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TESI DI DOTTORATO

Experimental study on bioactive molecules in contrasting dental diseases: microbiological assay, oral delivering and enamel remineralization

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Abstract

Introduction: The objective of this research program was to determine a new way in caries and erosion prevention. To achieve this target was analyzed the more effective anti-caries and anti-erosive bio-active molecules of natural origin, in order to elaborate a new novel chewing gum device. The under discussion molecules were plant polyphenols and casein-phosphopeptides.

Materials and methods: The research project has been divided in three parts: initially, was analyzed in vitro, through SEM inspection and pH analysis, the remineralizing effect of casein-phosphopeptides against cola drink dental erosion. In the second part, a microbiological assay of effects of Plantago lanceolata tea on oral bacteria was assessed: initially, the *P. lanceolata* extracts was chemical analyzed for its polyphenolic content; then, the MIC and MBC were determined for *P. lanceolata* extracts against cariogenic bacteria; subsequently, a controlled random clinical study was conducted on 44 volunteers to evaluate a *P. lanceolata-based* mouth rinse on cariogenic bacteria content in saliva. In the third part, in vitro experiments were performed using a specifically designed chewing apparatus to test the release in artificial saliva of Calcium (derived from CPP) and Quercetin included in gums, during the chewing time; while the in vivo experiments required a two sessions clinical trial, performed by 10 volunteers, in order to analyze the in vivo performance of the experimental chewing-gums.

Results: In the first phase experiments, dental enamel specimens subjected to cola-type drink showed wide areas of enamel dissolution, strongly different from samples treated adding CPP-ACP to the cola-type drinks. In the second phase of experiments, P. lanceolata extracts demonstrate good in vitro antimicrobial activity and in vivo trials showed that subjects treated with Plantago-based mouth rinse presented a significant

decrease in streptococci content in saliva. In the last phase experiments, results demonstrated that both Calcium derived from CPP-ACP and Quercetin were released during chewing; they were also both effectively released in the oral cavity during the in vivo chewing time.

Conclusions: Results of experimental protocols allow us to define that CPP-ACP and polyphenols, as Quercetin, could contrast dental caries and erosion in vitro and in vivo trials. Moreover, in vitro preliminary assay and the subsequent clinical trial demonstrate that experimental chewing gums are capable to release effective concentration of bioactive molecules into oral cavity, promoting the researched protective action against dental diseases.

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Overview on caries and erosion diseases prevention

In industrialized countries oral diseases, especially dental caries and dental erosion, are the most common of the paediatric chronic diseases. They are important public health topics and they are emerging public health problems in developing countries, because of their prevalence, the cost of treatment and their impact on individuals and the society [Hamissi et al., 2008] [Linnett and Seow, 2001] [Wiegand et al., 2006]. Oral diseases affect various aspects of children's quality of life, causing considerable pain, acute and chronic infections, eating and sleep difficulty, reduced body weight and loss of school days [Acs et al., 1992]. In addition, oral diseases affect not only physically but also psychologically, resulting in difficulty to socialize.

Regarding dental caries, the prevalence of this disease has declined in western countries in last decades [Campus et al., 2007]. Epidemiological studies have shown, particularly, that caries severity drastically decreased in the last thirty years; significantly gains in oral health have been observed over the last few years in countries like Germany, England, USA, Scandinavia, Scotland, Norway and Australia [Cypriano et al., 2008]. Among Norwegian adolescents, between 1985 and 2000, the DMFT declined by 49%; 10.2 +/- 0.75 to 5.2 +/- 0.78 [Birkeland et al., 2002]. Oral health in Germany improved considerably between the years 1994 and 2000. The mean DMFT values for Germany decreased from 2.44 in 1994-1995 to 1.81 in 1997 and 1.24 in 2000. Nevertheless, the results in different federal states showed a wide variation in caries prevalence [Pieper

and Schulte, 2004]. The values of the Italian DMFT (decayed, missing, filled teeth), for example, have decreased by almost 90% in 12 year-old children since the late 1970s: from 6.9 [Vogel et al., 1979] to 1.09 [Campus et al., 2006]. An important decline in the prevalence of dental caries may be attributed to preventive strategies, such as tooth-brushing, dietary control, topical and systemic use of fluorides and fissure sealants [Birkeland et al., 2002; Tinanoff et al.,2002]. The most recent epidemiological data suggest that this decline has remained constant in the last years [Marthaler, 2004], and in some areas, children of low socioeconomic status and immigrants from outside Western Europe, still, generally have higher caries disease levels [Tinanoff et al., 2002]. The inhomogeneous distribution of caries needs the identification of people with the highest caries risk from tender age to adopt new preventive strategies suitable for these groups [Hamissi et al., 2008]. For these reasons, caries epidemiology will remain an indispensable part of dental public health [Birkeland et al., 2002; Ferrazzano et al.,2006; Marthaler, 2004]. A recent study has set out new oral health objectives for the year 2020. The new goals are: to increase the proportion of caries-free 6-year-olds, to reduce the DMFT particularly the D component (decayed) at age 12 with special attention to high-risk group within the population and to reduce the number of teeth extraction due to dental caries at age 18 [Hobdell et al., 2003]. It's well known that dental caries has a multi-factorial etiology [Kutsch and Young, 2011]. It is caused by three main factors: carbohydrate, bacteria and susceptible teeth (the host) [Lenander-Lumikari and Loimaranta, 2000]. Among the three above mentioned factors, bacteria are considered to play a leading role in the development and progression of caries [Marsh; 1994]. Streptococci mutans and Lactobacilli casei are known as cariogenic oral bacteria [Loesche, 1986; Seminario et al., 2005]. S. mutans synthesize adherent, water-insoluble glucans from sucrose by the action of glycosyl-transferases (GTFs). The synthesis of

glucans determines the adherence of the cariogenic bacteria to tooth surface and contributes to the composition of the dental plaque polysaccharide matrix [Tanzer et al., 1974; Koo et al., 2002]. The virulence key factor is the ability of bacteria to produce organic acids from carbohydrate (anaerobic glycolysis) [Loesche, 1986]. The accumulation of acids in dental plaque reduces the pH value of the bio-film to less than 5.5 and causes decalcification of tooth enamel and consequentelly dental decay [Freedman and Tanzer, 1974]. L. casei are acidogenic in presence of carbohydrates; they are acid tolerant [Crossner et al., 1989] and they are often isolated from established carious lesions [Loesche and Syed,1973; Staat et al., 1975]. Since these bacteria are the main factors in dental caries development, it is necessary to use antibacterial substances capable of reducing their pathogenicity [Palombo, 2011].

Concerning dental erosion, as the progressive, irreversible loss of dental hard tissue, chemically etched away from the tooth surface by extrinsic and/or intrinsic acids [Petersen et al., 2005] without the involvement of bacteria, epidemiological studies have shown that prevalence of dental erosion in children varies widely between 2 and 57% [Linnett and Seow, 2001]. Also this disease appears particularly in industrialized countries, reflecting the wide availability and the frequent consumption of acidic beverages, fruit juices, carbonated beverages, wines, and sports drinks. A sample of 10 to 12-year-old children showed that tooth erosion was present in 30.4% of subjects and in 44.2% of same sample after three years. Deep enamel or dentin was eroded in 1.8% of 11 year-old sample and in 23.8% of same children after three years [El Aidi et al., 2010]. Another study showed that 14% of the 15 to 17-year-old children sample had more than 3 dental surfaces eroded and that the maxillary lingual surfaces were more affected than the buccal surfaces [Larsen et al., 2005]. Other researchers reported the

data collected from a sample of 12-year-old children: at baseline 4.9% of them showed enamel erosions or enamel and dentine erosions and after two years the subjects affected by this condition had risen to 13.1%; in other words, about 8.2% of children had developed tooth erosion in the following two years [Dugmore and Rock, 2003]. Moreover, another study showed that prevalence of tooth erosion in 2-7 year-olds was 32% and increased with age [Wiegand et al., 2006]. As above mentioned, dental erosion is a multifactorial condition. Acids that cause dental erosion may be extrinsic or intrinsic in origin [Lussi et al., 1993; Lussi and Jaeggi, 2008]. Typical acid sources come from diet, medications, occupational exposure and lifestyle activities, but dietary acids are the most extensively studied etiological agents and the most important extrinsic factors [Lussi et al., 2004]. Gastrointestinal disease is an example of the intrinsic factors, particularly regurgitation of acid into the mouth [Correr et al., 2009; de Carvalho Sales-Peres et al., 2007; Lussi and Jaeggi, 2008; Young, 2005]. Nowadays acidic drinks consumption is, probably, the principal extrinsic factor in young people [Ramalingam et al., 2005]. The erosive potential of acidic drinks depends mainly on the acid contained in the formulation, such as citric, maleic or phosphoric acid, which decreases the pH of the oral environment, but it also depends on the high titratable acidity, viscosity, and concentration of inorganic protective factors (calcium, phosphate, and fluoride) [Cochrane et al., 2009; Lussi and Jaeggi, 2008]. Preventive methods that may reduce the degree of tooth demineralization include increased acid resistance of the tooth structure, and the remineralization process, which requires calcium, phosphate and fluoride [Azarpazhooh and Limeback, 2008; Cochrane et al., 2008; Rios et al., 2008; Wiegand and Attin, 2003].

The research is providing for foods and vegetables naturally rich in bioactive compounds, already in use in traditional medicine, considering as good alternatives to synthetic molecules [Kim, 2005]. In fact, there is a growing awareness that some dietary components may affect the overall health. Suitably, the role of food as an agent for improving health has been recognized, initiating the development of new classes of food, known as functional foods [Honkanen, 2009]. The concept of functional foods is to improve the general conditions of the body and to decrease the risk of illness and disease [Siró et al., 2008]. Bioactive compounds present as natural constituents in food have the potential to provide health benefits, beyond the basic nutritional value of the product [Biesalski et al., 2009]. Most of the bioactive molecules are derived either directly or indirectly from naturally occurring sources, especially, terrestrial food plants and marine species or from animal products [Shahidi, 2009; Lordan et al., 2011]. Subsequently, these natural bioactive compounds are being incorporated in the form of ingredients in functional and novel foods, dietary supplements and even pharmaceuticals with the purpose of delivering specific oral health benefits. To date, numerous plant products have been analyzed for their effectiveness in the prevention of dental plaque formation. However, only a very small number of these natural products have been used for therapeutic applications. This limited use is due to various factors such as adequate effectiveness, stability, smell, taste and cost [Ferrazzano et al., 2009]. Vulnerary plants represent one of the largest legacies provided by folk medicine worldwide [Forrest, 1982]. The therapeutic activities of these plants are numerous and span widely. Some of them present astringent properties, other are endowed of anti-inflammatory power. Some plants, for example, may be immune-stimulant and/or possess recognized antimicrobial activity [Houghton et al., 2005]. For these reasons, vulnerary plants are an inexhaustible sources of research for such types of compounds,

especially those aimed at fighting microbial infections. Indeed, Brantner and Grein demonstrated that about 60% of plant extracts used in traditional medicine exhibit antibacterial actions [Brantner and Grein., 1994].

As above mentioned, another topic in dental caries prevention, is based on the use of bioactive principles derived from milk and milk products: the anti-caries activity was in vitro and in vivo attributed to the direct chemical effects of casein phosphopeptides, derived from proteolytic digestion of caseins, on calcium and phosphate ions content [McDougall, 1977; Reynolds, 1997; Reynolds et al., 2003; Walker et al., 2006]. For about 10 years, in industrial fields are commercialized anticaries products as paste or chewing gums containing synthetic CPP-ACP complexes, but not yet are enough commercialized products containing exclusively biomolecules of natural origin.

In conclusion, there is a need to developing new pharmaceutical devices, loaded with natural-derived molecules, to prevent carious and erosive lesions, or to arrest enamel demineralization, when the dental caries and erosion have already been started.

Capther I

Polyphenols and Casein-phospopeptides in Dentistry

I.1 Polyphenols

In recent years, there has been an increased interest in polyphenolic compounds found in plant foods. This is probably due to the mounting evidence that several of these may have beneficial effects on humans. Polyphenols constitute one of the most common and widespread groups of substances in plants. Simple phenols consist of a single substituted phenolic ring; flavones and their derivatives - flavanoids and flavanols - are phenolic structures containing one carbonyl group [Cowan, 1999]. Polyphenols occur in all vegetative plant organs, and also in flowers and fruits. Plants are the main source of the polyphenols daily intake in human diet, but other strong contributors are tea, coffee, cereals and fruit, due to their high consumption. Despite their wide distribution, the beneficial effects of dietary polyphenols have come to the attention of nutritionists only in the last years. The main factor responsible for the delayed research on polyphenols is the variety and the complexity of their chemical structure. The biological properties of polyphenols include antioxidant [Balz and Jane, 2003; Luczaj and Skrzydlewska, 2005], anticancer [Krishnan and Maru, 2004; Yamane et al., 1996; Zhang et al., 2002;] and anti-inflammatory [Sang et al., 2004] effects. Experimental studies strongly support a

role of polyphenols also in the prevention of cardiovascular disease, osteoporosis, diabetes mellitus and neurodegenerative diseases [Scalbert et al., 2005]. In the last years, polyphenols from some edible plants have attracted attention as potential sources of agents capable of controlling the growth of oral bacteria [Taguri et al., 2004]. Polyphenols could be able to influence the process of caries formation at crucial different stages. In fact, they have been shown to inhibit the adherence of mutans streptococci to saliva-coated hydroxyapatite [Smullen et al., 2007]. Polyphenols are able to interact with microbial membrane proteins, enzymes and lipids, thereby altering cell permeability and permitting the loss of protons, ions, and macromolecules [Ikigai et al., 1993]. It has been, in fact, demonstrated that when S. mutans was pretreated with Sunphenon, (a mixture, containing polyphenols), its cellular attachment to a saliva-treated hydroxyapatite surface was significantly reduced, showing that the phenomenon was a consequence of a specific interaction with the bacteria [Otake et al., 1991]. In addition, several works have demonstrated that polyphenols inhibit in vitro the glucosyltransferases activity of S. mutans (GTases) [Hattori et al.,1990; Kashket et al., 1985; Ooshima et al.,1993; Sakanaka et al., 1989]. Experiments also demonstrate the inhibition of salivary amylase activity by polyphenols. The effect on salivary amylase may contribute significantly to reduce the cariogenicity of starch-containing foods [Kashket and Paolino; 1988]. Following in vitro studies on plant extracts suggest an activity against several metabolic activities of mutans streptococci, resulting in a decrease in growth and virulence [Brighenti et al., 2008; Hirasawa et al., 2006; Otake et al., 1991; Matsumoto et al., 1999]. In particular, it has been proved that tea polyphenols can inhibit the adherence of Streptococcus mutans and Actinomyces viscosus to salivary acquired pellicle of the tooth surface [Xiao et al., 2000]. Smullen et al. have shown that the extracts from unfermented cocoa, green tea and red grape seeds have a bacteriostatic

effect on S. mutans. and reduce its adherence to glass [Smullen et al., 2007]. Studies on the development of anti-plaque agents for prevention of dental caries have investigated the effect of some tea preparations and their individual components on the glucan synthesis catalyzed by glucosyltransferase (GTF) from mutans streptococci. Extracts of green tea and polyphenol mixtures showed appreciable inhibition in the synthesis of insoluble glucan [Hattori et al., 1990]. Experiments demonstrate also the inhibition of salivary amylase activity by extracts of a commercial tea. The effect on salivary amylase may contribute significantly to reduce the cariogenicity of starch-containing foods [Kashket et al.,1985]. Besides to these in vitro evidences, there is a correlation between subjects with a diet rich in tea respect to subjects with a different diet [Signoretto et al., 2006; Jones et al., 1999]. In fact, tea has been considered a healthful beverages since ancient times and, actually, for its health benefits, tea has been included, in particulary green tea, in the group of beverages with bio-functional properties [Cabrera, 2006].

Hot infusions (herbal teas) represent by far the most popular type of consumption of herb to which medicinal properties have been acknowledged in traditional medicine [Evans, 2002]. Tsai et al. [2008] have demonstrated that several herbs commonly used as tea in Taiwan exhibited anti-cariogenic and anti-inflammatory properties [Tsai, 2008]. According to *European Medicines Agency* (EMA) [Committee on Herbal Medicinal Products (HMPC), 2011], herbal tea made from *P. lanceolata* leaves is used in European countries as a demulcent for the local treatment of oral or pharyngeal irritations and is considered a safe preparation with no known contraindications.

The genus *Plantago* (Plantaginaceae) encompasses approximately 275 species with a cosmopolitan distribution. Recent studies have confirmed that some *Plantago* species have considerable antiviral, anti-inflammatory, and antioxidant activities [Beara et al.,

2012; Galvez et al., 2005; Galvez et al., 2005]. Phytochemical studies have also shown that the *Plantago* genus contains a great amount of phenolic compounds (flavonoids and tannins). In particular, phenolic compounds seem to play a potential role in the control of bacterial growth and could prevent tooth decay, decreasing growth and virulence of pathogenic oral flora [Smullen et al., 2007]. A recent study has reported that *P. lanceolata* methanol/water extracts are rich in phenolic acids, with the benzoic acid derivatives hydroxybenzoate and 3,4,5-trihydroxybenzoate (gallic acid) being the most highly represented [Beara et al., 2012].

I.2 Casein phosphopeptides

As above mentioned, caries is a bacterial infectious disease. 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].

Casein phosphopeptides (CPPs) are phosphorylated casein-derived peptides produced by proteolytic digestion of s1-, s2-, and ß-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 demineralization and enhancing remineralization [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), for example during a carious or erosive process. That situation 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 demineralization and enhance the remineralization of early enamel caries [Reynolds et al., 2003]. On the basis of the generally accepted molecular formula for ACP [Ca3(PO4)2 - nH2O], 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].

Furtheremore, 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 remineralize 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 demineralization 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 remineralization of incipient enamel lesions by the topical application of Casein Phosphopeptide-Amorphous Calcium Phosphate (CPPACP) using laser fluorescence and scanning electron microscope showed high scores of remineralization [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 remineralization [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 remineralize 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 increases its ability to remineralize enamel subsurface lesions [Walker at al., 2006], the remineralization effect of others 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 protective effect against dental caries are the same as milk [Ferrazzano et al., 2007; Ferrazzano et al., 2008]: proteins content of yogurt are generally higher than that of milk because of the addition of non-fat dry milk during processing and concentration, which increases proteins content of the final product.

Other recent in vitro and in vivo 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].

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 determines colonization by less cariogenic bacteria.

Recents studies tested if adding casein phosphopeptide-stabilized amorphous calcium phosphate to the Powerade sport drink could be possible prevent erosive enamel lesions: enamel samples were analyzed at scanning electron microscope (SEM) after erosive immersion test with and without the protective biomolecules to evaluate the resulted surface profiles: it was assessed that CPP-ACP included in sport drinks significantly reduced the beverage's erosion effect on dental enamel without affecting the product's taste [Ramalingam et al., 2005].

Furthermore, another in vitro trial analyzed the microhardness of enamel surfaces after immersion in erosive soft drink-based solutions with or without CPP-ACP inclusion: it was assessed that sport drinks could reduce enamel erosion, showing a greater effect on enamel hardness [Panich and Poolthong, 2009].

Another study described, by evaluating at Atomic Force Microscope (AFM), the protective effect of CPP - ACP pastes (Tooth Mousse - GC) in prevention of enamel erosion; in vitro treatment of enamel surfaces was based on applications of CPP-ACP pastes: that treatment determined a mineral layer apposition that fills hydroxyapatite interprismatic cavities, in order to prevent subsequent acid attacks [Poggio et al., 2009]. Further studies have confirmed the CPP-ACP capability to prevent in vitro erosion due to acidic solutions or beverages, such as soft drinks [Tantbirojn et al., 2008; Ranjitkar et al., 2009; de Alencar et al., 2014; Somani et al., 2014], although it remains to deepen their in vivo effectiveness.

Capther II

Experimental study

The objective of this research program was to determine a new way in caries and erosion prevention.

To achieve this target was analyzed the more effective anti-caries and anti-erosive bio-active molecules of natural origin, in order to elaborate a new novel chewing gum device. The under discussion molecules were plant polyphenols and casein-phosphopeptides.

In the first period of the project it was analyzed the possibility, using the above mentioned molecules, to obtain in vitro a significant anticaries and antierosive effect. In the second period was assessed a clinical trial, using only molecoles which showed the most protective in vitro effects. For this aim, research project has been divided in three parts:

1: Remineralizing effect of casein-phosphopeptides and dental erosion.

2: In vitro and in vivo effects of Plantago lanceolata tea extracts on oral bacteria.

3: Release kinetics of active biomolecules included in a novel chewing gum.

II.1 Remineralizing effect of Casein-phosphopeptides and dental erosion

II.1.1 Abstract

Aim: Erosion of dental hard tissues induced by acidic dietary components is a high-prevalence finding, especially among children and adolescents. Acidic soft drinks are frequently implicated in dental erosion. The aim of this in vitro study was to assess if CPP-ACP preparation is capable of reducing enamel erosion caused by a cola-type drink.

Materials and methods: Twenty five sound human permanent premolars, extracted for orthodontic reasons in patients of 12-16 years old, were used. The roots were removed and the crowns were sectioned in order to obtain 3 enamel sections from each tooth. The specimens were immersed in: (A) cola-type drink; (B) cola-type drink plus CPP-ACP; (C) de-ionized water (control) for: 48 h, 24 h, 12 h, 6 h and 3 h, respectively. pH values were constantly monitored. Statistical analysis was performed using ANOVA. The enamel samples were evaluated for surface changes using scanning electron microscopy (SEM).

Results: Specimens subjected to cola-type drink (treatment A) showed wide areas of enamel dissolution, while the treatment B specimens showed a few areas of little enamel erosion, different from control samples. Adding CPP-ACP to the cola-type drinks influenced pH levels of the solutions, but always in the acidity range.

Conclusion: CPP-ACP provides protection against dental erosion from cola-type drinks in vitro. Therefore, further studies are necessary to evaluate if adding casein phosphopeptide-amorphous calcium phosphate complex to acidic cola drinks could reduce their erosive potential in vivo as well.

II.1.2 Aim

As above assessed, typical acid sources come from diet, medications, occupational exposure and lifestyle activities, but dietary acids are the most extensively studied aetiological agents and the most important extrinsic factors [Lussi et al., 2004]. Nowadays acidic drinks consumption is, probably, the principal extrinsic factor in young people [Ramalingam et al., 2005]. The aim of this in vitro study is to examine if synthetic CPP-ACP preparation is capable of reducing enamel erosion caused by a cola drink.

II.1.3 Materials and methods

Twenty-five human permanent premolars free of hypocalcification and/or caries, extracted for orthodontic reasons in patients aged 12-16 years, were used in this study. Enamel surfaces were cleaned with sterile gauze soaked in distilled water and stored at 4°C in sterile containers with 5% formalin solution. The roots were cut with a diamond disk (Diaflex H347- Horico Hopf, Ringleb & Co. & Cie.™, Berlin, Germany) mounted on a laboratory handpiece and the crowns were polished with pumice dust and non-fluoride toothpaste, using a circular brush with nylon bristles mounted on a dental handpiece (Intramatic Lux 3 20 LH, D481631 - KAVO, Biberach/Riß, Germany). The crowns were then sectioned with a diamond disk on a laboratory handpiece in order to obtain 3 enamel sections from each tooth. Samples surfaces were rinsed and stored as above described. One section of each tooth was immersed in: 100% cola-type drink

solution; 99.75% of cola-type drink plus 0.25% of synthetic CPP-ACP (GC Tooth Mousse™ - Recaldent™, Melbourne, Australia) solution; deionised water (control). Regardless of the treatment, the immersion times were 48 hours (Group 1), 24 hours (Group 2), 12 hours (Group 3), 6 hours (Group 4), 3 hours (Group 5). The experimental assessment of five different immersion times had the objective of simulating an in vivo oral chronic intake of cola drink in children, based on the salivary flow patterns analysed by Dawes [2008]. Each immersion time was divided in three equal cycles and at start and end of each cycle, the pH value was measured using a pHmeter (pHep® HI 96107 - Hanna instruments®, Modena, Italy) to evaluate pH variation during the erosive process and the buffering capacity of CPP-ACP paste added to the solution. The results underwent statistic elaboration with ANOVA testing, and the SPSS 19.0 software (SPSS Inc. Chicago, Illinois, USA). At the end of the experimental procedures, all specimens were dehydrated in gradual ethanol series, critical point dried (SPC-900/EX, The Bomar Co., Tacoma, USA), mounted in stubs, sputter-coated with gold (E 306, Edwards, UK) and observed under SEM (Quanta 200 - FEI Worldwide Corporate, Hillsboro, Oregon, USA) to evaluate enamel surface changes.

Erosion/remineralization experimental protocol

Five crowns for each of the 3 immersion groups were included in each of the 5 groups; the latter were characterized by a different immersion time: 48 hours (Group 1), 24 hours (Group 2), 12 hours (Group 3), 6 hours (Group 4) and 3 hours (Group 5), each immersion time was divided into three equal cycles each one, so that the solution was renewed twice. For each crown, the first sample (A) was immersed in 50 mL of erosive solution made of 100% cola-type drink at 37°C, with continuous mixing using an orbital

shaker (OS 20 – Bioscientifica srl, Rignano Flaminio, Italy). At start and end of each cycle of treatment, the pH value was assessed with a pHmeter. Each second sample (B) was immersed in 50 mL of solution made of 99.75% cola-type drink and 0.25% of synthetic CPP-ACP at 37°C, with continuous mixing using an orbital shaker. Again, at start and end of each cycle of treatment, the pH value was assessed with a pHmeter. Each third sample was immersed in 50 mL of deionized water as control (C) (Table 1).

Experimental protocol

	Immersion time	Treatment A	Treatment B	Treatment C
Group 1	48 hours	Cola drink	Cola drink + CPP	Deionised water
Group 2	24 hours	Cola drink	Cola drink + CPP	Deionised water
Group 3	12 hours	Cola drink	Cola drink + CPP	Deionised water
Group 4	6 hours	Cola drink	Cola drink + CPP	Deionised water
Group 5	3 hours	Cola drink	Cola drink + CPP	Deionised water

Table 1: summary chart of experimental protocol.

II.1.4 Results

Group 1 (immersion time: 48 h) SEM images of samples A, B and C are shown in Figure 1; pH values are shown in Table 2.

Group 2 (immersion time: 24 h) SEM images of samples A, B and C are shown in Figure 2; pH values assessed are shown in Table 2.

Group 3 (immersion time: 12 h) Significant SEM images of samples A and B are shown in Figure 3. The pH values assessed are reported in Table 2.

Group 4 (immersion time: 6 h) Significant SEM images of samples A and B are shown in Figure 4. The pH values are shown in Table 2.

Group 5 (immersion time : 3 h) Significant SEM images of samples A and B are shown in Figure 5. The pH values are shown in Table 2.

PH analysis

Group 1: 48h	Group 2: 24h	Group 3: 12h	Group 4: 6h	Group 5: 3h
Treatment A pH mean	Treatment A pH mean	Treatment A pH mean	Treatment A pH mean	Treatment A pH mean
t0: 2.64 ± (S.D.) 0.063 MIN: 2.5 MAX: 2.7	t0: 2.64 ± (S.D.) 0.051 MIN: 2.6 MAX: 2.7	t0: 2.67 ± (S.D.) 0.082 MIN: 2.6 MAX: 2.9	t0: 2.72 ± (S.D.) 0.068 MIN: 2.6 MAX: 2.8	t0: 2.71 ± (S.D.) 0.064 MIN: 2.6 MAX: 2.8
tf: 2.82 ± (S.D.) 0.115 MIN: 2.6 MAX: 3.0	tf: 2.78 ± (S.D.) 0.068 MIN: 2.7 MAX: 2.9	tf: 2.86 ± (S.D.) 0.083 MIN: 2.7 MAX: 3.0	tf: 2.79 ± (S.D.) 0.091 MIN: 2.7 MAX: 3.0	tf: 2.77 ± (S.D.) 0.070 MIN: 2.7 MAX: 2.9
Treatment B pH mean	Treatment B pH mean	Treatment B pH mean	Treatment B pH mean	Treatment B pH mean
t0: 2.72 ± (S.D.) 0.086 MIN: 2.6 MAX: 2.9	t0: 2.72 ± (S.D.) 0.094 MIN: 2.5 MAX: 2.8	t0: 2.75 ± (S.D.) 0.074 MIN: 2.6 MAX: 2.9	t0: 2.73 ± (S.D.) 0.070 MIN: 2.6 MAX: 2.8	t0: 2.7 ± (S.D.) 0.076 MIN: 2.6 MAX: 2.8
tf: 3.07 ± (S.D.) 0.188 MIN: 2.8 MAX: 3.4	tf: 3.19 ± (S.D.) 0.252 MIN: 2.9 MAX: 3.8	tf: 3.13 ± (S.D.) 0.149 MIN: 2.9 MAX: 3.4	tf: 2.81 ± (S.D.) 0.112 MIN: 2.6 MAX: 3.0	tf: 2.77 ± (S.D.) 0.080 MIN: 2.6 MAX: 2.9

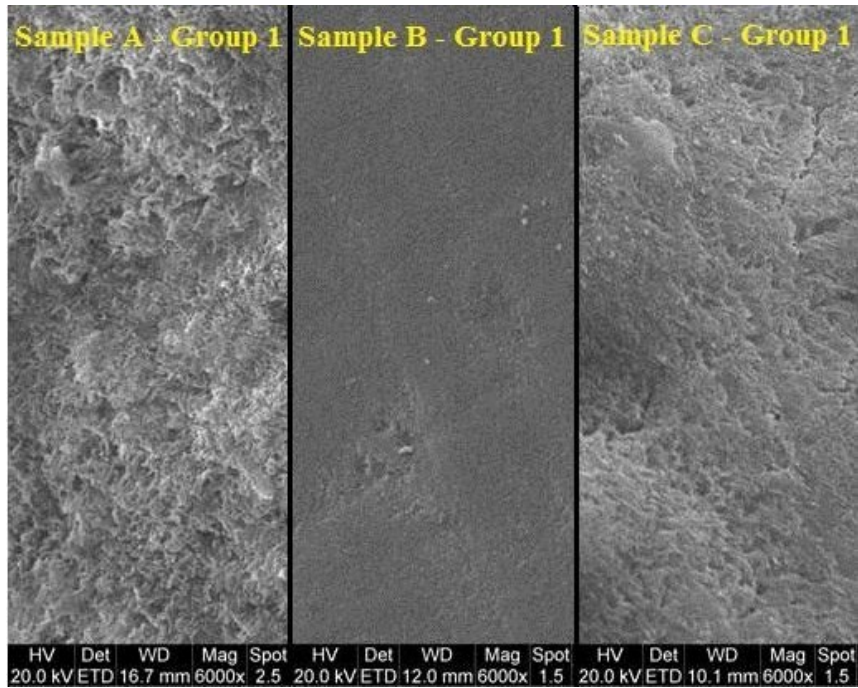Table 2: summary chart of pH analysis results.

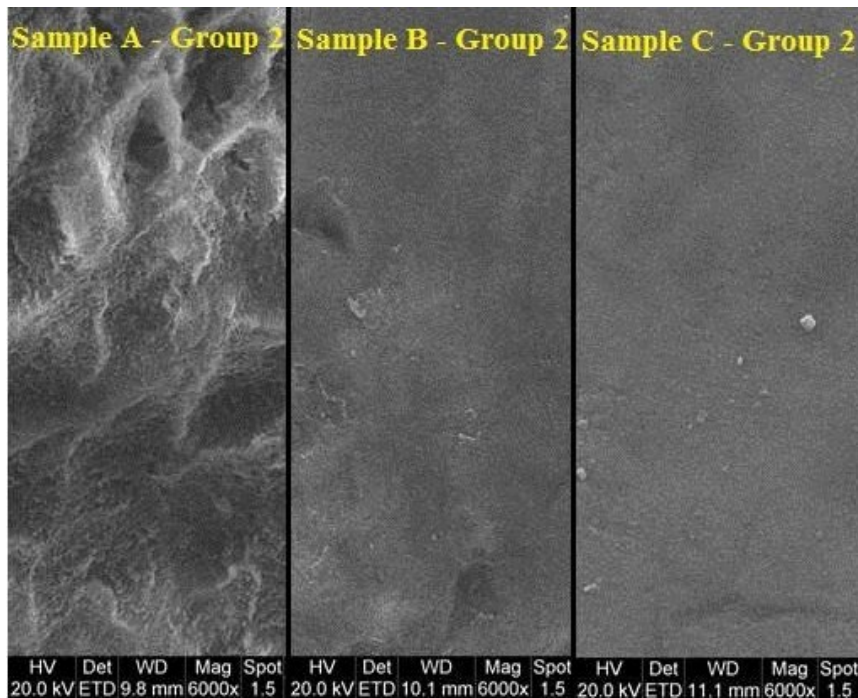Fig. 1: Group 1 samples images; 6000x.
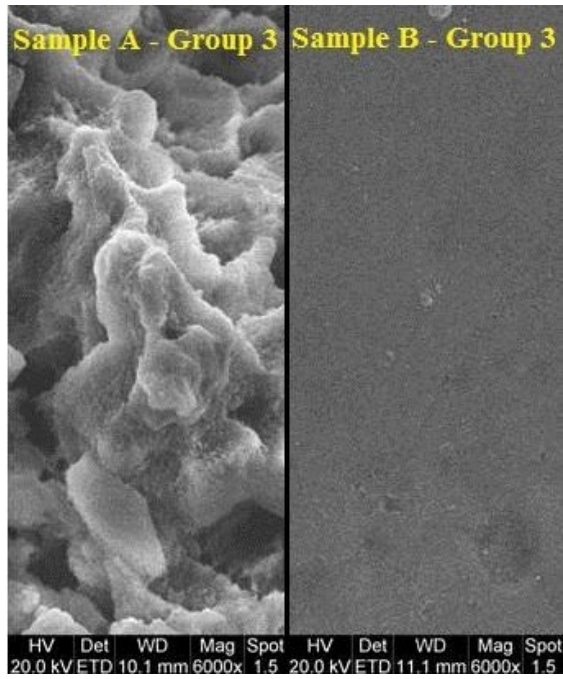

Fig. 2: Group 2 samples images; 6000x.

Fig. 3: Group 3 samples images; 6000x.

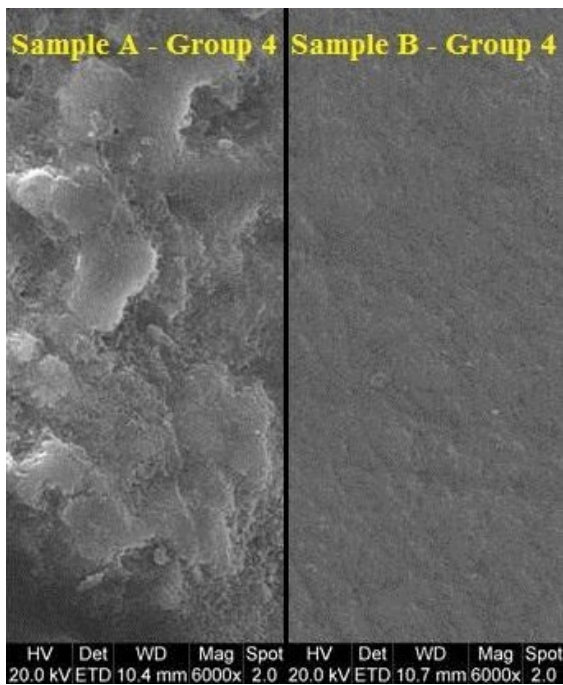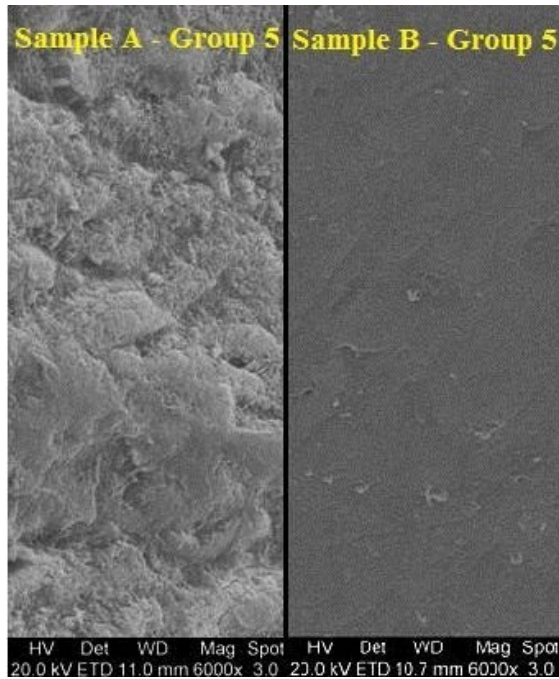Fig. 4: Group 4 samples images; 6000x.

Fig. 5: Group 5 samples images; 6000x.

II.1.5 Discussion and conclusions

The SEM images evaluation of each group showed interesting results.

In Group 1 (48 h), images of samples A show wide areas of enamel dissolution with loss of the macro-morphological mineral structure (Fig. 1), clearly different to the control treatment samples, that showed a smooth surface (Fig.1). Images of samples B showed few areas of little enamel demineralization due to the high immersion time of the samples into acidic solutions, characterised by irregular surface defects (Fig. 1).

In Group 2 (24h) it is possible to evaluate the high degree of enamel dissolution of samples A, with a characteristic etched-like aspect (Fig. 2); moreover, images of samples B showed a smooth, undamaged surface very similar to that in images of control (C) samples (Fig. 2). Group 3 (12 h) images confirmed that samples A are

characterised by wide areas of enamel dissolution, with a marked etched-like aspect (Fig. 3). These features are very different compared to the samples of samples B (Fig. 3), where homogeneous and undamaged enamel surfaces can be seen.

Moreover, the evaluation of SEM images of groups 4 (6 h) and 5 (3 h) showed that samples A and B had the same evidences of other groups, except that, reducing the immersion time, the enamel lesions on samples A are quite reduced (Fig. 4, 5); however, these images showed moderate surface dissolution, that cannot be seen in the images of samples B (Fig. 4, 5).

These considerations suggest that CPP have a positive effect in protecting and remineralising lesions caused by erosive process in vitro. ANOVA analysis of pH values produced interesting results. All pH values at start (t0) and end (tf) of each cycle in cola drink solutions (A), for each group, are in the acidic range lower than the pH threshold able to dissolute the hydroxyapatite crystals of enamel. Therefore these values explain the evident dissolution of enamel surfaces showed in the SEM images. Moreover, differences between pH values at start and end of each cycle in cola drink solutions show significant differences probably due to progressive loss of carbonates from the solution during immersion time (Group 1: $F = 28.350$ - $p < 0.001$; Group 2: $F = 41.160$ - $p < 0,001$; Group 3: $F = 41.458$ - $p < 0,001$; Group 4: $F = 5.147$ - $p = 0.031$; Group 5: $F = 5.968$ - $p = 0.021$) (F= distribution of Fisher-Snedecor).

The ANOVA analysis of pH values of solutions containing cola-type drink + CPP (B) showed that pH values, at start and end of each cycle, were always in the acidic values range, lower than the pH capable of dissolving enamel surfaces. pH values showed statistical significant differences at start and end of each cycle: this is probably related both to the mild basic action of CPPACP paste composition, and to the loss of carbonates from solutions during immersion time (Group 1: $F = 42.250 - p < 0.001$;

Group 2: F = 46.430 - p < 0.001 ; Group 3: F = 78.424 - p < 0.001 ; Group 4: F = 6.395 - p = 0.017 ; Group 5: F = 6.669 - p = 0.015).

It is interesting to note that, though pH values were always acidic, the SEM images of samples B showed few areas of surface defects, probably because these protective biomolecules in acidic conditions release calcium and phosphate ions in solution, protecting against enamel erosion. Therefore, it can be stated that the protective action of CPP-ACP complexes against erosion is actually due to the enamel remineralization process in solution and, at a minimal rate, to the CPP buffering capacity in erosive solutions.

This in vitro experimental protocol shows the protective effect of biomolecules, like synthetic casein phosphopeptide-stabilised amorphous calcium phosphate, against cola-type drink dental erosion. In particular, CPP-ACP complexes in vitro are able to reduce enamel dissolution and promote enamel remineralization during erosive process caused by cola-type drinks. Future researches should then be focused on in vivo trials to investigate the addition of remineralizing biomolecules to acidic drinks, in order reduce their erosive effects.

II.2 In vitro and in vivo effects of Plantago lanceolata tea extracts on oral bacteria

II.2.1 Abstract

Introduction: Plant extracts may be suitable alternative treatments for caries. Aims: to investigate the *in vitro* and *in vivo* antimicrobial effects of *Plantago lanceolata* herbal tea (from flowers and leaves) on cariogenic bacteria; to identify the major constituents of *P. lanceolata* plant.

Materials and Methods: The MIC and MBC against cariogenic bacteria were determined for *P. lanceolata* tea. Subsequently, a controlled random clinical study was conducted. Group A was instructed to rinse with a *P. lanceolata* mouth rinse, Group B received a placebo mouth rinse for seven days. The salivary colonisation of streptococci and lactobacilli was investigated prior to treatment and on the fourth and seventh days. Finally, the *P. lanceolata* tea was analysed for its polyphenolic content, and major phenolics were identified.

Results: *P. lanceolata* teas demonstrate good *in vitro* antimicrobial activity. The *in vivo* test showed that Group A subjects presented a significant decrease in streptococci compared to Group B. The phytochemical analysis revealed that flavonoids, coumarins, lipids, cinnamic acids, lignans, and phenolic compounds are present in *P. lanceolata* infusions.

Discussion and conclusions: *P. lanceolata* extract could represent a natural anti-cariogenic agent *via* an antimicrobial effect and might be useful as an ancillary measure to control the proliferation of cariogenic flora.

II.2.2 Aim

As above mentioned, alternative antimicrobial treatments, such as antibacterial compounds extracts from plants, reducing cariogenic microflora salivary counts, can be proposed to controlling this disease [Palombo, 2011; Ferrazzano et al., 2011; Ferrazzano et al. 2011; Ferrazzano et al., 2013].

In this study, an *in vitro* assessment of the antimicrobial activity of *P. lanceolata* tea against cariogenic bacterial strains of the species *Streptococcus* and *Lactobacillus* isolated from clinical samples was performed; subsequently, a mouth rinse made with an infusion of dried *P. lanceolata* leaves and flowers was tested *in vivo* for its effectiveness in reducing cariogenic microflora salivary counts. Finally, the *P. lanceolata* herbal tea was analysed for its polyphenolic content, and major phenolics were identified.

II.2.3 Materials and Methods

Chemicals and reagents

Phenolic compound standards were purchased from Sigma Aldrich (Milan, Italy). All the other chemicals and solvents were purchased from Fluka (Saint-Quentin Fallavier, France) at analytical or HPLC grade and were used as received.

Plant material and extract preparation

P. lanceolata was collected in June 2012 in the Royal Park of Capodimonte, Naples, Italy. The voucher specimen (No. 0134) was prepared and identified in Botany laboratories of Department of Plant Biology and deposited at the Herbarium of the Botanical Garden of the "Federico II" University of Naples, Italy.

Flowers, leaves and roots of *P. lanceolata* were dried separately at 55°C for 48 h in a Carlo ErbaE.28 CL.1 Oven and then ground to a coarse powder. A total of 2 g of powder of each part of the plant was suspended in 20 mL of *Amorosa*™ water (still mineral water, low in sodium and ideal for the preparation of baby and toddler food) at 100°C for 3 min. The preparation was infused for 8 min and then was cooled to room temperature, filtered and evaporated to complete dryness.

To prepare the mouth rinse, 200 g of pulverised material (flowers and leaves) were suspended in 2.0 L of *Amorosa* water following the same procedure previously described. The preparation was filtered, placed in a sterilised 3.0 L glass container, and stored at 4°C before use.

In vitro *antimicrobial tests*

The bacterial strains used for testing were *L. casei, S. bovis, S. mutans, S. mitis, S. parasanguinis, S. viridans* and *S. sobrinus.* The strains were from clinical specimens obtained at the Diagnostic Unit of Bacteriology and Microbiology of "Federico II" University of Naples. Bacteria were grown on Trypticase Soy Agar II with 5% Sheep Blood (TSS; Becton Dickinson, USA) plates at 37°C in 5% CO_2 for 48 h.

Antimicrobial Tests. The minimum inhibitory concentration (MIC) was measured using the standard microdilution method in 96-well polystyrene plates using Brain-Heart Infusion (BHI) medium. The starting inoculum was 5×10^5 CFU mL^{-1}, and the final

concentrations of the *P. lanceolata* infusion ranged from 4 to 0.025 mg mL^{-1}. To determine the minimal bactericidal concentration (MBC), 50 μL of bacterial suspension from the wells containing extract concentrations equal to or higher than the MIC were inoculated in 5 mL of sterile BHI medium and incubated for 24 h at 37°C under a 5% CO_2 atmosphere.

Biologic assays on human-lymphocytic cells

 We have chosen two human cell lines one p53 negative and one p53 positive.

The p53-null U937 human leukemic monocyte lymphoma and the p53-positive isogenic human lymphoblast TK6 cell lines were obtained from American Type Culture Collection (Rockville, MD). Both cells lines grew in Dulbecco's Modified Eagle Medium, 2 mM l-glutamine, 100 μg/mL streptomycin, 100 units/mL penicillin, and 10% Foetal Calf Serum (FCS). All media and cell culture reagents were purchased from Life Technologies (San Giuliano Milanese, Italy).

 Cell viability was assayed by using the Cell Proliferation Kit II (XTT, Roche, Milan Italy). This assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolic active cells; therefore, this conversion only occurs in viable cells.

The plant extracts, were provided to our laboratory in the relative extraction solvents having all a nominal concentration of 50 mg/ml. The extracts were dried under vacuum and re-dissolved in DMSO 10% in water. Samples were stored at -30°C until use. Before measurements, samples were brought to room temperature under agitation and added to culture media in a ratio 1 to 10. In details, 90 μl of suspensions of U937 or TK6 cells (containing ~1x104 cells in complete medium) were seeded into 96-well plates. Then, 10 μl of each extract (50 mg/ml in 10% DMSO) were added to each well

so that the final extract concentration was 5 mg/ml, while the DMSO content was reduced to 1%. Cells were incubated in these conditions at 37°C for 24 hours in 5% CO_2 atmosphere. Triplicate samples were prepared for any individual condition. As a positive control for cyto-toxicity we used Triclosan at low concentration (0.03% as compared with 3% used in toothpastes). This synthetic is a polychloro-phenoxy phenolan endowed with antibacterial and antifungal properties. For these reasons it is currently largely used in oral hygiene as additive of toothpastes to prevent gingivitis.

In vivo *antimicrobial tests*

The clinical study was conducted on a sample consisting of forty-four adolescents (24 males and 20 females) enrolled in the Department of Paediatric Dentistry at the "Federico II" University of Naples, Italy, ranging 12 to 18 years old.

The inclusion criteria were good general health (ASA I: Healthy person; ASA II: Mild systemic disease) and an agreement to comply with study procedures.

Subjects who had used antibiotics or mouth-rinses during the 14 days prior to the beginning of the study were excluded.

Only 2 subjects of the 44 were excluded for this reason. However, during the research period, two subjects chose to end their participation; thus, 40 subjects completed the entire protocol. Participation in the study was voluntary. All of the parents gave written informed consent after receiving verbal and written explanations of the experimental protocol and study aims. The protocol was approved by the Ethical Committee of the School of Dentistry, "Federico II" University of Naples, Italy.

A controlled random clinical study was conducted. The subjects were divided into 2 groups of twenty subjects, respectively (Group A and Group B). Patients were randomly

assigned to test and control groups using blocked randomisation from a computer-generated list.

Salivary counts of *S. mutans* and *L. casei* were estimated using a chair-side test that contained two agar surfaces. The blue mitis-salivarius-agar with bacitracin was used to detect the presence of streptococci, whereas the light culture medium, Rogosa agar, was used to evaluate that of lactobacilli.

No special dietary restrictions were imposed on the subjects, and no tooth brushing was allowed for at least 1 h after consuming lunch and dinner. All of the subjects were encouraged to maintain their normal oral hygiene habits. All subjects were given the same brush and tooth-paste for daily oral hygiene.

Saliva was inoculated on a dip-slide with selective media for streptococci and lactobacilli. After adding a $NaHCO_3$ tablet to the tube, the dip-slides were immediately cultivated at 37°C for 48 h. The tablet releases CO_2 on contact with moisture, creating favourable conditions for bacterial growth.

The colonies were identified using a stereomicroscope with ×10 magnification, and the culture density (CFU/mL) was visually compared with the aid of a chart provided by the manufacturer. Bacterial colonies were categorised as low ($<10^5$ CFU/mL of saliva) or high ($>$ or $= 10^5$ CFU/mL).

Two different mouth rinse formulations were prepared:

1) Experimental (Group A)

An experimental mouth rinse was prepared with an infusion of *P. lanceolata* leaves and flowers, as previously described.

2) Placebo (Group B)

The placebo mouth rinse was prepared with *Amorosa*™ water, coloured with food dye.

Experimental design

During the experimental period, after collecting the first saliva sample (T0), all of the Group A participants were asked to rinse with 10 mL of the experimental mouth rinse; instead, the participants of Group B were instructed to rinse with 10 mL of a placebo mouth rinse that did not contain phenolic substances, for 60 seconds after performing oral hygiene, 3 times a day (after breakfast, after lunch and before sleeping) for 7 days. On the 4th (T1) and 7th (T2) days of treatment, additional salivary samples were collected and immediately incubated, to calculate the density (CFU/mL) of *S. mutans* and *L. casei* for each subject.

Statistical analysis

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

Analysis of phenolics

Infusions of *P. lanceolata* whole plants and separated parts (flowers, leaves and roots) were subjected to different chemical tests to detect the presence of flavonoids [Chang et al., 2002], coumarins [Khan et al., 2011], lipids and cinnamic acids [Khoddami et al., 2013], and tannins [Sembratowicz et al., 2006].

Column chromatography (CC) was performed on a Merck Kieselgel 60 (230–400 mesh). HPLC was performed on a Shimadzu LC-10AD using a Shimadzu RID-10A UV–VIS detector. A semi-preparative HPLC was performed using an RP18

(LiChrospher 10 μm, 250 × 10 mm i.d., Merck) column with a flow rate of 1.2 mL min^{-1}, using an injection volume of 200 □L and a binary mixture of water/formic acid 0.1% (99:1) and methanol (solvents A and B, respectively). The gradient was as follows: 0 min, 70% A/30% B, reaching 35% A/65% B after 12 min, then reaching 100% B after 25 min, which was maintained for 12 min. A 10 min equilibration time was used between analyses.

^{1}H- and ^{13}C-NMR spectra were recorded on a Varian INOVA-500 NMR instrument (^{1}H at 499.6 MHz and ^{13}C at 125.62 MHz), referenced with deuterated solvents (CDCl$_3$ or CD$_3$OD) at 25°C. Proton-detected heteronuclear correlations were measured using a gradient heteronuclear single-quantum coherence (HSQC), optimised for $^1J_{HC}$ = 155 Hz, and a gradient heteronuclear multiple bond coherence (HMBC), optimised for $^nJ_{HC}$ = 8 Hz.

MS spectra were obtained using a HP 6890 spectrometer equipped with an MS 5973 N detector. HR-ESI-MS/MS was performed using a Q-TRAP model API-2000 LC-MS/MS system equipped with a heated nebuliser source and using the Analyst software (Applied Biosystems).

Determination of total phenolic content

Total phenolic content was determined spectrophotometrically according to the Folin–Ciocalteu's method with slight modifications [22]. Briefly, 1.0 mL of properly diluted samples, calibration solutions or blank solution were pipetted into separate test tubes (20 mL) and 1.0 mL of Folin-Ciocalteu's reagent was added to each. The mixture was mixed well and allowed to equilibrate. After exactly 10 min, 10 mL of milliQ water and 3.0 mL of Na$_2$CO$_3$ solution (w= 5%) were added and the volume was made up with milliQ water. The mixture was swirled and put in a water bath at 40°C for 20 min.

Then, the tubes were rapidly cooled on ice, and the colour generated was read at its maximum absorption. The measurements were compared to a standard curve of prepared gallic acid solutions (50, 100, 150, 250, 500 mg L^{-1}) and expressed as milligrams of gallic acid equivalents. All measurements were performed in triplicate, the absorbance was measured in 1-cm cuvettes, and for calibration solutions and blank preparation, a methanolic solution at the same concentration as the samples was used.

II.2.4 Results

Determination of P. lanceolata infusion phenolic content and identification of major constituents

A preliminary analysis was performed to identify the major constituents of the whole *P. lanceolata* plant. Flavonoids, coumarins, lipids, cinnamic acids, lignans, and phenolic compounds are present in infusions of *P. lanceolata* (Table 1).

Table 1

Phytochemical	Average amount (µg/g of DW)
Flavonoids	358 ± 20
Coumarins	9 ± 0.5
Lipids	1120
Cinnamic acid content	200
Lignans	-
Phenolic content	1368 ± 54

Table 1: Average amount of the main classes of compounds in the infusion of the whole *Plantago lanceolata* plant.

The hot water infusion of the entire *P. lanceolata* (flowers, leaves and roots) contains different classes of phenolics, including flavonoids, coumarins, lipids, cinnamic acids at a range of concentrations (Table 2).

Table 2

Phytochemical	Flowers	Leaves	Roots
Flavonoids	+++	++	-
Coumarins	+	+	+
Lipids	-	-	+
Cinnamic acid content	++	+++	+
Lignans	-	-	-
Phenolic content	++	+++	+

Table 2: Results of the phytochemical analysis of the infusion of flowers, leaves and roots of *Plantago lanceolata*.

Key: +++: High concentration, ++: Medium concentration, +: Low concentration, -: Absent

In the lyophilised infusion, the level of phenolic compounds was 40.5 ± 1.3 mg GA equiv/g DW, as revealed by HPLC analysis. The level of total phenolics, as detected by Folin–Ciocalteu's method, was higher (89.0 ± 2.5 mg GA equiv/g DW); the difference can be explained by the presence of protein, ascorbic acid or reducing sugars in *P. lanceolata*, which contribute to higher Folin–Ciocalteu values. An LC/MS/MS technique was applied to the hot infusion prepared from *P. lanceolata* leaves (yield of 5.4% of the air-dried raw material mass), leading to the isolation of several polyphenols, including flavonoids, of which the most abundant were apigenin (**14**), luteolin (**15**), and luteolin-7-*O*-glucoside (**16**). Quercetin-3-rutinoside (rutin, **2**), quercetin-3-*O*-D-galactopyranoside (hyperoside, **3**), 3,5,7,4-tetrahydroxyflavonol (kaempferol, **4**), 3,5,7,3,4-pentahydroxyflavone (quercetin, **5**), and 3,5,3,4-tetrahydroxy-7-methoxyflavonol (rhamnetin, **6**) were also present, while naringenin (**17**) was present

only in trace amounts, in agreement with the data that Beara et al. (2012) reported for methanol extracts (fig.1). Other phenolic compounds with lower molecular weights were identified as vanillic (**10**) and gallic (**1**) acids, and three derivatives of the latter: 3-*O*-galloyl-4,6-hexahydroxydiphenoyl-D-glucose (**7**), 2,3-di-*O*-galloyl-D-glucose (**8**), and 1,2,3-tri-*O*-galloyl-D-glucose (**9**), which was the most abundant [Makhmudov et al., 2011]. Furthermore, a significant amount of cinnamic acids including cinnamic (**11**), caffeic (**12**) [Beara et al., 2012] and chlorogenic (**13**) acids was measured (fig. 2).
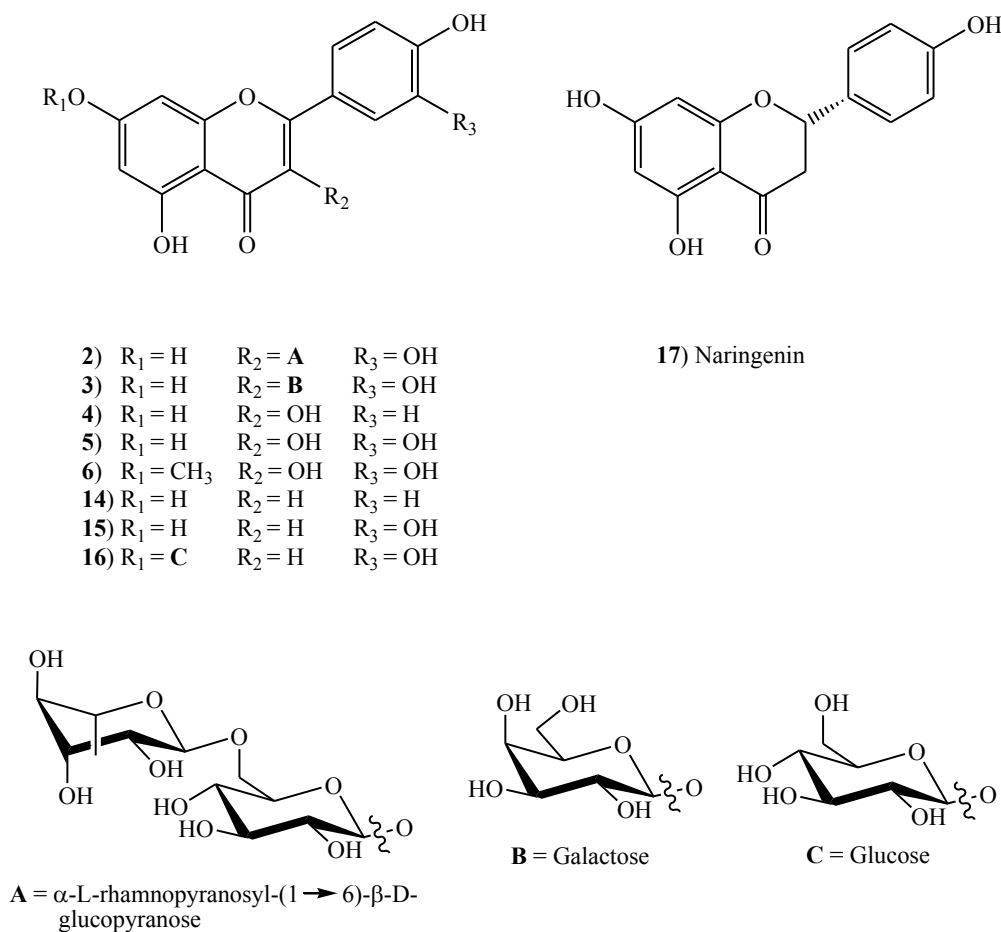
Figure 1

2) $R_1 = H$	$R_2 = A$	$R_3 = OH$
3) $R_1 = H$	$R_2 = B$	$R_3 = OH$
4) $R_1 = H$	$R_2 = OH$	$R_3 = H$
5) $R_1 = H$	$R_2 = OH$	$R_3 = OH$
6) $R_1 = CH_3$	$R_2 = OH$	$R_3 = OH$
14) $R_1 = H$	$R_2 = H$	$R_3 = H$
15) $R_1 = H$	$R_2 = H$	$R_3 = OH$
16) $R_1 = C$	$R_2 = H$	$R_3 = OH$

17) Naringenin

A = α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranose

B = Galactose

C = Glucose

Figure 1: general structures of flavonoids isolated from *P. lanceolata*.

Figure 2

1) $R_1 = OH$ $R_2 = H$
10) $R_1 = H$ $R_2 = OCH_3$

11) $R_1 = H$ $R_2 = H$ $R_3 = H$
12) $R_1 = H$ $R_2 = OH$ $R_3 = OH$
13) $R_1 = D$ $R_2 = OH$ $R_3 = OH$

D

7) $R_1 = E$

E

8) $R_1 = H$ $R_2 = E$ $R_3 = E$
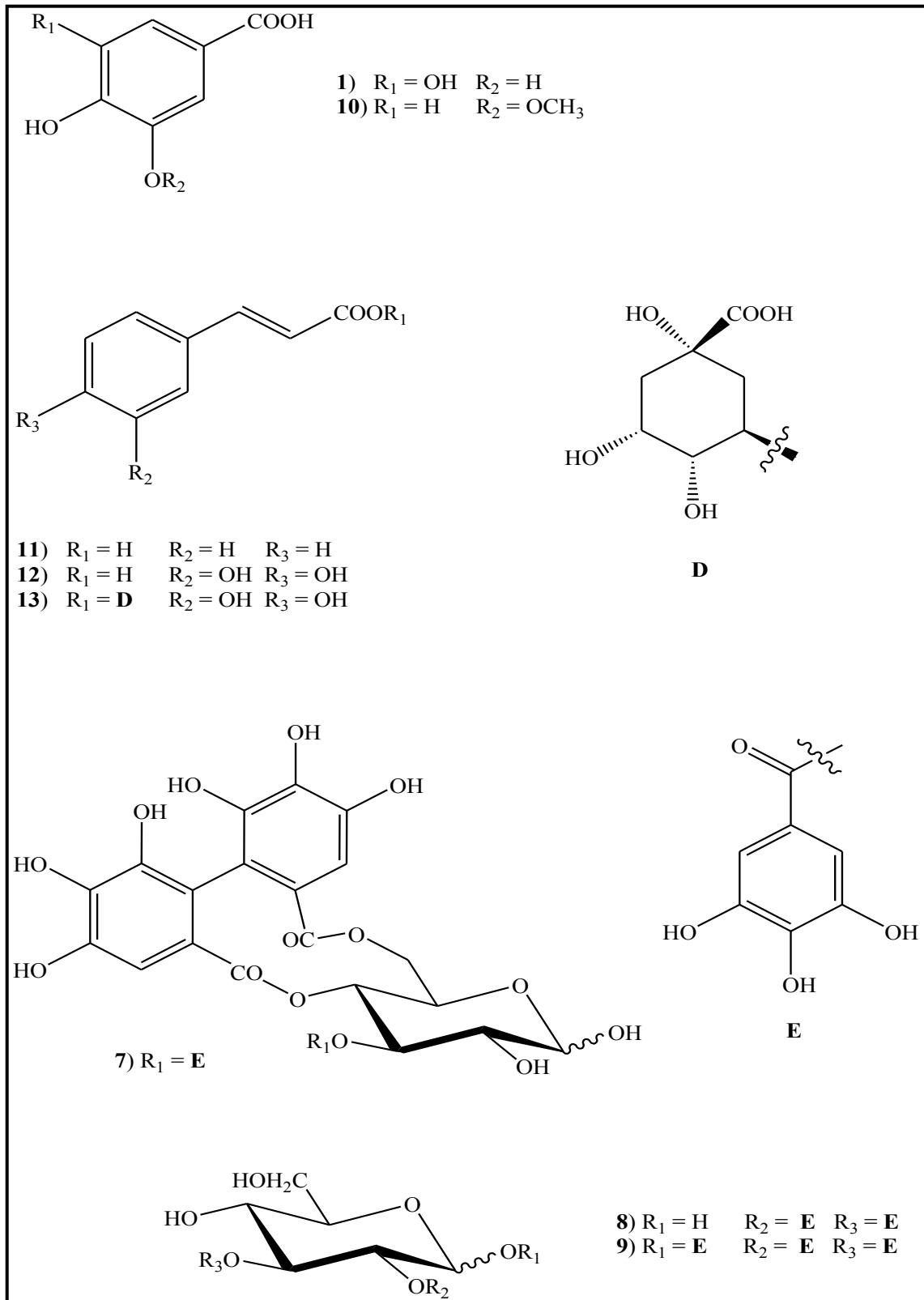**9)** $R_1 = E$ $R_2 = E$ $R_3 = E$

Figure 2: cinnamic derivatives of *P. lanceolata*.

In vitro *antimicrobial tests*

An infusion of dried flowers and leaves was used to assess the *in vitro* antimicrobial activity of *P. lanceolata*, expressed in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (Table 3). Strains belonging to several species of *Streptococcus*, namely *S. bovis*, *S. mitis*, *S. sobrinus*, *S. mutans*, *S. parasanguinis* and *S. viridans* and one strain of *L. casei*, that were isolated from clinical specimens were selected for the bioassay. The infused *P. lanceolata* was assayed at concentrations ranging from 4 to 0.025 mg/mL. The susceptibility of the strains to the tea varied considerably; however, concentrations between 250 μg/mL and 2.0 mg/mL showed antimicrobial activity, affecting the viability of all bacteria tested. As far as concerns the effects on human cell lines of P. lanceolata infusion, we have found that the viability of p53 and U 937 cell lines treated at concentration between 125 μg/mL and 4 mg/mL does not decrease in comparison with the respective controls. This indicates the absence of cytotoxicity at concentrations higher than that used for clinical tests.

Table 3

Species	MIC90 (mg/ml)	MBC (mg/ml)
Streptococcus bovis	0,50	1
Streptococcus mitis	0,25	0,50
Streptococcus mutans	0,50	1
Streptococcus sobrinus	0,50	1
Streptococcus parasanguinis	1	2
Streptococcus viridans	0,50	0,50
Lactobacillus casei	0,75	2

Table 3: *in vitro* antimicrobial activity of *Plantago lanceolata*, expressed in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

In vivo *assessment of antimicrobial activity of* P. lanceolata *infusions*

For the test group the differences in streptococci concentration (CFU/mL) between T0 and T1 and between T0 and T2 were significant ($p<0.001$ for both); the difference between T1 and T2 was not significant. The differences in lactobacilli concentration (CFU/mL) were not significant between any two time points.

For the control group the differences in the density (CFU/mL) of streptococci and lactobacilli were not significant.

The difference in the streptococci density between test and control groups was not significant at T0 but was significant at T1 and T2, whereas the differences in the lactobacilli concentration were not significant (Table 4).

Table 4

	Streptococci		Lactobacilli	
	Test	Control	Test	Control
T0	85,7%	85,7%	85%	90%
T1	35,7%	78,6%	60%	80%
T2	28,6%	85,7%	65%	75%

Table 4: density of the *Streptococci mutans* CFU (> or = 10^5 CFU/mL) and *Lactobacilli casei* CFU (> or = 10^5 CFU/mL) at T0, T1, T2.

II.2.5 Discussion and conclusions

P. lanceolata infusions demonstrated good *in vitro* antimicrobial activity, particularly against streptococci. The biocide potential of some species of the *Plantago* genus is well known: *P. major* extracts, for example, caused strong alterations in the cell wall

structure of different gram positive bacteria [Sharifa et al., 2008]. Also *P. lanceolata* extracts in different solvents exhibited mild antimicrobial activity, most likely due to the presence of flavonoids and terpenes [Nostro et al., 2000]. Interestingly, the water extracts of *P. lanceolata* produced the best anti-microbial effects, exhibiting weak or moderate antimicrobial activity against *S. aureus*, *S. epidermidis*, *S. marcescens* and *Proteus vulgaris* [Pehlivan Karakaş et al., 2012]. Moreover, Bazzaz et al. (2003) reported that the pressed juice of fresh *P. lanceolata* has a bactericidal effect.

The *in vivo* test demonstrated that subjects who used a *P. lanceolata* mouth rinse presented a significant decrease in streptococci compared to subjects treated with the placebo, whereas only a slight (not significant) decrease was observed for lactobacilli counts following the treatment.

Apart from the strain-specific differences, a possible explanation for the poor reduction of lactobacilli with respect to streptococci is that the latter normally grow on exposed surfaces that are easily accessible, whereas lactobacilli and related species recover in shed retentive areas [Cildir et al., 2009], that have limited contact with the mouth rinse.

Furthermore, in the present study, only the short-term (seven days) effect of Plantago Lanceolata against oral streptococci and lactobacilli was assessed in vivo. The promising results shown suggest that further studies need to be undertaken to establish whether the capability to reduce mutans streptococci salivary counts will be long lasting, and whether no resistance will occur. In addition, the long-term patient acceptability and compliance needs to be assessed.

The phenolic content and the major phenolic compounds of the whole plant infusion, as well as that of isolated leaves, flowers and roots of *P. lanceolata*, was determined. The results of analysis showed that all extracts have a high phenolic content. Nine flavonoids, three cinnamic acids, two compounds with a C6-C1 skeleton, and three of

their glycosylated derivatives were identified. In particular, the leaf extract is the richest in polyphenols, followed by that of the flowers and, to a lesser extent, the roots. A significant difference in the flavonoid content was also detected, the highest flavonoid content occurring in the flower extract, followed by the leaves, whereas none was detected in the roots. In accordance with previous studies on *P. lanceolata* phenolic constituents [Beara et al., 2012], our analyses also detected the presence of cinnamic acids in all extracts, but most prevalently in the leaves. The content of coumarins was lower than the other phenolics, however, and constant in the three types of extracts. In contrast, the lipid content was markedly low and only present in the root extract. Finally, no lignans were found in any of the extracts that were analysed. The anti-streptococcal activity of *P. lanceolata* infusions could be related to the phenolic substances occurring in the infusion and is most likely the result of the synergistic activity of the many phenolics that were isolated. Chlorogenic and caffeic acids inhibit the growth of *S. mutans* [Almeida et al., 2006], and the presence of significant concentrations of apigenin, an effective inhibitor of GTFs from *S. mutans* and *S. sanguinis*, is also noteworthy. The adherence of bacterial cells to the tooth surface is important for the development of carious lesions, and interference with the mechanisms of adherence can prevent dental caries. Furthermore, as suggested by Özan, the phenolic substances found in plant extracts most likely act on the microbial membrane or the surface of the cell wall, causing structural and functional damage [Özan et al., 2007]. However, further research is needed to identify which pure compound could be responsible for the antibacterial properties and to determine its potential for use in pharmaceutical industries.

P. lanceolata mouth rinse might offer a new option for the microbiological control of dental caries because the infusion is easily obtained, inexpensive and exhibits beneficial

effects. There is an urgent need for alternative prevention and treatment options that are safe, effective and economical, given the incidence of oral disease and the adverse effects (as tooth and tongue discoloration and taste disorder) of certain antibacterial agents that are currently used in dentistry.

Based on these considerations, a *P. lanceolata* infusion might be useful as an ancillary measure to control the proliferation of oral cariogenic flora. Further studies, particularly *in vivo* and *in situ* studies, are needed to establish conclusive evidence of the effectiveness of *P. lanceolata* extract against dental caries, either alone or in combination with conventional therapies, for improving oral health.

II.3 Release kinetics of bioactive molecules included in a novel chewing gum

II.3.1 Abstract

There is a growing interest in the use of anticariogenic and antierosive agents to prevent demineralization and promote remineralization of dental enamel for the prevention of oral diseases. The purpose of this study was to develop a novel chewing-gum containing Casein-Phosphopeptides-Amorphous Calcium Phosphate complexes (CPP-ACP) and Quercetin (Qt) and evaluate their release using *in vitro* and *in vivo* experiments.

In vitro and *in vivo* experimental protocols were designed to test the percentages of Ca ions from CPP-ACP and Qt released, with time and delivery rate from this specific chewing gum. The *in vitro* experiments were performed using a specifically designed chewing apparatus to test the release of Ca and Qt in artificial saliva in function of chewing time; while the *in vivo* experiments required a two sessions clinical trial, performed by 10 volunteers in order to analyze the in vivo performance of the experimental chewing-gums: the residual CPP-ACP and Qt amounts presents in chewed gums and in volunteers saliva were analyzed.

This study demonstrated that both Calcium derived from CPP-ACP and Qt were released during chewing, although in vitro Calcium was released from the chewing gum in a slower and more controlled mechanism than Qt. They were also both effectively released in the oral cavity during the in vivo chewing time, showing an effective anticaries and antierosive concentration in volunteers saliva.

This research concludes that CPP-ACP and Quercetin included in experimental chewing gums could be efficiently released in vitro and in vivo during the mastication process in order to contrast carious and erosive diseases.

II.3.2 Aim

In biomedical and pharmaceutical industry, there is a growing interest in products for "drug delivery" as substitute functions (artificial plasma and tissue) and as matrix for slow release of active substances. Chewing gum can be considered a suitable drug delivery system for dental prevention. In fact chewing gums are solid, single-dose preparations that can contain one or more active ingredients that are released by chewing [European Pharmacopoeia, 2002].

In our study, experimental chewing gums were realized with two type of active principles, Casein Phosphopeptide-amorphous calcium phosphate (CPP-ACP) complex of dairy origin, and Quercetin (Qt), both known to have an anti-caries activity and the first complex also anti-erosive activity.

Gum formulations with different amounts of active principles were prepared with the aim to:

- provide chewing gums with good organoleptic and technological properties (pleasant mouth-feeling, chewing volume, anti-adhesive properties to teeth).

- develop an experimental protocol designed to verify the chewing gum's ability to release the drug dose and to assess its delivery rate.

- In order to simulate human chewing behavior, a specific self-made apparatus was employed and the released Calcium and Quercetin concentration were monitored at different chewing time, with the described apparatus.

- A two sessions clinical trial was performed with volunteers in order to analyze the in vivo performance.

II.3.3 Materials and methods

Chewing gums production, biochemical and microbiological analysis were made at Institute of Food Science of CNR (Consiglio Nazionale delle Ricerche), Avellino, Italy.

Determination of Quercetin content

A preliminary study was assessed to analyze the antimicrobial activity of Quercetin, using two different Qt concentrations, Qt1 (0.5 mg/ml) and Qt2 (1.0 mg/ml), that were dissolved in 20% dimethyl sulphoxide (DMSO, Sigma) for later experiments.

Test bacteria strain *S. mutans ATCC 25175* was kindly provided by the America Type Culture Collection (ATCC) Manassas, Virginia, USA. Strain was stored at -80 °C in tryptone yeast extract (TPY) containing 50% glycerol (v/v). After resuscitation on TPY agar for 48 h, typical colonies were re-inoculated in fresh TPY broth and incubated at 37°C under anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2) for 18 h. The concentration of bacteria in each suspension was adjusted to approximately 1×10^8 CFU/ml.

MIC and MBC assay - Antimicrobial activity of Quercetin:

Minimum Inhibitory Concentration (MICs) of Qt against *S. mutans* was determined by micro-dilution technique using the National Committee for Clinical and Laboratory Standards (NCCLS) method [National Committee for Clinical Laboratory Standards, 1997]. A series of 2-fold dilutions of Qt was prepared in a volume of 100µl per well in 96-well micro-liter plates. TPY broth without antibacterial agent was used as negative

control, TPY broth with 20% DMSO as solvent control and clorexidine (0.05%) as positive control.

After addition of 100 µl of bacterial cell suspension (1x108 CFU/ml) into each well, the plate was vibrated slightly for 1 min. Bacteria were then incubated at 37°C in an anaerobic atmosphere (80% N_2, 10% CO_2 and 10% H_2) for 48 h. The growth of bacteria in each well was then determined and MIC was defined as the lowest concentration at which no visible growth (limpid in the well) against a black background was observed. After determining the MIC, bacteria from wells that exhibited no growth were sub-cultured onto TPY agar and incubated at 37°C for 48 h in an anaerobic atmosphere (80% N_2, 10%CO_2 and 10% H_2). Minimum Bactericidal Concentration (MBC) was defined as the lowest concentration at which no microorganism growth on the agar was detected. All assays were performed in triplicate and repeated at least three times.

The concentration of Qt necessary to inhibit and to kill *S. mutans* ranged from 5.0 to 10.0 mg/ml. On the basis of these results, 5.0 (Qt1) and 10.0 mg (Qt2) of Qt were used per 1 g of gum.

Determination of CPP-ACP content

A number of studies have demonstrated CPP-ACP to have anticariogenic and antierosive activity in laboratory, animal and human experiments [Reynolds et al.,1999; Shen et al., 2001; Ramalingam et al., 2005]. According to Walker *et al.* the addition of 2.0–5.0 g CPP-ACP ⁄ l to milk increases its ability to remineralize enamel subsurface lesions [Walker et al., 2006].

In our study tree different percentage of CPP-ACP were used 25, 50 and 100 mg per gum, as shown in Table 1.

Chewing gum formulation

The previously identified amounts of CPP-ACP and Quercetin were added to the gum-base. The different formulations were tested with the aim to provide chewing gums with good organoleptic and technological properties (Table 1).

The gum-base was obtained from Gum Base Company S.p.A. (Lainate, Italy); flavouring agents were supplied from Officina degli Aromi S.r.l. (Milano, Italy); sweeteners were obtained from Sigmar Italia (Almè, Italy). The excipients (magnesium stearate and talc) were obtained from Carlo Erba (Milan, Italy). Qt and CPP-ACP were supplied from KFO France Co. Ltd. All other chemicals were of analytical grade and used as obtained commercially.

The gum-base was a mixture of products containing different percentages of gum to balance the chewing gum hardness and texture. Gum-base, flavouring agents, sweeteners, CPP, Qt and co-adjuvant excipients were mixed in a laboratory mixer (HulaMixer™ Sample Mixer) suitable for mixing small batches of powders or dry granules. Sweeteners and flavours were used to obtain a final pleasant taste. Preferably high intensity and not cariogenic sweeteners were selected.

Co-adjuvant excipients (talc, magnesium stearate) were added to the mixture to optimize the compression process. At this point, chewing gums, with their weighed amounts of different mixtures, were produced by progressively filling the die of a single-punch tableting machine (Matrix 2.2 A, Ataena Srl, Ancona, Italy) and then compressed, at room temperature.

Table 1

	CPP5-Qt0.5	CPP5-Qt1.0	CPP10-Qt0.5	CPP10-Qt1.0	CPP20-Qt0.5	CPP20-Qt1.0
Gum-base	73.5%	73.0%	68.5%	68.0%	58.5%	58.0%
CPP	5.0%	5.0%	10.0%	10.0%	20.0%	20.0%
Quercetin	0.5%	1.0%	0.5%	1.0%	0.5%	1.0%
flavouring agents and sweeteners	20.0%	20.0%	20.0%	20.0%	20.0%	20.0%
Co-adjuvant excipients	1.0%	1.0%	1.0%	1.0%	1.0%	1.0%

Table 1. Chewing gum composition, expressed in percentage of the components is reported.

Preparation of artificial saliva

Artificial saliva was used in the *in vitro* release studies in an attempt to simulate actual conditions of use. The ingredients of the artificial saliva were as follows: sodium chloride, 0.844 g; potassium chloride, 1.200 g; calcium chloride dihydrate, 0.193 g; magnesium chloride hexahydrate, 0.111 g; potassium phosphate dibasic, 0.342 g; bidistilled water to make to 1000 mL. The solution was stirred until complete dissolution of the salts, and the pH was adjusted with hydrochloric acid solution to pH 5.7 [Cilurzo et al., 2003].

In vitro release study

In vitro release study of Calcium and Quercetin from chewing gums was carried out using a self-made *in vitro* chewing release apparatus (Fig. 1).
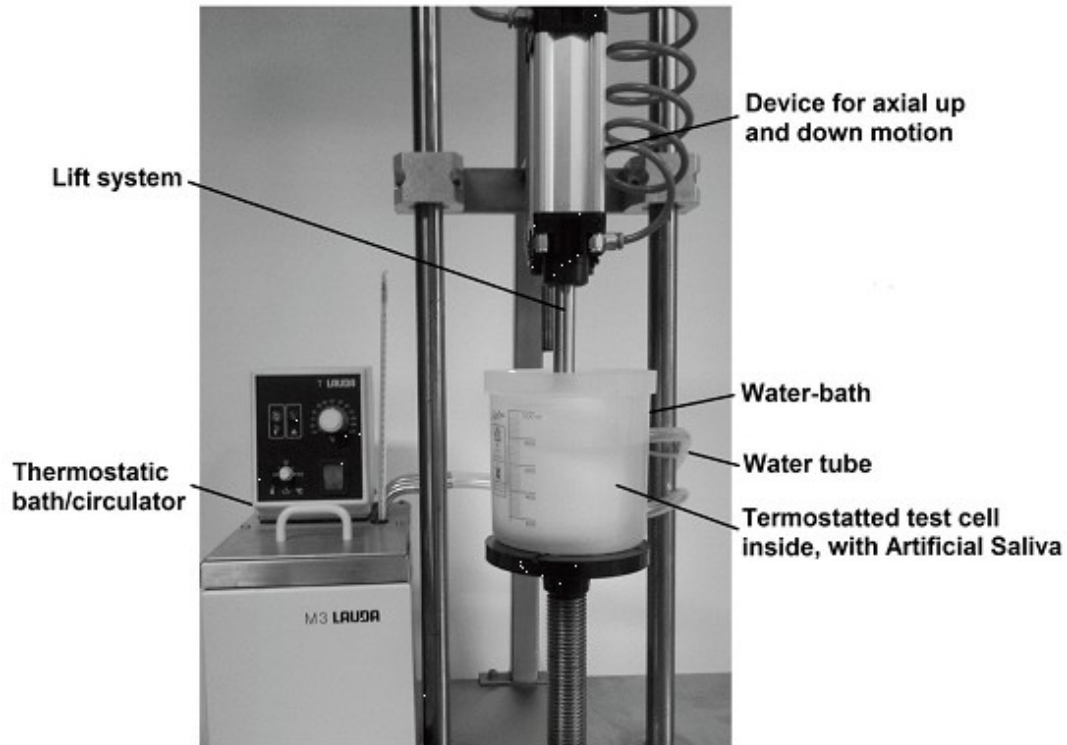
Fig. 1: Photographs of chewing apparatus and thermostatted test cell. The gum is placed between upper and lower surfaces.

This apparatus consisted of a thermostatted cell in which a vertically oriented piston holding an upper chewing plate was mounted; a second chewing plate was mounted on the lower cell surface. The cells were filled with 50 ml of artificial saliva and the chewing gum was loaded onto the lower chewing surface.

The chewing procedure consisted of up and down strokes of upper surface providing the mastication of the chewing gum. The average temperature of test was controlled at 37°C and the chew frequency was 50±2 strokes per min [Liljewall, 1992].

At predetermined time intervals, 500µl of supernatant were removed. Into the cell, it was replaced fresh artificial saliva after each sampling. The average release was calculated for the assessment of Calcium and Quercetin concentrations. The mixture was stored at +4°C until analysis.

In vivo release study

In vivo chewing was performed by a group of 10 young volunteered, five male and five female, of mean age 16.3 years. They were recruited among patients followed by Department od Paediatric Dentistry of Azienza Ospedaliera Universitaria Policlinico Federico II of Naples. All volunteers were healthy children without oral disease, no evidence of dry mouth or salivary gland disorders, no contemporary orthodontic treatment and with normal chewing ability.

The participants, also signed an informed consent to participate in the study, which was approved by the Ethical Committee of the "Federico II" University of Naples.

The participants were asked not to eat, drink or chew gums for at least 1 h before the test. The experimental procedure was divided in two sessions:

- In the I Session, each volunteer masticated 1 g of experimental gum for given periods of time (5, 10, 20 and 30 min). All partecipants chewed each gum made with different biomolecules content.

  Then, the chewed gums were stored and used for the chemical assessment of residual Calcium and Quercetin concentrations. The chewed samples were stored at +4°C until analysis. Each sample type was analyzed in triplicate.

- In the II Session, each volunteer swallowed a saliva sample before the test to analyze the Ca content at baseline. Then, they chewed 1 g of gum for 30 min, at their natural chewing frequency. During this time, the participants collected

separate samples, swallowing their saliva after 5, 10, 20 and 30 min [Shen et al., 2001].

For this session were used only gums made with 10% of CPP and 0.5 or 1.0 % of Quercetin; all participants chewed each kind of gum made with different Qt content. The volumes of the saliva samples were recorded and the flow rates was calculated for each period of chewing, then pH of samples was measured with a glass electrode pH-meter in the end of each period [Cross et al., 2005]. The saliva samples were then stored at +4°C for the chemical assessment of Calcium an Quercetin.

Determination of Calcium and Quercetin residual content

The Calcium concentration was determined by atomic absorption spectrophotometer (SpectrAA 200, Varian, Mulgrave – Victoria, Australia) with the flame method fed by air/acetylene using the following flows: air, 13.5 l/min; acetylene, 1.5 l/min. A Ca/Mg multi-element hollow-cathode lamp (Varian) was used as the source of radiation. The instrumental conditions were: selected wavelength, 422.7 nm; slit width, 0.5 nm; lamp current, 10 mA. Standard solutions of calcium (0.5 and 5.0 mg/l) were prepared for the calibration curve. Lanthanum chloride (1%, w/v) was added as a modifier of the matrix. Samples were ashed in Lenton muffle furnace (Lenton, United Kingdom).

The assessment of Quercetin content was performed according to the colorimetric method reported by Woisky and Salatino and modified by Chang *et al.*, [Woisky and Salatino,1998; Chang et al., 2002].

The amount of Quercetin was determined after lyophilization and the residues were re-suspended in methanol/water (80:30, v/v) solution. Quercetin pure standard was used to make the calibration curve. Ten milligrams of quercetin was dissolved in 80% methanol and then diluted to 25, 50 and 100 μg/ml. The diluted standard solutions (0.5 ml) were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a spectrophotometer UV-DU 730 (Beckman Coulter, Brea, CA). The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Similarly, 0.5 ml of methanol extracts of samples were reacted with aluminum chloride for determination of Quercetin content as above described.

Statistical Analysis

For each variable assessed, means and standard deviation of values were calculated. The effect of time interaction on all collection sessions was analyzed as the differences from baseline by one-factor analysis of variance (ANOVA); when significant effects occurred, Duncan's post-hoc test was performed. Before ANOVA analysis, data were tested for normality using Cochran's test and for homogeneity of variance using Shapiro-Wilk's test. A P-value <0.05 was considered significant. Statistical analysis were performed using STATISTICA for Windows® version 8.0 (Statsoft Inc., Tulsa, OK, USA).

II.3.4 Results and discussion

As above explicated, two series of experiments were performed in order to evaluate the drug release kinetic: *in vitro* and *in vivo*.
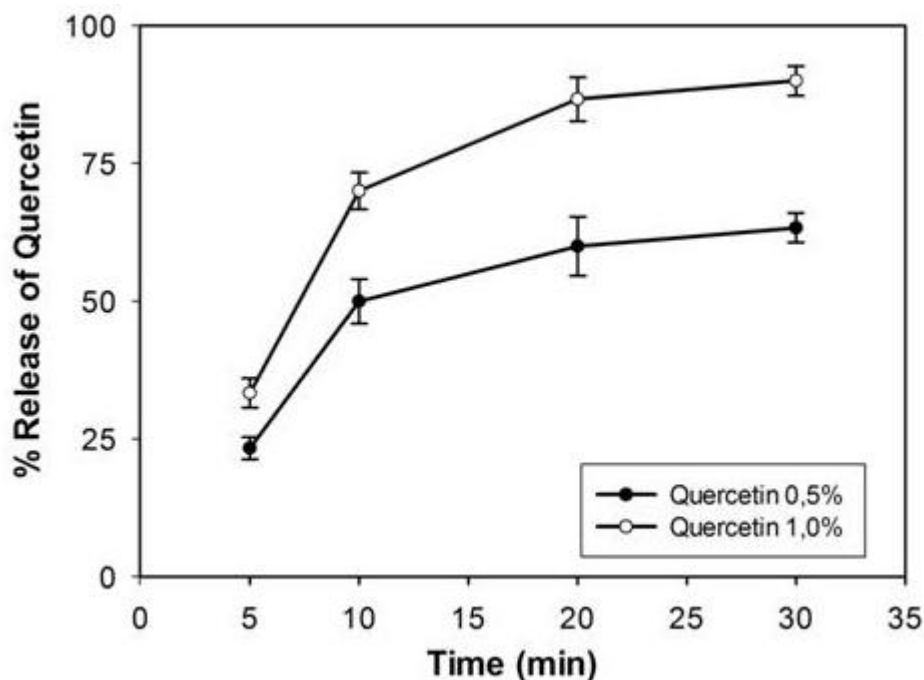
In Vitro release from chewing gums

In vitro release study of Calcium (Ca) and Quercetin (Qt) from chewing gums was carried out using an experimental *in vitro* chewing release apparatus.

The *in vitro* release profiles of Ca from chewing gums containing different amounts of CPP-ACP (5, 10 and 20% per gum) is shown (Fig. 2). The percentage of Ca released from the three gums was 24–26% at 5 min, 30–45% at 10 min, 58–72% at 20 min, and 59–74% at 30 min. The gums containing 20% of CPP-ACP showed a slightly higher percentage of release than the gums containing 5 and 10% of CPP-ACP. The amount of released Calcium was proportional to the loading level of the gum formulations. Totally, about 74% of Ca was released after 30 min.
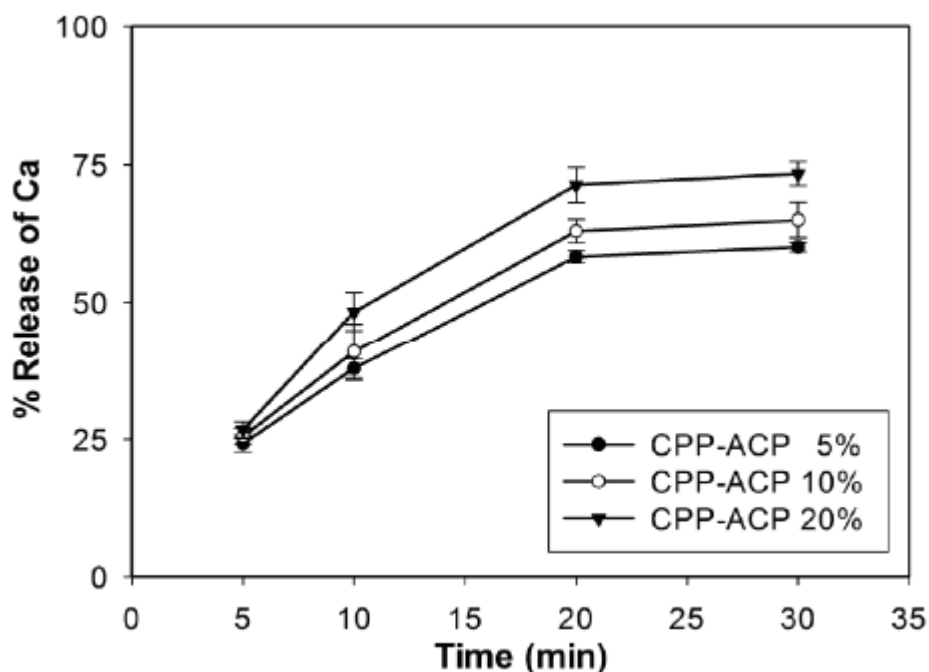
Fig. 2: In vitro release of Ca from gums in artificial saliva at 37°C. The chewing gums containing different CPP-ACP loadings (5, 10, 20 %) were studied using a chewing apparatus. The values represent the average and standard deviation of three independent experiments.

The percentage of Qt released as a function of mastication time from chewing gums containing different amounts (0.5 and 1%) of antioxidant is reported (Fig. 3). It is evident that the percentage of delivered Qt increased progressively as a function of chewing time. In any case, data shows that in 30 min of chewing Qt is delivered in a larger amount (70–90%) than Calcium. In fact, Qt was quickly released after 10 minutes of mastication, due to the higher solubility in the artificial saliva, while formulations containing less soluble principles (CPP-ACP) required longer mastication time (30 min) for their release.

Fig. 3: In vitro release of Quercetin from gums in artificial saliva at 37°C. The chewing gums containing different Quercetin loadings (0.5 or 1.0 %) were studied using a chewing apparatus. The values represent the average and standard deviation of three independent experiments.

In Vivo Session I

The amounts of Calcium contained in the chewed gum, with the same formulations analyzed *in vitro,* is reported in function of chewing time (Fig. 4).

The Ca amount contained in the chewed gum was complementary to that measured in the in *vitro* tests for each time point. The Ca concentration of three gum formulations showed 77–89% at 5 min, 52–70% at 10 min, 20–39% at 20 min, and 18–35% at 30 min.

The percentage of residual Qt in the chewed gum at two different amounts (0.5 and 1%) is reported in function of mastication time (Fig. 5). The percentage of Qt decreased

progressively as a function of the chewing time. In fact, the Qt concentration of the two different formulations showed 62–73% at 5 min, 27–48% at 10 min, 9–17% at 20 min, and 8–12% at 30 min, indicating that the Qt was released mainly in the first 10 minutes in accordance with *in vitro* patterns.
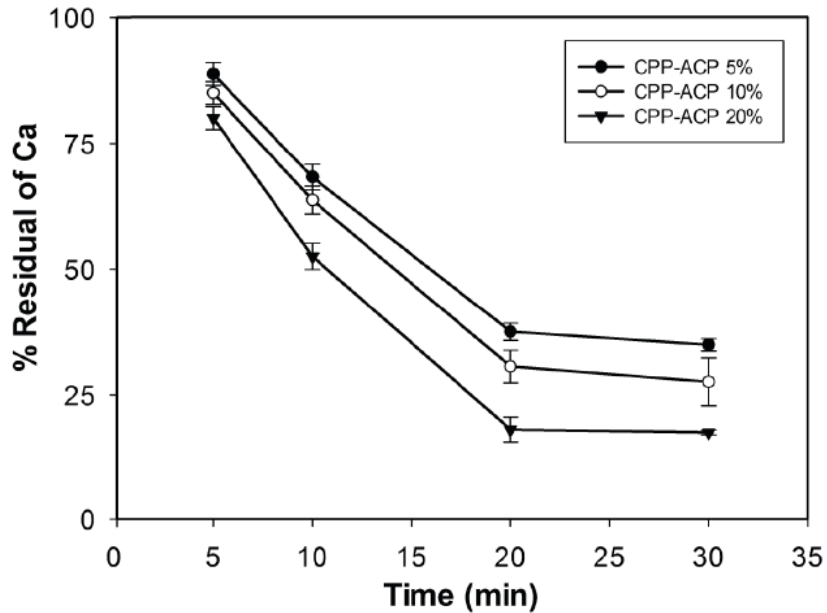
Fig. 4: In vivo release of Ca from gum formulations by a chew-out study. Volunteers chewed each gum containing 5, 10 or 20 % of CPPACP for the predetermined times and the residual amount of Ca was analyzed by atomic absorption spectrophotometer. The values represent the average and standard deviation of three independent experiments.
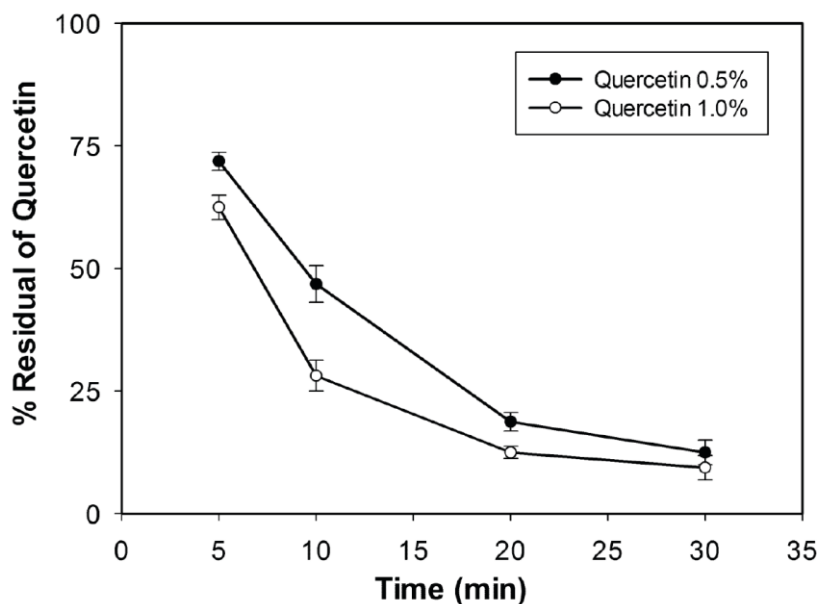
Fig. 5: In vivo release of Quercetin from gum formulations by a chew-out study. Volunteers chewed each gum containing 0.5 or 1.0 % of Quercetin for the predetermined times and the residual amount was extracted and analyzed. The values represent the average and standard deviation of three independent experiments.

In Vivo Session II

Salivary flow rate an pH

The mean flow rate of the collection of saliva during the first 5 min of chewing was 2.18 mL/min; it decrease at 1.71 mL/min and 1.33 mL/min respectively after 10 min and 20 min of chewing periods and fell to value of about 1.04 mL/min after 30 min (Fig. 6).
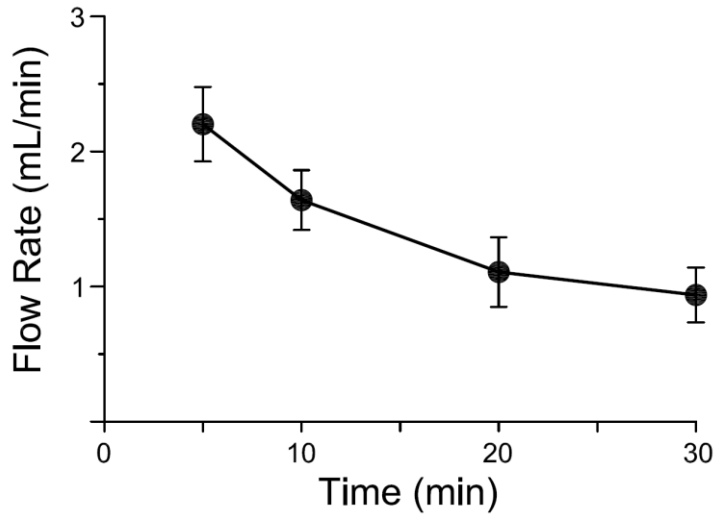
Figure 6 - Salivary flow rate (mean ± S.D.) over a 30 min period.

The pH increased from a mean of 6.84 at the end of 5 min collection of saliva to a mean of 6.95 after 10 min sample and then increased slightly to a mean of 7.35 over the 30 min collection period (Fig. 7). The statistical analysis showed that there were no significant differences in flow rate and in pH; that evidence prove how CPP-ACP complexes contrast demineralization process without any buffering capacity.
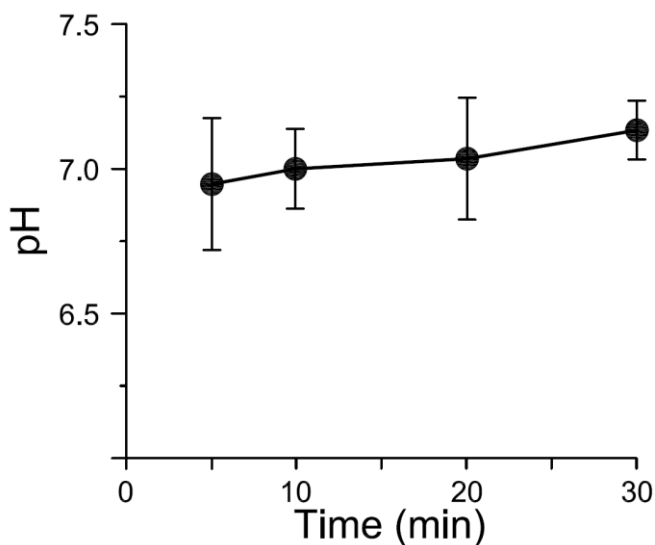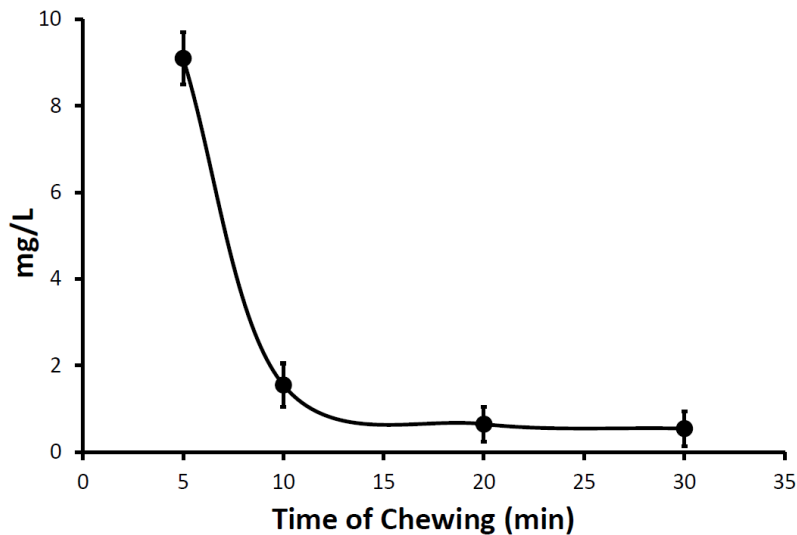

Figure 7 - Salivary pH (mean ± S.D.) over a 30 min period.

Calcium ad Quercetin content

Initially the Ca content of the saliva was evaluated and subtracted from the quantity released during chewing. The amount of Ca contained in the saliva samples collected from 10 participants in function of chewing time is reported in Figure 8.

Figure 8 - *In vivo* release of Ca (mean ± S.D.) from gum formulations by a chew-out study.

The Ca released from gum formulation was 9.09 mg/L during the first 5 min. collections of saliva, fell to value of 1.55 mg/L after 10 min. and finally decrease slowly at 0.65 mg/L and 0.54 mg/L after 20 min. and 30 min., respectively. The data show that Ca was quickly released after 5 minutes of chewing.

The Qt released as a function of mastication time from chewing gums containing different amounts (0.5 and 1.0%) of antioxidant is reported in Figure 9.
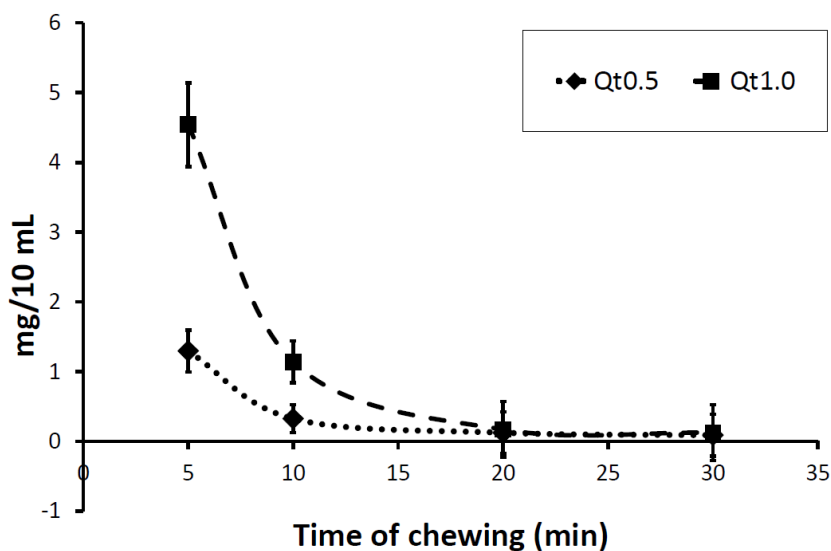
Figure 9 - *In vivo* release of Quercetin (mean ± S.D.) from different gum formulations by a chew-out study.

It is evident that the delivered $Qt_{0.5}$ decreased progressively as a function of the chewing time; in fact $Qt_{0.5}$ was 1.29 mg/10mL over 0—5 period of chewing, 0.32 mg/10mL over 5—10 period, 0.12 mg/10mL over 10—20 period and 0.09 mg/10mL over 20—30 period. Instead $Qt_{1.0}$ after the first 5 min collections of saliva was 4.54 mg/10mL, then fell quickly to 1.14 mg/10mL over 5—10 period and finally decreased slowly to 0.17 mg/10mL and 0.13 mg/10mL over 10—20, 20—30 periods respectively.

II.3.5 Conclusions

The *in vitro* and *in vivo* experiments to test the different Ca and Qt's release rate from a gum in the artificial saliva or in the chewed gums, revealed different release rates for the two substances.

Calcium was released from the chewing gum in a slower and more controlled manner than Quercetin. This study demonstrated that both Calcium derived from CPP-ACP and Qt were released during chewing; although, in vitro, Calcium was released from chewing gums in a slower and more controlled mechanism than Qt. They were also both effectively released in the oral cavity during the in vivo chewing time, showing an effective anticaries and antierosive concentration in volunteers saliva.

This research concludes that CPP-ACP and Quercetin included in new experimental chewing gums could be efficiently released in vitro and in vivo during the mastication process in order to contrast carious and erosive diseases.

Chapter III

General Conclusions

This project analyzed the capability of plant polyphenols and casein-phophopeptides to promote an anti-caries and anti-erosive action.

Results of experimental protocols allow us to define that CPP-ACP and polyphenols, as Quercetin, could contrast dental caries and erosion in vitro and in vivo trials. Regarding the protective role of CPP-ACP against soft drink erosion, within the limits of an in vitro assay and a SEM qualitative surfaces inspection, the above mentioned molecules play an active role in reduction of dental demineralization, but research should improve studies of this protective action, analyzing the quantitative variations of Calcium and Phosphate ions from enamel surface of samples, using novel imaging techniques.

Moreover, the capability of Plantago Lanceolata extracts to contrast oral cariogenic bacteria is well shown in above explicated protocol results, without any risk for young or adult humans. Between the various polyphenols presents in the extracts, it was choose to include Quercetin into the experimental chewing gums, because its well known scientific background, which demonstrates the safe and effective use in paediatric people.

The real news of this project in scientific community is the idea of adding in experimental chewing gums not only a remineralizing molecule as CPP-ACP, but also a

antibacterial polyphenol as Quercetin. In vitro preliminary assay and the subsequent clinical trial demonstrate that experimental chewing gums are capable to release effective concentration of bioactive molecules into oral cavity, promoting the researched protective action against dental diseases.

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