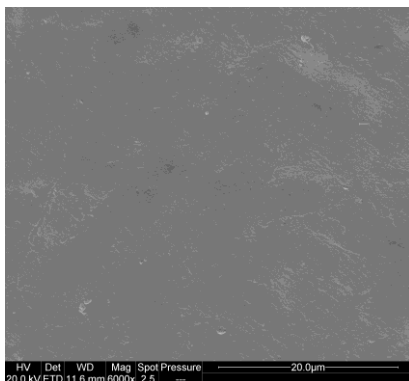


Fig. 21: Sound enamel surface and yogurt subgroup α_3 (6000x).

Subgroup α_4

The enamel specimens from subgroup α_4 did not show any erosion or demineralisation of enamel surface. A mineral sediment with a trend of wavy lines was clearly visible at low magnification (600x) (Fig. 22).

At higher magnification (6000x) it can be clearly seen that the surfaces were covered with an amorphous deposit, which obscured completely the underlying prism structure. In all the specimens this mineral sediment entirely filled the previously created lesions (Fig. 23).

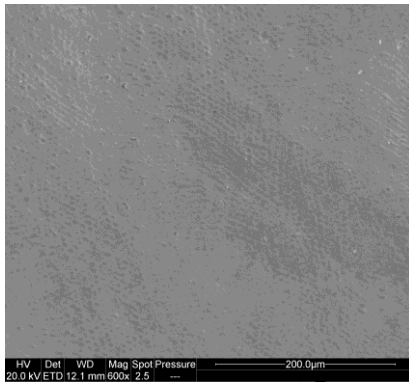


Fig. 22: Remineralised enamel surface with yogurt subgroup α_4 (600x).

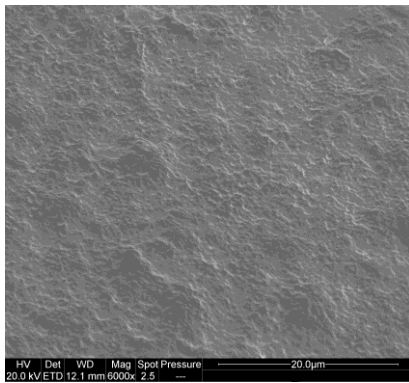


Fig. 23: Remineralised enamel surface with yogurt subgroup α_4 (6000x).

Specimens from Group B

Subgroup β_1

SEM images of the enamel specimens from subgroup β_1 were suggestive of sound and smooth surface (Fig. 24).

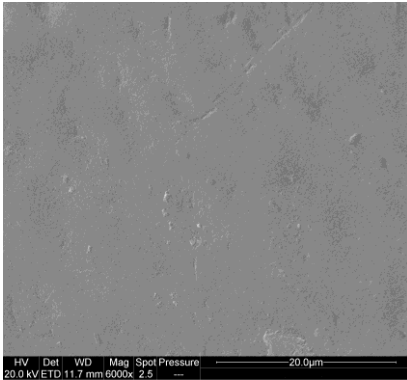


Fig. 24: Sound enamel surface subgroup β_1 (6000x).

Subgroup β_2

Characteristic etching patterns were found on the surfaces of the specimens from subgroup β_2 . Acid-etching of sound enamel created microporosities from 4-20 μm in diameter (Fig. 25, 26).

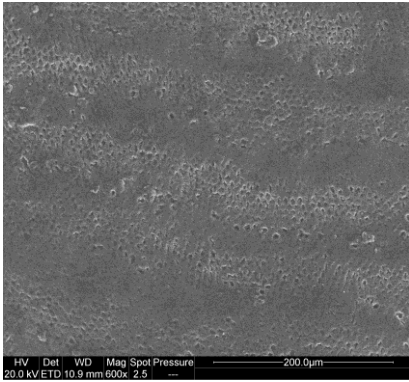


Fig. 25: Demineralised enamel surface subgroup β_2 (600x).

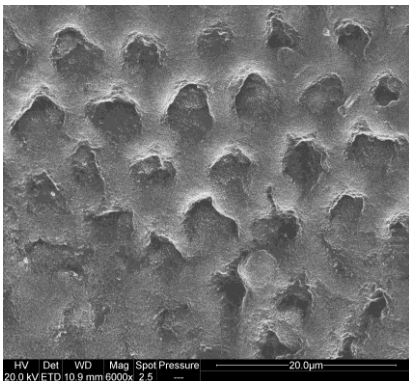


Fig. 26: Demineralised enamel surface subgroup β_2 (6000x).

Subgroup β_3

The enamel specimens from subgroup β_3 presented, to the SEM observation, a coating on the surface that covered and completely filled the previously created erosions (Fig. 27, 28).

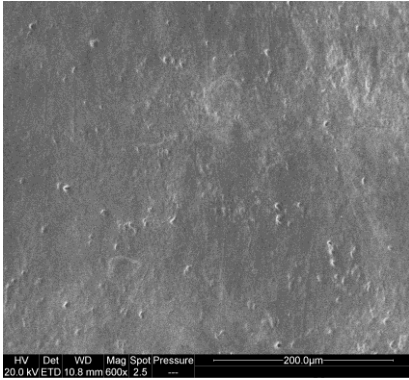


Fig. 27: Remineralised enamel surface with GC Tooth Mousse subgroup β_3 (600x).

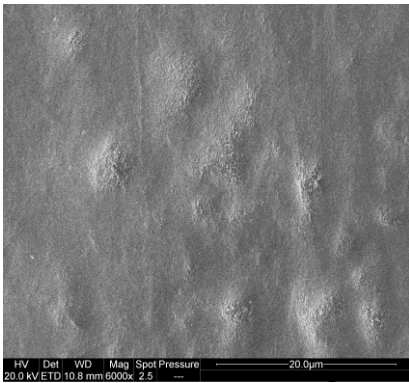


Fig. 28: Remineralised enamel surface with GC Tooth Mousse subgroup β_3 (6000x).

Subgroup β_4

The enamel specimens from subgroup β_4 showed strong superficial alterations, loss of enamel and pronounced holes from 5-25 μm in diameter, higher than the typical lesions created by the acid solution used for the demineralization protocol. In particular, the surfaces appeared disorganized, without any distinct etching pattern (Fig. 29, 30).

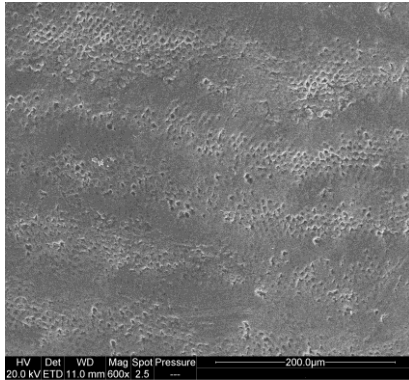


Fig. 29: Demineralised enamel surface with placebo subgroup β_4 (600x).

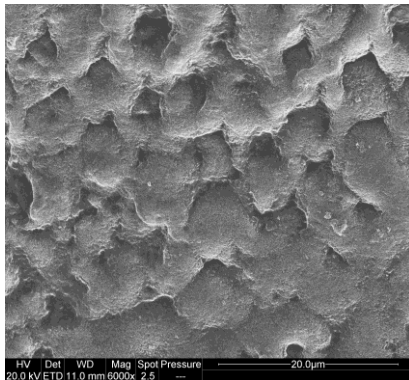


Fig. 30: Demineralised enamel surface with placebo subgroup β_4 (6000x).

DISCUSSION

SEM analysis provided useful information about structural changes occurring on the enamel surfaces during the test period.

In particular, the effectiveness of CPPs was clearly visible from the images of the specimens demineralised for four days at pH 4.8 and then subjected to the treatment with natural and synthetic CPPs, in which the previously created microporosities were covered and filled by an amorphous mineral sediment. In fact, the inorganic

components contained in high concentrations in CPP-ACP acted to enhance remineralisation of the enamel structure.

The increased enamel remineralisation found *in vivo* in this study by natural and synthetic CPPs was consistent with previous studies showing the anti-cariogenic and remineralisation potential of CPP-ACP in solution and the proposed mechanism of the localization of amorphous calcium phosphate on the tooth surface by the CPPs, depressing enamel demineralisation and enhancing remineralisation [Rahiotis and Vougiouklakis, 2007; Reynolds, 1997; Reynolds et al., 1995].

It can be stated that both yogurt and GC Tooth Mousse were a valid product that exhibited anti-caries effect, even if due to SEM limits, the study didn't show significant differences between natural and synthetic CPPs effects as SEM analysis provided only structural-morphologic images, but didn't permit to have information about quantitative changes.

Furthermore, the intent of this *in vivo* model was also to mimic what occurred in the natural caries process, and to give clinically relevant informations about remineralisation mechanism in a short period of time, without causing irreversible tissue changes in the natural dentition.

It should be emphasized that this *in vivo* system could be considered, such as suggested by Zero, as a bridge between the natural uncontrolled clinical situation and the highly controlled laboratory situation [Zero, 1995]. The main advantage of this model was the use of a 'natural' environment, consisting of tooth substrate; formation or presence of dental plaque with cariogenic potential; carbohydrate challenge, provided by the subject's normal diet, oral microflora, host factors such as saliva.

Enamel specimens fixed in the mouth had also the advantage that subject compliance with wearing the appliance is not a factor. Model systems that use the subjects' normally worn partial dentures ensure a level of certainty that the subjects will be compliant with the study requirements. Furthermore, the enamel specimens, mounted on the buccal sites, were well tolerated by subjects.

The remineralisation potential of CPP-ACP highlighted the importance of using milk-derived peptides against dental caries in the normal diet (delivered by yogurt) and in the oral daily care (delivered by commercial tooth paste).

Future studies should, then, be focused on the evaluation of the potential synergic effects of natural and synthetic CPPs, in order to create a new preventive strategy based both on promoting the consumption of protective factors naturally present in food and on utilizing these bio-active principles in personal hygiene products to reduce cariogenicity.

Part 4

In vivo short-term effect of commercial plain fruit yogurt on the number of salivary mutans streptococci and lactobacilli.

ABSTRACT

Aim To investigate whether short-term consumption of commercial plain fruit yogurt would affect the levels of salivary mutans streptococci and lactobacilli in young patients.

Materials and methods 28 volunteers (age range from 10 to 18 years) were recruited and divided in 2 groups (test group and control group). The 14 subjects from test group were instructed to eat a pot of commercial plain fruit yogurt (125 gr) (Vitasnella, Danone- Italy), purchased from a local supermarket, twice a day for 2 weeks. To the 14 subjects from control group no special dietary restrictions were imposed. Salivary mutans streptococci and lactobacilli were enumerated with chair-side kits at baseline(t0), after 1 week(t1), and after 2 weeks(t2).

Results A decline of mutans streptococci counts was observed in the test group respect to the control group. No effects on the levels of salivary lactobacilli were noted.

Discussion Short term daily consumption of commercial yogurt may have a beneficial effect on oral health, reducing the salivary count of mutans streptococci, which have been suggested to be the most prominent factor in dental caries.

MATERIALS AND METHODS

The study was carried out in the Department of Paediatric Dentistry of the University of Naples “Federico II”, Italy.

For this study 28 subjects were recruited with an age range from 10 to 18 years with the following criteria. Inclusion criteria were: good general health (ASA I-II) and agreement to comply with study procedures. An intra-oral examination confirmed that each member had no periodontal disease or other oral pathology. Exclusion criteria were: milk protein allergies, use of antibiotics or probiotics within the 2-week washout period prior to the study.

Participation was voluntary. After parents and patients had been given verbal and written explanations of the experimental protocol and the study aims, written informed consent was signed by them prior to the start of the study. Permission was received from the appropriate authorities. The study protocol was in accordance with the Helsinki Declaration of Human Rights.

At the baseline (t₀), sampling of stimulated whole saliva was carried out, in order to calculate the density of the CFU (CFU/ml) of mutans streptococci and lactobacilli for each subject prior to the start of treatment.

After thorough rinsing with water, the subjects were asked to chew on a piece of paraffin wax for 5 minutes and the saliva was collected directly into a graded test

tube. The secretion rate was calculated as millilitre/minute. The counts of salivary mutans streptococci and lactobacilli were estimated with a chair-side test (CRT, Ivoclar Vivadent AG, Schaan, Liechtenstein) according to the manufacturer's instructions. Saliva was inoculated on a dip-slide with selective agar media for mutans streptococci and lactobacilli. After adding a NaHCO₃ tablet to the tube, the dip-slides were immediately cultivated at 37°C for 48 hours. The colonies were identified by morphology with the aid of a stereomicroscope with ×10 magnification and the density of the CFU (CFU/ml) was visually compared with the aid of a chart provided by the manufacturer (Fig. 31).

Then, the subjects were split randomly into test and control groups.

The 14 subjects from test group were instructed to eat a pot of commercial plain fruit yogurt (125 gr) (Vitasnella, Danone- Italy), purchased from a local supermarket, twice a day for 2 weeks and were asked to complete a diary documenting their eating yogurt habits. The commercial starter cultures, according to information provided by the manufacturer, were *S. thermophilus* (minimum declared concentrations of 1×10^8 and 1×10^6 cfu/g on dates of manufacture and expiry, respectively) and *L. bulgaricus* (minimum declared concentrations of 3×10^6 and 1×10^6 cfu/g on dates of manufacture and expiry, respectively) with overall minimum declared concentrations of 2×10^8 and 1×10^7 cfu/g on dates of manufacture and expiry, respectively. The subjects were advised to eat the yogurt at lunchtime and at dinnertime and no tooth brushing was allowed for at least 1 h after eating yogurt.

To the 14 subjects from control group no special dietary restrictions were imposed. Saliva samples were re-collected again after 1 week (t1) and at the end of the experiment after 2 weeks (t2), in order to calculate the density of the CFU (CFU/ml)

of mutans streptococci and lactobacilli for each subject during (t1) and immediately after (t2) the treatment.

The counts of salivary mutans streptococci and lactobacilli at t1 and t2 were estimated as described previously.



Fig. 31: chart provided by the manufacturer.

At the end of the treatments the data were processed with the Statistical Package for Social Sciences (version 10.0, SPSS Inc., Chicago, Illinois, USA). A regression binary logistic analysis was made. Statistical significance level was established at $p < 0.05$.

RESULTS

The stimulated saliva secretion rate exceeded 1.4 ml/minute for all subjects.

Test group

At baseline (t0), regarding mutans streptococci , 78.6 per cent of the subjects had levels $\geq 10^5$ CFU, while 21.4 per cent of the subjects had levels $< 10^5$ CFU (Fig. 32).

Regarding lactobacilli, 21.4 per cent of the subjects had levels $\geq 10^5$ CFU, while 78.6 per cent of the subjects had levels $< 10^5$ CFU (Fig. 33).

After 1 week (t1), regarding mutans streptococci, 35.7 per cent of the subjects had levels $\geq 10^5$ CFU, while 64.3 per cent of the subjects had levels $< 10^5$ CFU (Fig. 32).

Regarding lactobacilli, no one of the subjects had levels $\geq 10^5$ CFU, while 100 per cent of the subjects had levels $< 10^5$ CFU (Fig. 33).

After 2 weeks (t2), regarding mutans streptococci, 42.9 per cent of the subjects had levels $\geq 10^5$ CFU, while 57.1 per cent of the subjects had levels $< 10^5$ CFU (Fig. 32).

Regarding lactobacilli, 7.1 of the subjects had levels $\geq 10^5$ CFU, while 92.9 per cent of the subjects had levels $< 10^5$ CFU (Fig. 33).

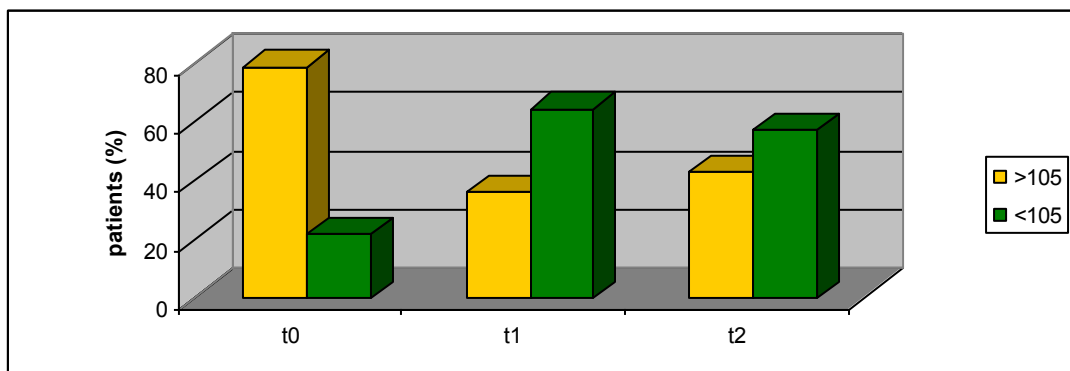


Fig. 32: variation in streptococci mutans density of the CFU (CFU/ml) at t0, t1, t2 (test group).

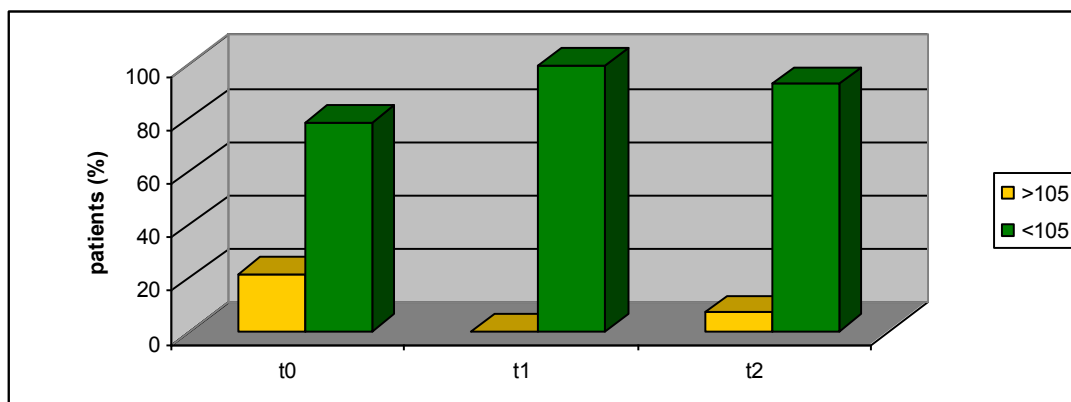


Fig. 33: variation in lactobacilli density of the CFU (CFU/ml) at t0, t1, t2 (test group).

Control group

At baseline (t0), regarding mutans streptococci, 85.7 per cent of the subjects had levels $\geq 10^5$ CFU, while 14.3 per cent of the subjects had levels $< 10^5$ CFU (Fig. 34). Regarding lactobacilli, 21.4 per cent of the subjects had levels $\geq 10^5$ CFU, while 78.6 per cent of the subjects had levels $< 10^5$ CFU (Fig. 35).

After 1 week (t1), regarding mutans streptococci, 78.6 per cent of the subjects had levels $\geq 10^5$ CFU, while 21.4 per cent of the subjects had levels $< 10^5$ CFU (Fig. 34). Regarding lactobacilli, 14.3 of the subjects had levels $\geq 10^5$ CFU, while 85.7 per cent of the subjects had levels $< 10^5$ CFU (Fig. 35).

After 2 weeks (t2), regarding mutans streptococci, 78.6 per cent of the subjects had levels $\geq 10^5$ CFU, while 21.4 per cent of the subjects had levels $< 10^5$ CFU (Fig. 34). Regarding lactobacilli, 21.4 of the subjects had levels $\geq 10^5$ CFU, while 78.6 per cent of the subjects had levels $< 10^5$ CFU (Fig. 35).

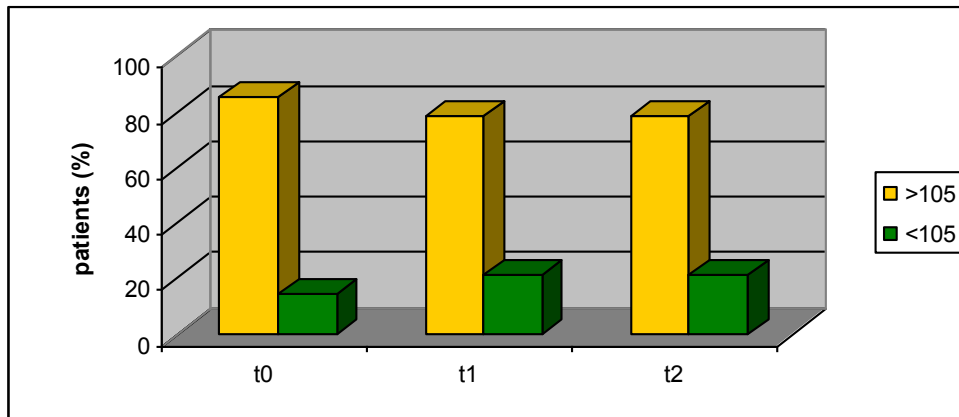


Fig. 34: variation in streptococci mutans density of the CFU (CFU/ml) at t0, t1, t2 (control group).

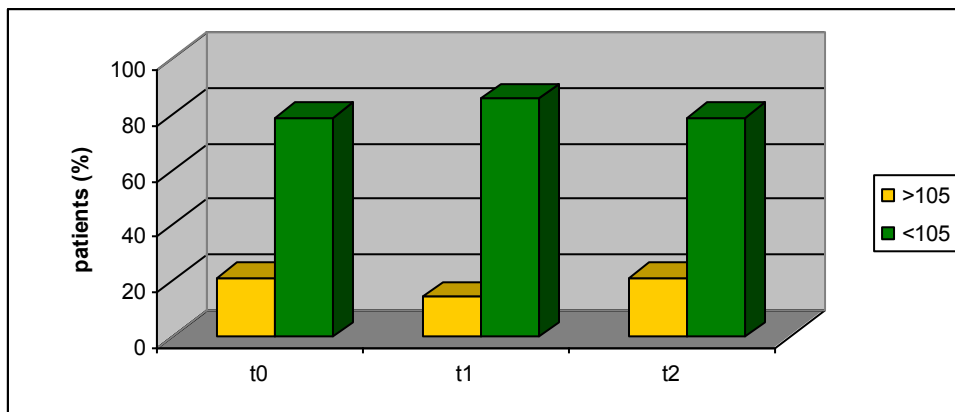


Fig. 35: variation in lactobacilli density of the CFU (CFU/ml) at t0, t1, t2 (control group).

Statistical elaboration showed that subject from control group (not consuming yogurt for two weeks) have a risk 3.511 (odds ratio) greater than subjects from test group (daily consuming yogurt for two weeks) of having a density of the CFU (CFU/ml) of salivary mutans streptococci $\geq 10^5$ (sig.0.012).

Instead, regarding salivary lactobacilli, the differences in the density of the CFU (CFU/ml) between control and test groups were not statistically significant (sig. 0.220).

Here below are reported some images of the chair-side test (CRT, Ivoclar Vivadent AG, Schaan, Liechtenstein) used for test (Fig. 36, 37) and control (Fig. 38, 39) groups, respectively.

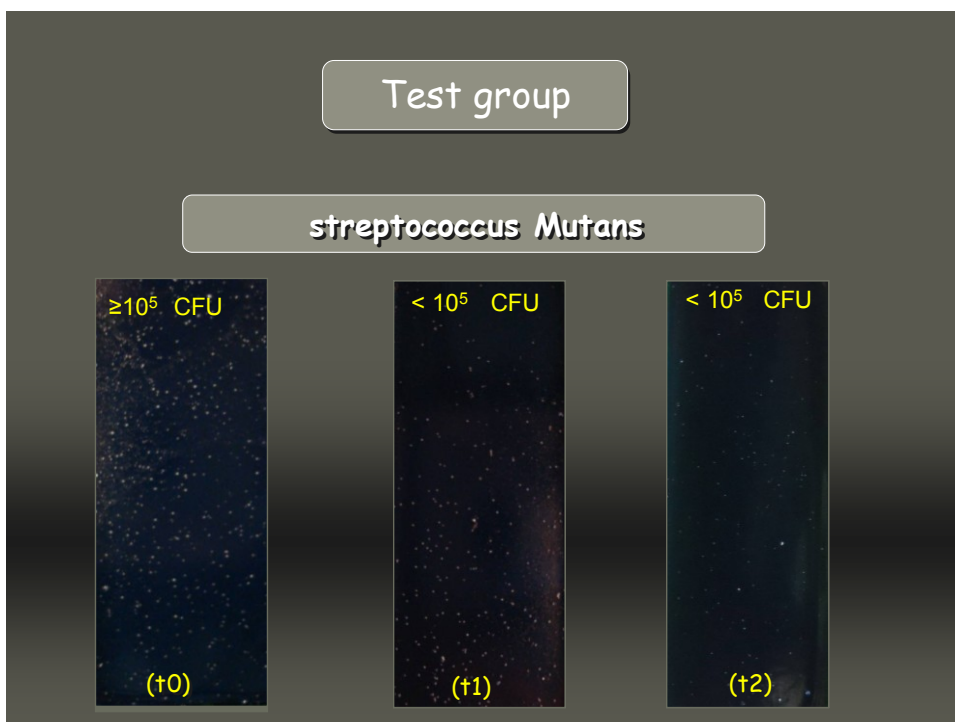


Fig. 36: density of the streptococci mutans CFU (CFU/ml) at t0, t1,t2 (test group).

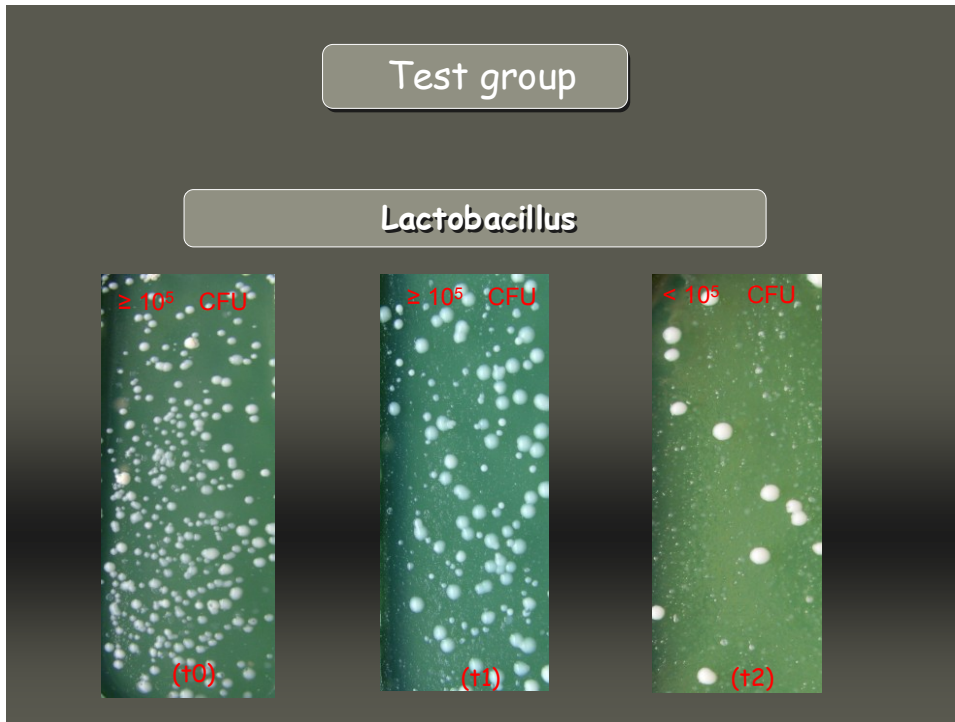


Fig. 37: density of the lactobacilli CFU (CFU/ml) at t0, t1,t2 (test group).

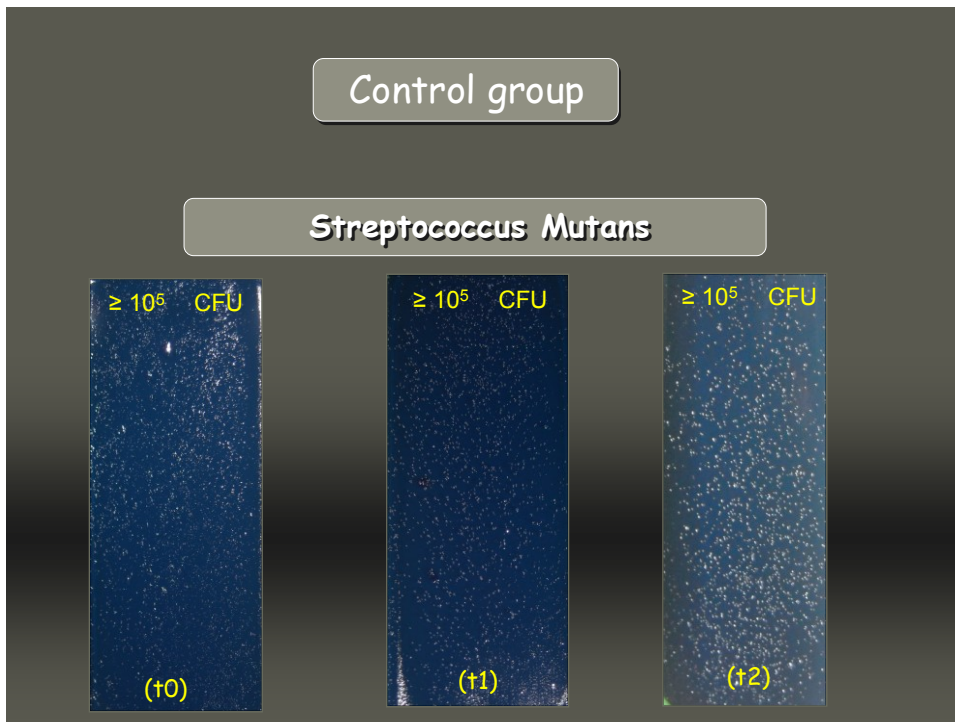


Fig. 38: density of the streptococci mutans CFU (CFU/ml) at t0, t1,t2 (control group).

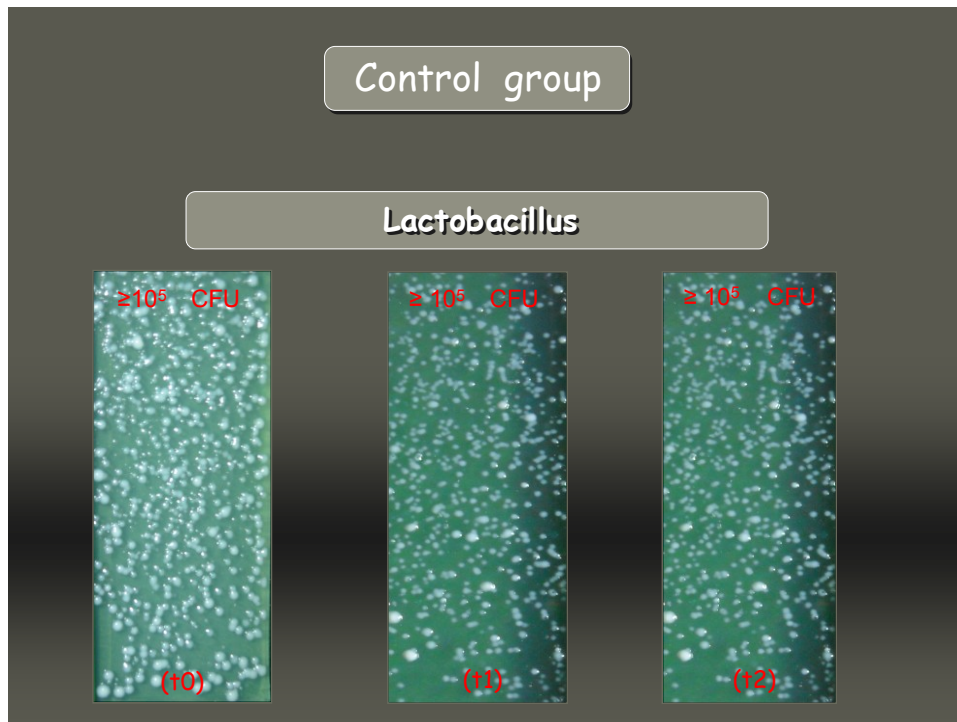


Fig. 39: density of the lactobacilli CFU (CFU/ml) at t0, t1,t2 (control group).

DISCUSSION

Since caries is an infectious disease occurred to tooth [Hamada and Slade, 1980; Loesche, 1986], application of antibiotics for prevention of this illness has been investigated [Caufield and Dasanayake, 2001].

Antibiotics that may be given orally or systemically for the prevention of caries may enter the oral cavity via saliva and gingival crevicular fluid and lead to an imbalance in the oral microbiota.

However, prolonged treatments with antibiotics suppress the resident bacterial population resulting in induction and overgrowth of antibiotic-resistant bacteria and other opportunistic pathogens such as *Candida albicans*. Thus, prevention of caries

based on antibiotic administration, even though effective in theory, is unfeasible for microbiological and economic reasons [Stewart, 2002].

Microbial resistance to antibiotics raises the need to develop novel compounds and approaches for microbial control of dental diseases.

For this reason, there is an increase in the number of investigations on new and natural substances in order to evaluate their activity and possible application for the prevention and treatment of dental caries[Aimutis, 2004].

In this field natural products derived from milk (such as yogurt) could be very useful since there is a relationship between their consumption and the decrease of dental caries [Levine, 2001].

The results from the present study showed that consumption of commercial plain fruit yogurt for 2 weeks decreased the mutans streptococci counts in saliva in young patients.

Our findings are in accordance with Petti et al., who have also shown in a randomized clinical trial that yogurt consumption does have some activity against the salivary microflora [Petti et al., 2001].

In particular, such has reported by Petti et al., the reduction of the mutans streptococci counts in the test group respect to the control group should be explained with the capability of casein and casein derivatives to inhibit the bacterial adherence and/or with the bactericidal activity of bacteriocins (e.g. lacticin 3147) present in dairy products with selective activity on streptococcus mutans [Petti et al., 2008].

These mechanisms could be both efficient and it is also believable that casein and casein derivates and microorganisms contained in yogurt have synergistic anti-mutans activity.

Instead, no effects on the levels of salivary lactobacilli were noted following the consumption of commercial plain fruit yogurt for 2 weeks .

As reported by Cildir et al., a probable reason is that mutans streptococci usually grow on exposed surfaces, easy accessible for the yogurt, while lactobacilli are recovered in shed retentive areas with limited contact with the ingested yogurt [Cildir et al., 2009]. To conclude, in the light of these results it is possible to affirm that short term daily consumption of commercial yogurt may have a beneficial effect on oral health, reducing the salivary count of mutans streptococci, which have been suggested to be the most prominent factor in dental caries.

Further long-term studies involving more subjects are needed to clarify if this approach is an alternative strategy for reducing the severity of this illness only by changing food behaviours.

Part 5

In vitro effectiveness of TMR (transverse micro-radiography) in assessing dental enamel remineralisation by artificial saliva.

ABSTRACT

Aim to evaluate the in vitro effectiveness of TMR (transverse micro-radiography) in assessing dental enamel remineralisation by artificial saliva.

Materials and Methods 16 bovine dental enamel sections were coated with an acid-resistant nail-varnish save for an exposed window on the buccal surface. All sections were immersed in a demineralising solution for 18h. Then 2 slices from each section were cut for analysing with TMR to assess the characteristics of the baseline lesions. For the remineralisation procedure, the sections were divided into 2 groups (Group 1: water; Group 2: artificial saliva). All sections were immersed in their respective solutions for 30mins 3 times a day for 6 weeks. At the end of this procedure the sections were prepared for the TMR analysis and then analysed. Data were statistically elaborated. **Results** The difference in the mean mineral loss for the artificial saliva group between demineralisation and remineralisation procedures is statistically significant ($F=12.614$; $p<0.001$). **Discussion** TMR was used successfully to analyse the demineralisation and remineralisation processes. Artificial saliva

saturated with respect to calcium and phosphate salts may possibly reduce the degree of demineralisation.

MATERIALS AND METHODS

This study was carried out in the Department of Clinical Dental Sciences, School of Dentistry, The University of Liverpool, Edwards Building, Liverpool, UK, under the supervision of Prof. S.M. Higham.

Preparation of enamel specimens

16 bovine teeth were extracted from freshly slaughtered cattle and placed immediately in 0.1 % thymol solution (GPR reagent BDH Poole, Dorset, UK).

All teeth were debrided of soft tissue remnants and gently abraded with pumice (Kemdent, fluor of pumice, grade: extra-fine oral) using a rotary brush. Wet and dry sandpaper (English Abrasives P320A, English Abrasives & Chemicals Ltd, London, UK- medium and fine coarseness) and Sof-lex discs (3M Corporation, Oxfordshire, UK- fine and extra fine) were then used to polish the buccal surface of the teeth to remove the outermost ridged enamel and any remnants of pellicle to leave a smooth plateau of buccal enamel (Fig. 40). The prepared teeth were dried and inspected using QLF (quantitative light induced fluorescence) to further assess the teeth for irregularities, staining and enamel malformations.

Then, the teeth were sectioned using a rotary diamond disc (Fine superflex diamond disc, Hawley Russell & Baker Ltd, Hertfordshire, UK) to remove the root below the cemento-enamel junction (Fig. 41).

All samples were mounted on a glass rod with greenstick compound (Kerr Inc, Orange, California,USA) (Fig. 42).

From each crown, an enamel area was isolated with transparent acid resistant nail varnish (Max Factor Nailfinity, Weybridge, UK) to leave a 5mm x 5mm exposed window on the buccal surface. The windows were positioned in the area that represented the most homogenous enamel as indicated by preliminary QLF imaging.



Fig. 40: bovine incisor.



Fig. 41: bovine incisor crown.

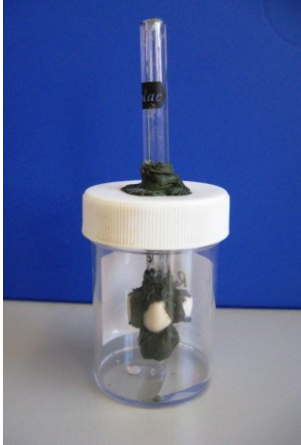


Fig. 42: experimental pot containing enamel sections.

Demineralising systems

All the samples were immersed in 40ml of a demineralising solution (2.2mM KH_2PO_4 , 50mM acetic acid, 2.2mM CaCl_2 , 0.05ppmF at pH 4.5) for 18 hours. A small magnetic stirrer (VWR International s.r.l., Milano, Italy) was added to ensure gentle agitation on a Stuart Stirrer (Bibby Sterilin Ltd, Stone, and Staffordshire, UK) set at 150rpm.

Slices Preparation for TMR baseline lesion analysis

At the end of the demineralisation procedure, all teeth were withdrawn and rinsed thoroughly for 3 mins with distilled water and dried.

2 slices from each tooth were obtained using a water-cooled diamond saw (Well, Walter Ebner, Le Locle, Germany) (fig. 43) with a 0.17 diameter wire for analysing with TMR to assess the characteristics of the baseline lesions.

The cut sections were approximately 80 μm thick and were mounted on a brass anvil with nail varnish (Fig. 44), and polished on a diamond disc to give a planoparallel specimen of 80 μm thickness (Fig. 45).

Sections were removed from the anvils by soaking in acetone and stored in labelled 2ml containers in distilled water prior to begin radiographed.



Fig. 43: water cooled diamond saw.



Fig. 44: brass anvil.

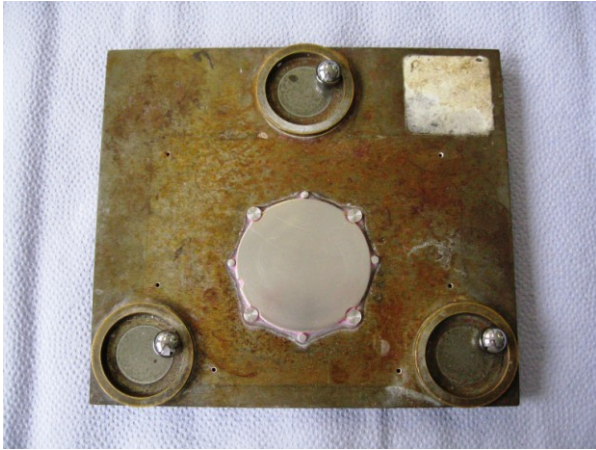


Fig. 45: diamond disc.

TMR imaging and analysis

The sections were mounted on a labelled microradiographic plate-holder which housed an aluminium stepwedge. This was comprised of ten layers of 25 μm thick steps. The microradiographs were taken at 20mins exposure on Kodak high-resolution plates (type 1A) using a Cu(K α) X-ray source (Philips B.V., Eindhoven, The Netherlands) operating at 25 Kv and 10mA with a focus-specimen distance of 30cm. The plates were developed with standard techniques.

The microradiographs were subjected to examination and image analysis in a Leica Leitz DMRB optical microscope (Leiza, Wetzlar , Germany).The image was captured at a magnification of 20 x 0.40 via a CCD video camera (Viglen PC, London, UK).

The lesions variables, mineral loss (Vol % μm), lesion width (μm) and lesion depth (μm), were assessed by TMRW v.1.22 (Inspektor Research System BV, Amsterdam, The Netherlands) software.

Remineralising systems

For the remineralisation procedure, the specimens were divided into 2 groups (8 samples each), which subsequently underwent 2 different chemical treatments:

Group 1: water (as control);

Group 2: artificial saliva.

All samples were mounted using greenstick compound and glass rods and placed in a 50 ml containers.

Group 1

The samples from group 1 were immersed in a control solution, containing deionised water.

Group 2

The samples from group 2 were treated with an artificial saliva solution containing: (methyl-p-hydroxybenzoate(2.00g/l), sodium carboxymethylcellulose (10.0 g/l), KCl(8.38mM), MgCl₂.6H₂O(0.29mM), CaCl₂.2H₂O(1.13mM), K₂HPO₄·3 H₂O (4.62 mM), KH₂PO₄(2.40 mM),0.05 ppmF, pH 7.2).

Each experimental test solution measured 40mls.

The remineralisation experimental procedure ran for 6 weeks. All groups were immersed in their respective solutions for 30mins 3 times a day. Gentle agitation was provided by the Stuart Stirrer described previously. Following immersion in the test solution, the samples were rinsed for 3mins with copious amounts of distilled water before being placed between dippings in a 40mls of artificial saliva under gentle agitation. All the test solutions were renewed twice a week. The artificial saliva storage solution was also changed twice weekly.

At the end of this procedure all the samples were prepared for the TMR analysis and then analysed as described previously.

Statistical Package for Social Sciences software (SPSS 10.0, Chicago, Illinois, USA) was used for analysing data. Descriptive statistics including mean, standard deviation, minimum and maximum values were calculated. The TMR data results were analysed with a one way ANOVA model. Values of $p < 0.05$ were accepted as statistically significant.

RESULTS

Group 1 (distilled water)

Statistical analysis showed that the mean mineral loss after demineralisation (baseline lesion) was $2550.49 \pm$ (SD) 388.81 (maximum value 3350.00; minimum value 1680.00) (Fig. 46). The mean lesion depth after demineralisation (baseline lesion) was $79.34 \pm$ (SD) 7.48 (maximum value 94.70; minimum value 66.80) (Fig. 47). The mean lesion width after demineralisation (baseline lesion) was $67.09 \pm$ (SD) 5.95 (maximum value 80.40; minimum value 57.50) (Fig. 48). After remineralising procedure the mean mineral loss was $2282.21 \pm$ (SD) 570.43 (maximum value 4430.00; minimum value 1430.00) (Fig. 49). The mean lesion depth was $81.30 \pm$ (SD) 9.55 (maximum value 101.1; minimum value 53.8) (Fig. 50). The mean lesion width was $62.91 \pm$ (SD) 28.65 (maximum value 90.4; minimum value -139.7) (Fig. 51).

Group 2 (artificial saliva)

Statistical analysis showed that the mean mineral loss after demineralisation (baseline lesion) was $2447.56 \pm (\text{SD}) 477.30$ (maximum value 3370.00; minimum value 1380.00) (Fig. 52). The mean lesion depth after demineralisation (baseline lesion) was $82.70 \pm (\text{SD}) 9.88$ (maximum value 101.60; minimum value 66.00) (Fig. 53). The mean lesion width after demineralisation (baseline lesion) was $62.74 \pm (\text{SD}) 32.58$ (maximum value 79.70; minimum value -146.1) (Fig. 54). After remineralising procedure the mean mineral loss was $2110.32 \pm (\text{SD}) 568.62$ (maximum value 3790.00; minimum value 950.00) (Fig. 55). The mean lesion depth was $79.91 \pm (\text{SD}) 14.43$ (maximum value 114.4; minimum value 35.0) (Fig. 56). The mean lesion width was $65.77 \pm (\text{SD}) 15.16$ (maximum value 100.7; minimum value -12.0) (Fig. 57).

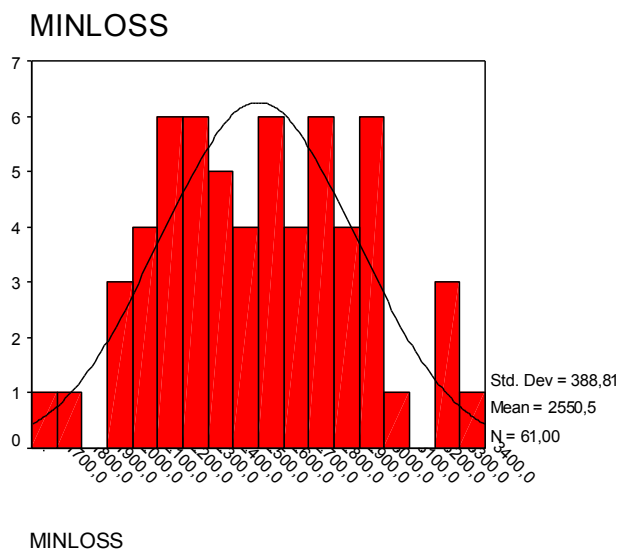


Fig. 46: graphic distribution of the mean mineral loss after demineralisation (baseline lesion (group 1)).

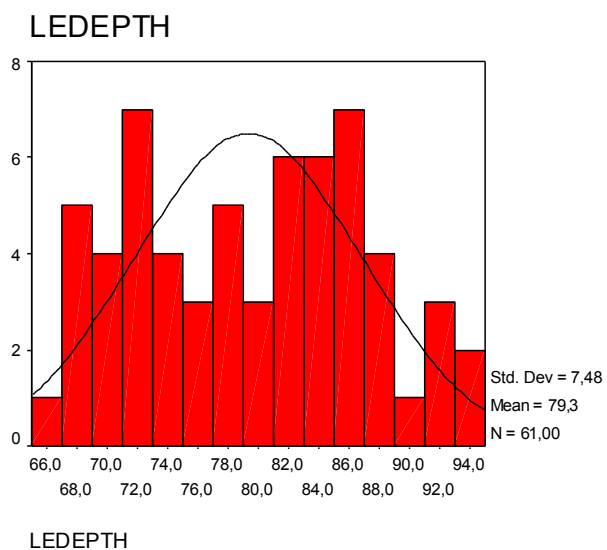


Fig. 47: graphic distribution of the mean lesion depth after demineralisation (baseline lesion (group 1)).

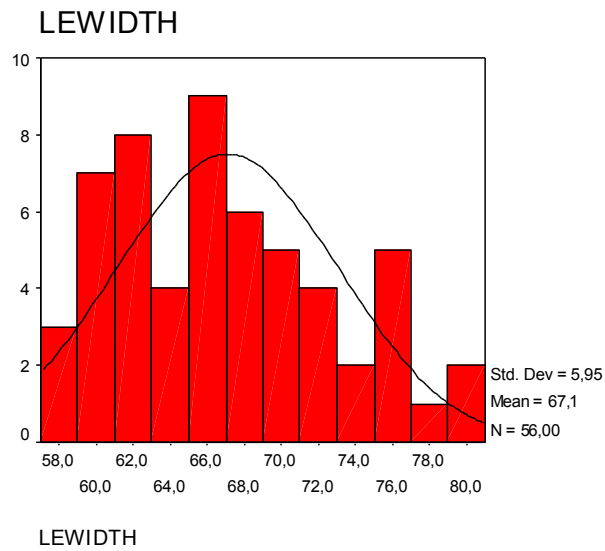


Fig. 48: graphic distribution of the mean lesion width after demineralisation (baseline lesion (group 1)).

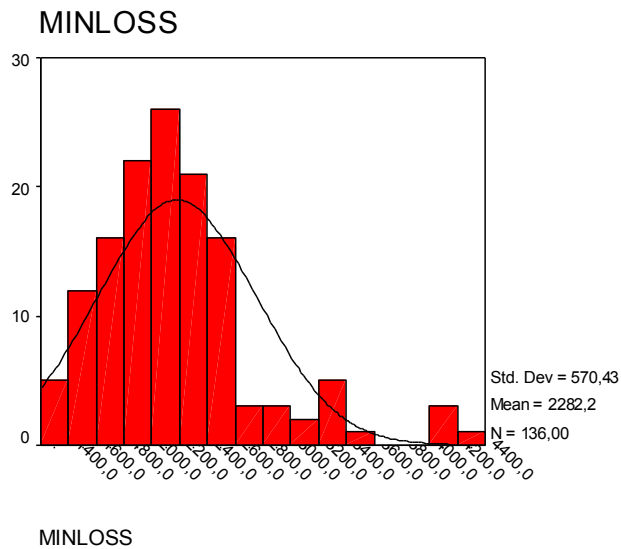


Fig. 49: graphic distribution of the mean mineral loss after remineralisation procedure (group 1).

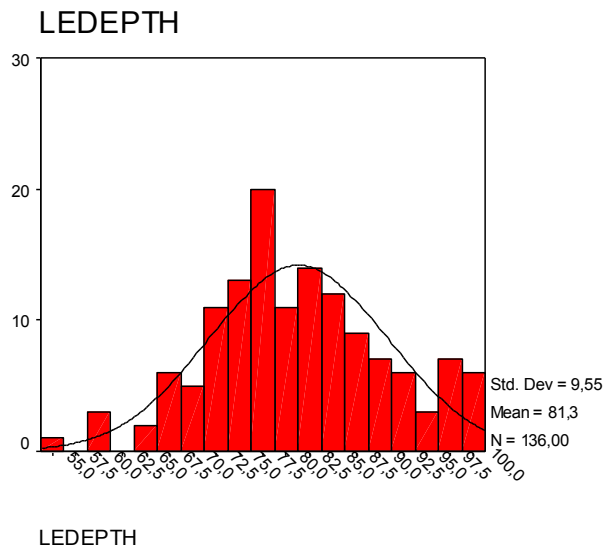


Fig. 50: graphic distribution of the mean lesion depth after remineralisation procedure (group 1).

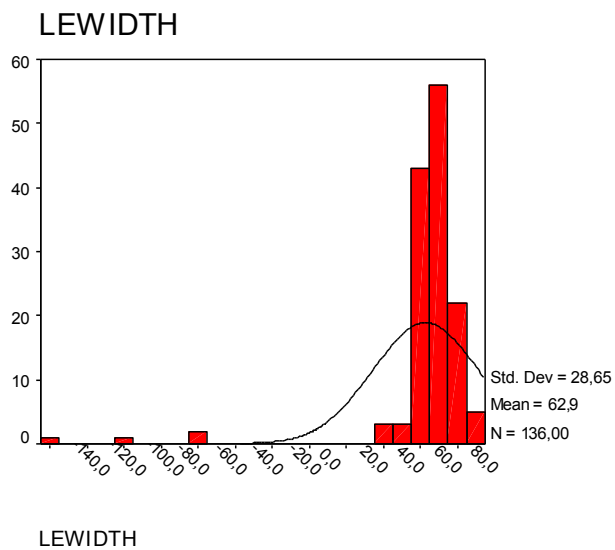


Fig. 51: graphic distribution of the mean lesion width after remineralisation procedure (group 1).

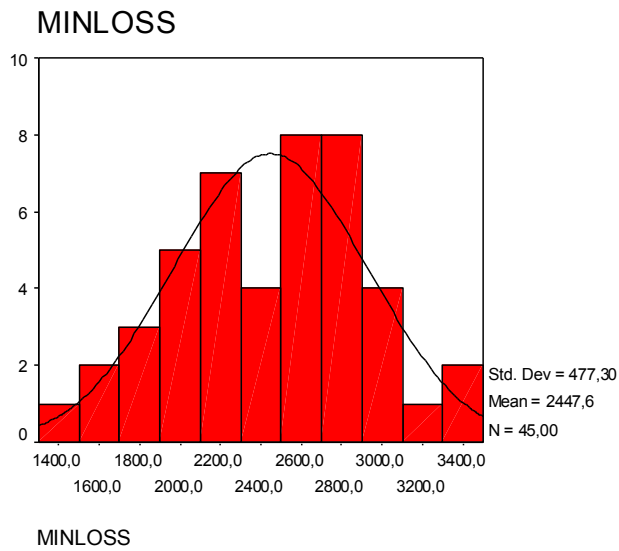


Fig. 52: graphic distribution of the mean mineral loss after demineralisation (baseline lesion (group 2)).

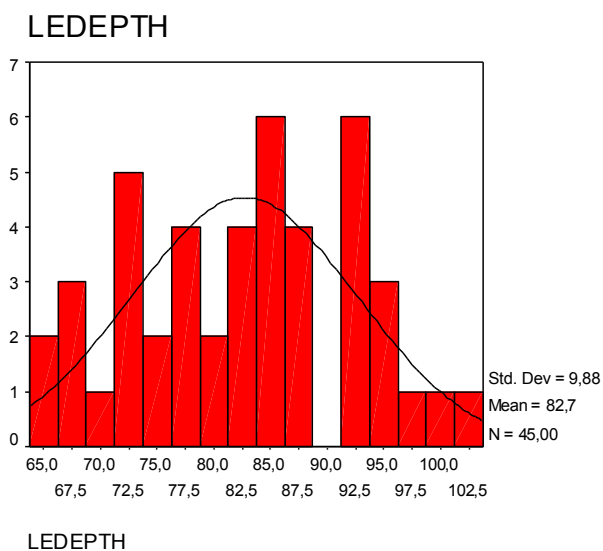


Fig.53: graphic distribution of the mean lesion depth after demineralisation (baseline lesion (group 2)).

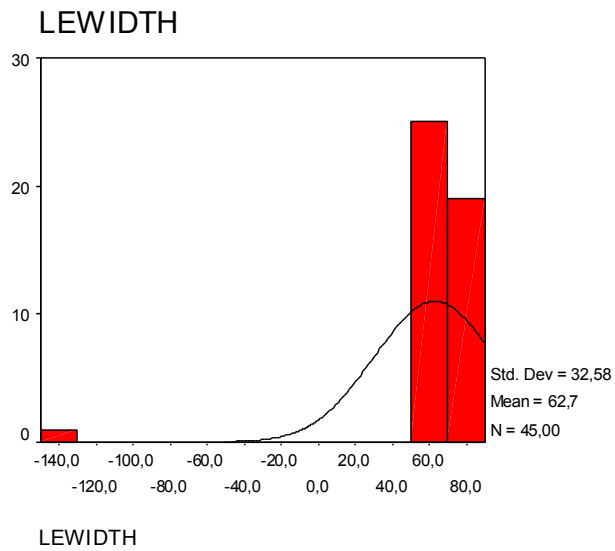


Fig. 54: graphic distribution of the mean lesion width after demineralisation (baseline lesion (group 2)).

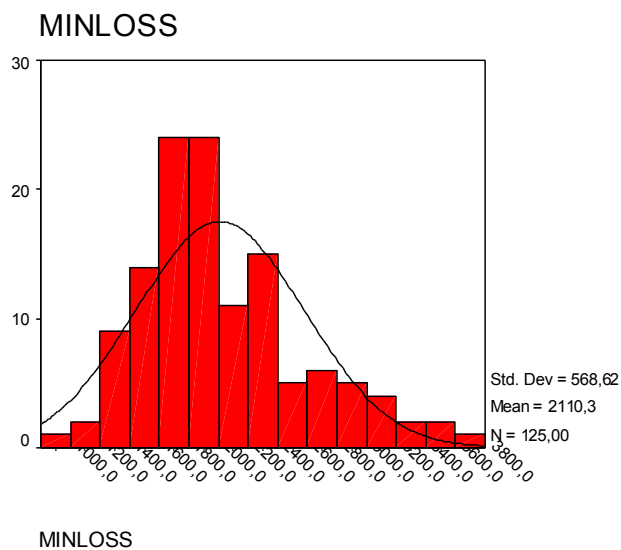


Fig. 55: graphic distribution of the mean mineral loss after remineralisation procedure (group 2).

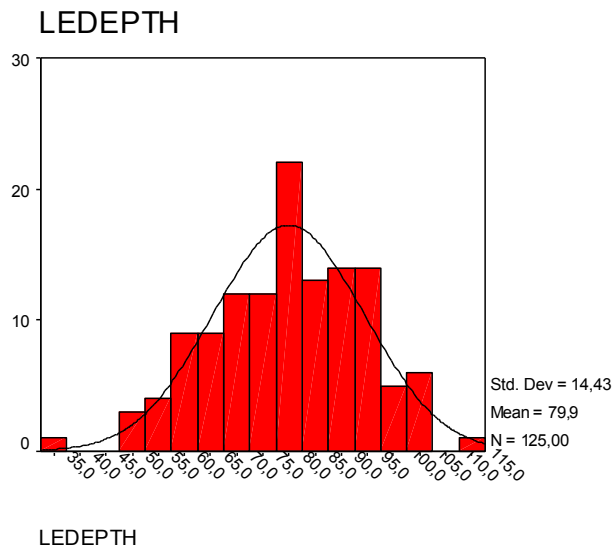


Fig. 56: graphic distribution of the mean lesion depth after remineralisation procedure (group 2).

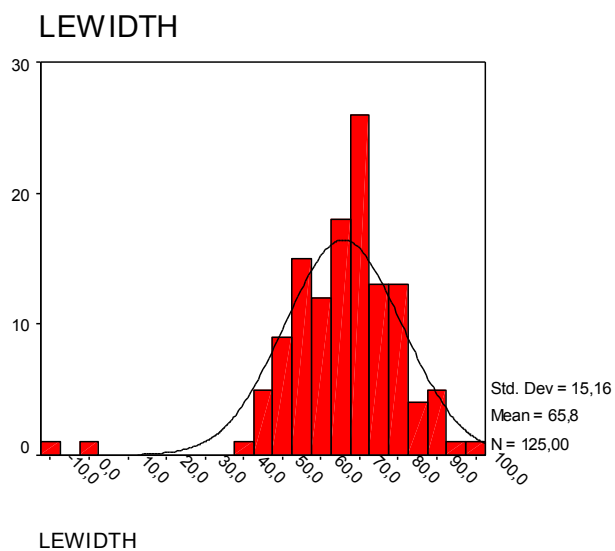


Fig. 57: graphic distribution of the mean lesion width after remineralisation procedure (group 2).

Here below are reported some TMR images recorded after demineralization and remineralization procedures, respectively for group 1 and 2 (Fig. 58, 59, 60, 61).

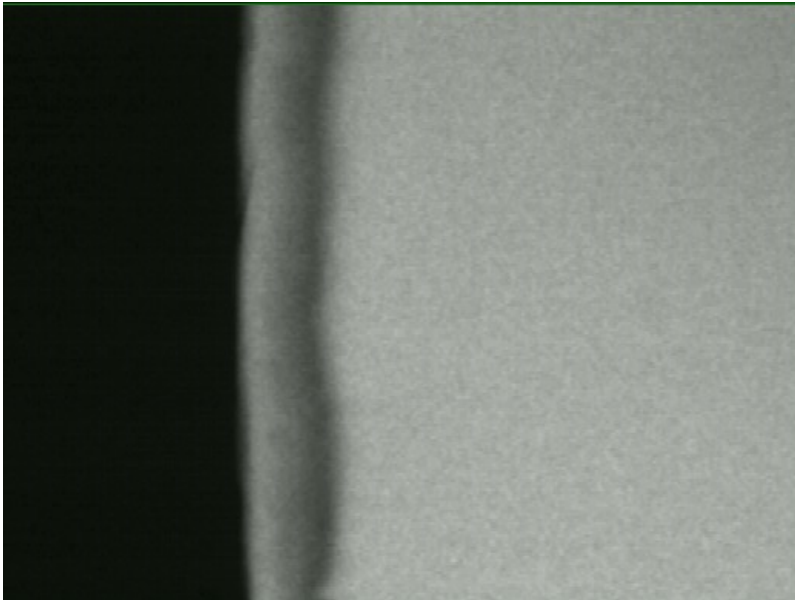


Fig. 58: TMR image recorded after demineralisation (baseline lesion) (group 1).

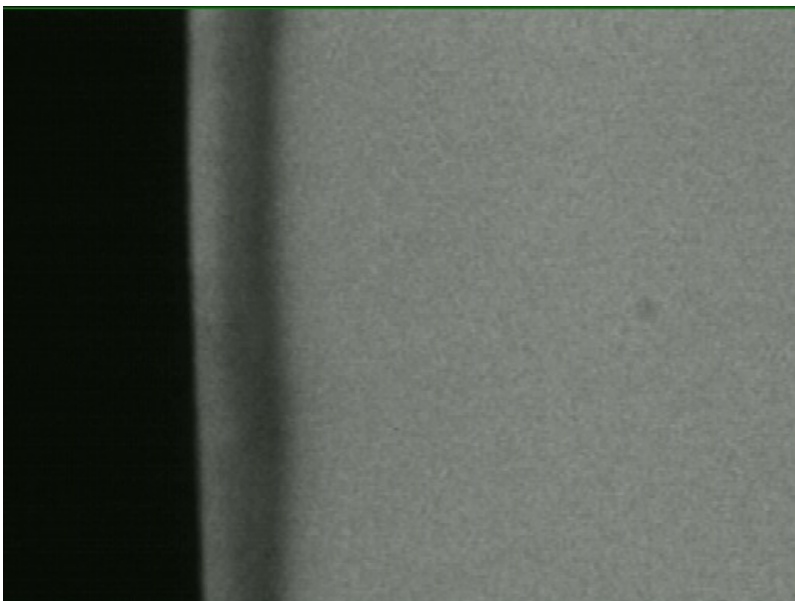


Fig. 59: TMR image recorded after remineralisation procedure (group 1).

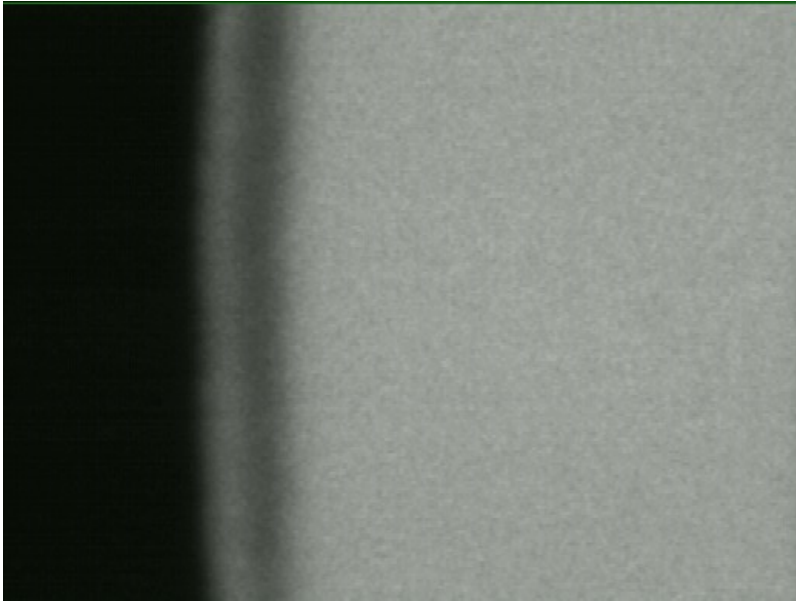


Fig. 60: TMR image recorded after demineralization (baseline lesion) (group 2).

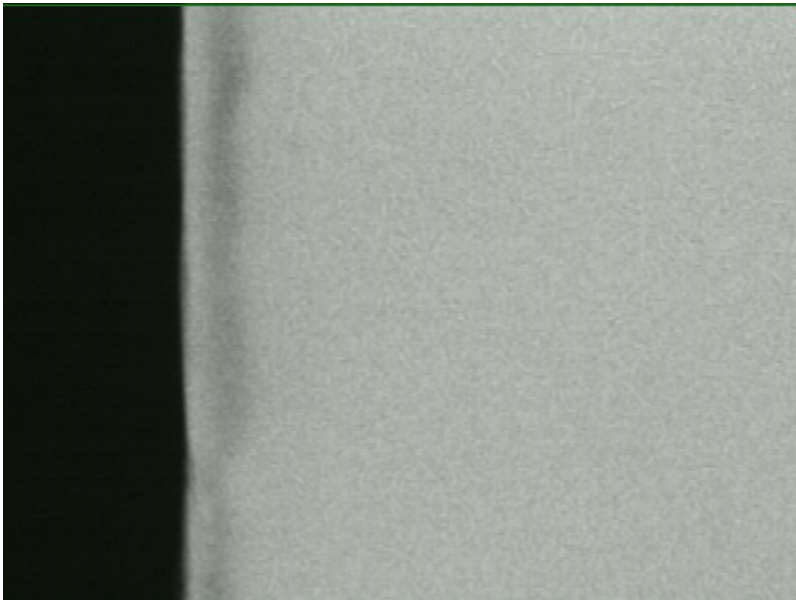


Fig. 61: TMR image recorded after remineralisation procedure (group 2).

DISCUSSION

The elaboration of the results obtained showed that the difference in the mean mineral loss for the group 1 between demineralisation and remineralisation procedures is statistically significant ($F = 11.153$; $p < 0.001$).

The difference in the mean lesion depth for the group 1 between demineralisation and remineralisation procedures is not statistically significant ($F = 2.005$; $p > 0.001$).

The difference in the mean lesion width for the group 1 between demineralisation and remineralisation procedures is not statistically significant ($F = 1.172$ $p > 0.001$).

The difference in the mean mineral loss for the group 2 between demineralisation and remineralisation procedures is statistically significant ($F = 12.614$; $p < 0.001$).

The difference in the mean lesion depth for the group 2 between demineralisation and remineralisation procedures is not statistically significant ($F = 1.436$; $p > 0.001$).

The difference in the mean lesion width for the group 2 between demineralisation and remineralisation procedures is not statistically significant ($F = 0.682$; $p > 0.001$).

It is unclear the reason for which the reduction in mineral loss for both groups after remineralisation treatments was not commensurate with an improvement in lesion depth and lesion width. This apparent contradiction may be explained such suggested by Dowker et al. In fact, these authors describe the non-uniformity of initial lesion formation across the faces of the exposed enamel windows. In particular, the volume (related to lesion depth and lesion width) may expand at different rates in the enamel. When examined as a whole, such differences within the data may become less obvious or even not seen [Dowker et al., 2003].

The mineral increase seen in the two groups has been due to the presence of fluoride, calcium and phosphate ions in the artificial saliva, used both as test solution in the group 1 and as storage solution for the groups 1 and 2.

The mechanism of action of the in vitro remineralisation observed is in accordance with the hypothesis suggested by ten Cate and Arends.

These authors theorize that during remineralisation initial enamel lesions accumulate mineral by direct deposition of hydroxyapatite. This is indicated by increasing concentrations of calcium and phosphate detected by x-ray diffraction [ten Cate and Arends, 1980].

Bovine incisors were chosen for this study. They are frequently used for in vitro cariogenicity testing as they are readily available and show a higher degree of enamel consistency when compared to human enamel.

In an interesting research the relative rates of enamel demineralisation between bovine, human deciduous and human permanent enamel were compared.

The findings from this study revealed that lesion progression was three times faster in bovine enamel than in permanent human enamel.

This could be due to the more porosity of bovine enamel, respect to human permanent enamel.

As such, bovine enamel behaves in a similar manner to deciduous human enamel and is an appropriate substrate for in vitro de- and remineralisation studies [Featherstone and Mellberg, 1981].

The creation of carious lesions in vitro was found to be a technique sensitive procedure. Many variables have to be controlled: pH, volume and concentration of solutions, level of agitation, careful managing of samples.

Furthermore, this study has demonstrated that conventional transverse microradiographic techniques can be applied for the accurate measurement of the mineral loss and gain from thin sections following demineralisation and remineralisation procedures.

TMR may be considered as the gold standard in direct quantification of mineral change and lesion characterization.

However, being the TMR a destructive-sample technique, it was only possible to assess the mineral content of the samples at the end of demineralization procedure (by cutting thin slices from each main enamel block) and at the end of the remineralisation procedure.

In conclusion, the in vitro model used in this investigation had the advantage of controlling many conditions such as concentrations, temperature, pH and exposure of samples to test solutions.

However, the in vitro situation has the disadvantage of being of limited relevance to real caries process due to absence of microorganisms and plaque.

In future, it would be interesting to add a bacterial microflora and biofilms to this model to better mimic the oral ecosystem.

Furthermore, enamel specimens in situ could be used to more accurately replicate the real clinical situation.

REFERENCES

Addeo F, Chianese L, Sacchi R, Musso SS, Ferranti P, Malorni A. Characterization of the oligopeptides of Parmigiano-Reggiano cheese soluble in 120 g trichloroacetic acid/l. *J Dairy Res* 1994;61(3):365-74.

Adolfsson O, Meydani SN, Russell RM. Yogurt and gut function. *Am J Clin Nutr* 2004;80(2):245-56. Review.

Ahola AJ, Yli-Knuuttila H, Suomalainen T, Poussa T, Ahlström A, Meurman JH, Korpela R. Short-term consumption of probiotic-containing cheese and its effect on dental caries risk factors. *Arch Oral Biol* 2002;47(11):799-804.

Aimutis WR. Bioactive properties of milk proteins with particular focus on anticariogenesis. *J Nutr* 2004;134(4):989S-95S.

Aiuchi H, Kitasako Y, Fukuda Y, Nakashima S, Burrow MF, Tagami J. Relationship between quantitative assessments of salivary buffering capacity and ion activity product for hydroxyapatite in relation to cariogenic potential. *Aust Dent J* 2008;53(2):167-71.

Al-Malik MI, Rehbini YA. Prevalence of dental caries, severity, and pattern in age 6 to 7-year-old children in a selected community in Saudi Arabia. *J Contemp Dent Pract* 2006;7(2):46-54.

Amaechi BT, Higham SM. In vitro remineralisation of eroded enamel lesions by saliva. *J Dent* 2001;29(5):371-6.

Angmar B, Carlstrom D, Glas JE. Studies on the ultrastructure of dental enamel. IV. The mineralization of normal human enamel. *J Ultrastruct Res* 1963;8:12-23.

Arends J, Christoffersen J, Christoffersen MR, Schuthof J. Influence of fluoride concentration on the progress of demineralization in bovine enamel at pH 4.5. *Caries Res* 1983;17(5):455-7.

Arends J, Ruben JL, Inaba D. Major topics in quantitative microradiography of enamel and dentin: R parameter, mineral distribution visualization, and hyper-remineralization. *Adv Dent Res* 1997;11(4):403-14.

Arends J, ten Bosch JJ. Demineralization and remineralization evaluation techniques. *J Dent Res* 1992;71 Spec No:924-8.

Arneberg P, Ogaard B, Scheie AA, Rølla G. Selection of *Streptococcus mutans* and lactobacilli in an intra-oral human caries model. *J Dent Res* 1984;63(10):1197-200.

Azarpazhooh A, Limeback H. Clinical efficacy of casein derivatives: a systematic review of the literature. *J Am Dent Assoc* 2008;139(7):915-24.

Bouhallab S, Bouglé D. Biopeptides of milk: caseinophosphopeptides and mineral bioavailability. *Reprod Nutr Dev* 2004;44(5):493-8.

Bourlioux P, Pochart P. Nutritional and health properties of yogurt. *World Rev Nutr Diet* 1988;56:217-58.

Caglar E, Sandalli N, Twetman S, Kavaloglu S, Ergeneli S, Selvi S. Effect of yogurt with *Bifidobacterium* DN-173 010 on salivary mutans streptococci and lactobacilli in young adults. *Acta Odontol Scand* 2005;63(6):317-20.

Cai F, Shen P, Morgan MV, Reynolds EC. Remineralization of enamel subsurface lesions in situ by sugar-free lozenges containing casein phosphopeptide-amorphous calcium phosphate. *Aust Dent J* 2003;48(4):240-3.

Caufield PW, Dasanayake AP, Li Y. The antimicrobial approach to caries management. *J Dent Educ* 2001;65(10):1091-5.

Chianese L, Caira S, Pizzolongo F, et al. Production of a probiotic yogurt with increased levels of bioactive peptides. Proceedings of the IDF Seminar on Aroma and Texture of Fermented Milk, Kolding, Denmark. IDF, ISBN. 2002;290-301.

Cildir SK, Germec D, Sandalli N, Ozdemir FI, Arun T, Twetman S, Caglar E. Reduction of salivary mutans streptococci in orthodontic patients during daily consumption of yoghurt containing probiotic bacteria. *Eur J Orthod* 2009;31(4):407-11.

Clare DA, Swaisgood HE. Bioactive milk peptides: a prospectus. *J Dairy Sci* 2000;83(6):1187-95.

Cross KJ, Huq NL, Stanton DP, Reynolds EC. NMR studies of a novel calcium, phosphate and fluoride delivery vehicle- α (S1)-casein(59-79) by stabilized amorphous calcium fluoride phosphate nanocomplexes. *Biomaterials* 2004;25(20):5061-9.

Cross KJ, Huq NL, Palamara JE, Perich JW, Reynolds EC. Physicochemical characterization of casein phosphopeptide-amorphous calcium phosphate nanocomplexes. *J Biol Chem* 2005;280(15): 15362-9.

Crossner CG, Claesson R, Johansson T. Presence of mutans streptococci and various types of lactobacilli in interdental spaces related to development of proximal carious lesions. *Scand J Dent Res* 1989;97(4):307-15.

Cypriano S, Hoffmann RH, de Sousa Mda L, Wada RS. Dental caries experience in 12-year-old schoolchildren in southeastern Brazil. *J Appl Oral Sci* 2008;16(4):286-92.

Damen JJ, Exterkate RA, ten Cate JM. Reproducibility of TMR for the determination of longitudinal mineral changes in dental hard tissues. *Adv Dent Res* 1997;11(4):415-9.

de Almeida CM, Petersen PE, Andre SJ, Toscano A. Changing oral health status of 6- and 12-year-old schoolchildren in Portugal. *Community Dent Health* 2003;20(4):211-6.

Dowker SE, Elliott JC, Davis GR, Wassif HS. Longitudinal study of the three-dimensional development of subsurface enamel lesions during in vitro demineralisation. *Caries Res* 2003;37(4):237-45.

Elli M, Callegari ML, Ferrari S, Bessi E, Cattivelli D, Soldi S, Morelli L, Goupil Feuillerat N, Antoine JM. Survival of yogurt bacteria in the human gut. *Appl Environ Microbiol* 2006;72(7):5113-7.

Featherstone JD. The science and practice of caries prevention. *J Am Dent Assoc* 2000;131(7):887-99.

Featherstone JD, Mellberg JR. Relative rates of progress of artificial carious lesions in bovine, ovine and human enamel. *Caries Res* 1981;(1):109-14.

Ferranti P, Barone F, Chianese L, Addeo F, Scaloni A, Pellegrino L, Resmini P. Phosphopeptides from Grana Padano cheese: nature, origin and changes during ripening. *J Dairy Res* 1997;64(4):601-15.

Ferrazzano GF, Cantile T, Ingenito A, Chianese L, Quarto M. New strategies in dental caries prevention: experimental study on casein phosphopeptides. *Eur J Paediatr Dent* 2007;8(4):183-7.

Ferrazzano GF, Cantile T, Quarto M, Ingenito A, Chianese L, Addeo F. Protective effect of yogurt extract on dental enamel demineralization in vitro. *Aust Dent J* 2008;53(4):314-9.

Ferrazzano GF, Scaravilli MS, Ingenito A. Dental and periodontal health status in Campanian children and relation between caries experience and socio-economic behavioural factors. *Eur J Paediatr Dent* 2006;7(4):174-8.

Ferro R, Besostri A, Olivieri A, Stellini E, Mazzoleni S. Preschoolers' dental caries experience and its trend over 20 years in a North-East Italian Health District. *Eur J Paediatr Dent* 2007;8(4):199-204.

Flink H. Studies on the prevalence of reduced salivary flow rate in relation to general health and dental caries, and effect of iron supplementation. *Swed Dent J Suppl* 2007;(192):3-50, 2 p preceding table of contents.

García-Albiach R, José M, de Felipe P, Angulo S, Morosini MI, Bravo D, Baquero F, del Campo R. Molecular analysis of yogurt containing *Lactobacillus delbrueckii*

subsp. *bulgaricus* and *Streptococcus thermophilus* in human intestinal microbiota. *Am J Clin Nutr* 2008;87(1):91-6.

Gray M, Morris AJ, Davies J. The oral health of South Asian five-year-old children in deprived areas of Dudley compared with White children of equal deprivation and fluoridation status. *Community Dent Health* 2000;17(4):243-5.

Guggenheim B, Neeser JR, Golliard M, Schüpbach P. Salivary pellicle modified by milk components mediated caries prevention. 1995. 41st ORCA Congress Abstracts, pag 182.

Hallett KB, O'Rourke PK. Caries experience in preschool children referred for specialist dental care in hospital. *Aust Dent J* 2006;51(2):124-129.

Hamada S, Koga T, Ooshima T. Virulence factors of *Streptococcus mutans* and dental caries prevention. *J Dent Res* 1984;63(3):407-11.

Hamada S, Slade HD. Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol Rev* 1980;44(2):331-84.

Hamissi J, Ramezani GH, Ghodousi A. Prevalence of dental caries among high school attendees in Qazvin, Iran. *J Indian Soc Pedod Prev Dent* 2008;26 Suppl 2:S53-5.

Hardie JM, Thomson PL, South RJ, Marsh PD, Bowden GH, McKee AS, Fillery ED, Slack GL. A longitudinal epidemiological study on dental plaque and the development of dental caries--interim results after two years. *J Dent Res* 1977;56 Spec No:C90-8.

Herod EL. The effect of cheese on dental caries: a review of the literature. *Aust Dent J* 1991;36(2):120-5.

Iijima Y, Cai F, Shen P, Walker G, Reynolds C, Reynolds EC. Acid resistance of enamel subsurface lesions remineralized by a sugar-free chewing gum containing casein phosphopeptide-amorphous calcium phosphate. *Caries Res* 2004;38(6):551-6.

Iijima Y, Takagi O, Ruben J, Arends J. In vitro remineralization of in vivo and in vitro formed enamel lesions. *Caries Res* 1999;33(3):206-13.

Jacobsen P, Young D. The use of topical fluoride to prevent or reverse dental caries. *Spec Care Dentist* 2003;23(5):177-9.

Keyes PH, Jordan HV. Factors influencing the initiation, transmission and inhibition of dental caries. In: *Mechanisms of hard tissue destruction*. 1963. Harris RS, editor. New York, NY: Academic Press, pp. 261-283.

Kumar M, Sequeira PS, Peter S, Bhat GK. Sterilisation of extracted human teeth for educational use. *Indian J Med Microbiol* 2005;23(4):256-8.

Kumar VL, Itthagaran A, King NM. The effect of casein phosphopeptide-amorphous calcium phosphate on remineralization of artificial caries-like lesions: an in vitro study. *Aust Dent J* 2008; 53(1):34-40.

Larsen MJ, Pearce EI. Saturation of human saliva with respect to calcium salts. *Arch Oral Biol* 2003;48(4):317-22.

Larsen MJ, Richards A. The influence of saliva on the formation of calcium fluoride-like material on human dental enamel. *Caries Res* 2001;35(1):57-60.

Levine RS. Milk, flavoured milk products and caries. *Br Dent J* 2001;191(1):20.

Llena C, Forner L, Baca P. Anticariogenicity of casein phosphopeptide-amorphous calcium phosphate: a review of the literature. *J Contemp Dent Pract* 2009;10(3):1-9.

Loesche WJ. Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev* 1986;50(4):353-80.

Loesche WJ, Syed SA. The predominant cultivable flora of carious plaque and carious dentine. *Caries Res* 1973;7(3):201-16.

Manton DJ, Walker GD, Cai F, Cochrane NJ, Shen P, Reynolds EC. Remineralization of enamel subsurface lesions in situ by the use of three commercially available sugar-free gums. *Int J Paediatr Dent* 2008;18(4):284-90.

McDougall WA. Effect of milk on enamel demineralization and remineralization in vitro. *Caries Res* 1977;11(3):166-72.

Merritt J, Qi F, Shi W. Milk helps build strong teeth and promotes oral health. *J Calif Dent Assoc* 2006;34(5):361-6.

Meydani SN, Ha WK. Immunologic effects of yogurt. *Am J Clin Nutr* 2000;71(4):861-72. Review.

Milciuviene S, Bendoraitiene E, Andruskeviciene V, Narbutaite J, Sakalauskiene J, Vasiliauskiene I, Slabsinskiene E. Dental caries prevalence among 12-15-year-olds in Lithuania between 1983 and 2005. *Medicina (Kaunas)* 2009;45(1):68-76.

Morgan MV, Adams GG, Bailey DL, Tsao CE, Fischman SL, Reynolds EC. The anticariogenic effect of sugar-free gum containing CPP-ACP nanocomplexes on approximal caries determined using digital bitewing radiography. *Caries Res* 2008;42(3):171-84.

Neville MC, Keller RP, Casey C, Allen JC. Calcium partitioning in human and bovine milk. *J Dairy Sci* 1994;77(7):1964-75.

- Ngo H, Ruben J, Arends J, White D, Mount GJ, Peters MC, Faller RV, Pfarrer A. Electron probe microanalysis and transverse microradiography studies of artificial lesions in enamel and dentin: a comparative study. *Adv Dent Res* 1997;11(4):426-32.
- Nikawa H, Makihira S, Fukushima H, Nishimura H, Ozaki Y, Ishida K, Darmawan S, Hamada T, Hara K, Matsumoto A, Takemoto T, Aimi R. Lactobacillus reuteri in bovine milk fermented decreases the oral carriage of mutans streptococci. *Int J Food Microbiol* 2004;95(2):219-23.
- Nishikawara F, Nomura Y, Imai S, Senda A, Hanada N. Evaluation of cariogenic bacteria. *Eur J Dent* 2007;1(1):31-9.
- Pai D, Bhat SS, Taranath A, Sargod S, Pai VM. Use of laser fluorescence and scanning electron microscope to evaluate remineralization of incipient enamel lesions remineralized by topical application of casein phospho peptide amorphous calcium phosphate (CPP-ACP) containing cream. *J Clin Pediatr Dent* 2008;32(3):201-6.
- Parvez S, Malik KA, Ah Kang S, Kim HY. Probiotics and their fermented food products are beneficial for health. *J Appl Microbiol* 2006;100(6):1171-85.
- Petersen PE. Sociobehavioral risk factors in dental caries –international perspectives. *Community Dent Oral Epidemiol* 2005;33(4):274-279.
- Petridou E, Athanassouli T, Panagopoulos H, Revinthi K. Sociodemographic and dietary factors in relation to dental health among Greek adolescents. *Community Dent Oral Epidemiol* 1996;24 (5):307-11.
- Petti S, Tarsitani G, D'Arca AS. A randomized clinical trial of the effect of yoghurt on the human salivary microflora. *Arch Oral Biol* 2001;46(8):705-12.
- Petti S, Tarsitani G, Simonetti D'Arca A. Antibacterial activity of yoghurt against viridans streptococci in vitro. *Arch Oral Biol* 2008;53(10):985-90.
- Rahiotis C, Vougiouklakis G. Effect of a CPP-ACP agent on the demineralization and remineralization of dentine in vitro. *J Dent* 2007;35(8):695-8.
- Ramalingam L, Messer LB, Reynolds EC. Adding casein phosphopeptide-amorphous calcium phosphate to sports drinks to eliminate in vitro erosion. *Pediatr Dent* 2005;27(1):61-7.
- Rasić JL, Kurmann JA. Bifidobacteria and their role. Microbiological, nutritional-physiological, medical and technological aspects and bibliography. *Experientia Suppl* 1983;39:1-295.

- Reynolds EC. Remineralization of enamel subsurface lesions by casein phosphopeptide-stabilized calcium phosphate solutions. *J Dent Res* 1997;76(9):1587-95.
- Reynolds EC. Anticariogenic complexes of amorphous calcium phosphate stabilized by casein phosphopeptides: a review. *Spec Care Dentist* 1998;18(1):8-16. Review.
- Reynolds EC, Cain CJ, Webber FL, Black CL, Riley PF, Johnson IH et al. Anticariogenicity of calcium phosphate complexes of tryptic casein phosphopeptides in the rat. *J Dent Res* 1995;74(6):1272-9.
- Reynolds EC, Cai F, Shen P, Walker GD. Retention in plaque and remineralization of enamel lesions by various forms of calcium in a mouthrinse or sugar-free chewing gum. *J Dent Res* 2003;82(3):206-11.
- Saint-Eve A, Lévy C, Martin N, Souchon I. Influence of proteins on the perception of flavored stirred yogurts. *J Dairy Sci* 2006;89(3):922-33.
- Selwitz RH, Ismail AI, Pitts NB. Dental caries. *Lancet* 2007;369(9555):51-9. Review.
- Shahani KM, Chandan RC. Nutritional and healthful aspects of cultured and culture-containing dairy foods. *J Dairy Sci* 1979;62(10):1685-94.
- Shen P, Cai F, Nowicki A, Vincent J, Reynolds EC. Remineralization of enamel subsurface lesions by sugar-free chewing gum containing casein phosphopeptide-amorphous calcium phosphate. *J Dent Res* 2001;80(12):2066-70.
- Silva MF, Burgess RC, Sandham HJ, Jenkins GN. Effects of water-soluble components of cheese on experimental caries in humans. *J Dent Res* 1987;66(1):38-41.
- Simonsen RJ. Pit and fissure sealant: review of the literature. *Pediatr Dent* 2002;24(5):393-414.
- Skeie MS, Riordan PJ, Klock KS, Espelid I. Parental risk attitudes and caries related behaviors among immigrant and western native children in Oslo. *Community Dent Oral Epidemiol* 2006;34(2):103-113.
- Staat RH, Gawronski TH, Cressey DE, Harris RS, Folke LE. Effects of dietary sucrose levels on the quantity and microbial composition of human dental plaque. *J Dent Res* 1975;54(4):872-80.
- Stewart PS. Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol* 2002;292(2):107-13.

Sudjalim TR, Woods MG, Manton DJ, Reynolds EC. Prevention of demineralization around orthodontic brackets in vitro. *Am J Orthod Dentofacial Orthop* 2007;131(6):705.e1-9.

Sundby A, Petersen PE. Oral health status in relation to ethnicity of children in the municipality of Copenhagen, Denmark. *Int J Paediatr Dent* 2003;13(3):150-7.

ten Bosch JJ, Angmar-Månsson B. A review of quantitative methods for studies of mineral content of intra-oral caries lesions. *J Dent Res* 1991;70(1):2-14.

ten Cate JM, Arends J. Remineralization of artificial enamel lesions in vitro: III. A study of the deposition mechanism. *Caries Res* 1980;14(6):351-8.

Truin GJ, Van Rijkom HM, Mulder J, Van't Hof MA. Caries trends 1996-2002 among 6- and 12-year-old children and erosive wear prevalence among 12-year-old children in the Hague. *Caries Res* 2005;39(1):2-8.

Van Houte J, Gibbons RJ, Pulkkinen AJ. Ecology of human oral lactobacilli. *Infect Immun* 1972;6(5):723-9.

van Ruyven FO, Lingström P, van Houte J, Kent R. Relationship among mutans streptococci, "low-pH" bacteria, and iodophilic polysaccharide-producing bacteria in dental plaque and early enamel caries in humans. *J Dent Res* 2000;79(2):778-84.

Vered Y, Zini A, Livny A, Mann J, Sgan-Cohen HD. Changing dental caries and periodontal disease patterns among a cohort of Ethiopian immigrants to Israel: 1999-2005. *BMC Public Health* 2008;8:345.

Walker G, Cai F, Shen P, Reynolds C, Ward B, Fone C, Honda S, Koganei M, Oda M, Reynolds E. Increased remineralization of tooth enamel by milk containing added casein phosphopeptide-amorphous calcium phosphate. *J Dairy Res* 2006;73(1):74-8.

White DJ, Faller RV, Bowman WD. Demineralization and remineralization evaluation techniques--added considerations. *J Dent Res* 1992;71 Spec No:929-33. Review.

Wyne AH. Caries prevalence, severity, and pattern in preschool children. *J Contemp Dent Pract* 2008;9(3):24-31.

Zero DT. In situ caries models. *Adv Dent Res* 1995;9(3):214-30.