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XXIX CICLO

ANTIMICROBIAL ACTIVITY AND CHEMICAL CHARACTERIZATION OF THE SARDINIAN PLANTS *CITRUS LIMON* CV. *POMPIA* CAMARDA, *VITIS VINIFERA* L. CV. CANNONAU, *THYMUS HERBA-BARONA* LOISEL AND *PISTACIA LENTISCUS* L.

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1. Oral ecosystem

a. Oral cavity features

The oral cavity represents the starting point of the digestive processes and it also corresponds to the first tract of the respiratory system; it gets involved in food, air, microparticles, and microorganisms transition.
It represents a perfect habitat for the microbic colonization thanks to phisical-chemical factors, nutritional factors and to the nature of its diffent surfaces. It is in these terms that we can talk about an ecosystem: an habitat where microorgnisms transform and recycle substances (Teti and Mattina, 2002).
Despite the potential movement of microorganisms between sites, the characteristic biological and physical properties of each site in human body determine specific environmental conditions, which allow the growth of a well defined microflora (Wilson, 2005). This is true for bacteria living in the skin, digestive and reproductive tracts, as well as in the mouth *etc*. This observation shows that the properties of the habitat are discriminatory in the ability of organisms to colonize, grow and become minor or major members of a community (Marsh et al., 2011).
The oral cavity is a morpho-physiologically heterogeneous, dynamic environment with biotic and abiotic factors influencing with different intensity various specialized surfaces of its particular compounds (tongue, teeth, gums, *etc.*).
The mouth is warm and moist, it is maintained at a temperature of around 35–37°C, which is suitable for the growth of a distinctive collection of microorganisms (viruses, mycoplasma, bacteria, Archaea, fungi and protozoa) (Marsh and Martin, 2009). Most sites (mucosal or plaque) yielded 20–30 different predominant species, and the number of species per individual mouth ranged from 34 to 72 (Aas et al., 2005).
Among these factors, temperature, pH of saliva (related to food substances), oral care agents and specific diet may impact oral cavity ecology. This environment creates an open system with dynamic ecological conditions promoting its colonization with many microbiota and influences their species composition (Zawadzki et al., 2016).
Then, also the mouth, as other habitats within the body has a characteristic microbial community that provides benefits for the host.

b. Oral microbiota features

Humans are colonized by myriads of microorganisms in various parts of the body, such as the skin, the mouth, the vagina and the gastrointestinal tract. Furthermore, our microbiota is not only comprised of bacteria, but also of archaea and eukaryotes such as protozoa, fungi and nematodes. Even viruses, collectively termed the virome, can be found in the microbiota (Virgin, 2014).
The term “microbiota” describes the total collection of organisms of a geographic region or a time period. In the context of human health the term microbiota was first used to describe the gingival crevice (Socransky et al., 1953).

The term “microbiome” was originally used to refer to the collection of the genomes of the microbes in a particular ecosystem and termed by Nobel laureate Joshua Lederberg (1925–2008) (Hooper and Gordon, 2001).
The oral microbiota represents an important part of the human microbiota, and includes several hundred to several thousand diverse species. It is estimated, that a minimum of 700 species occur in the human cavity, from at least 12 phyla (Wade, 2013), including even *Archaea*.
Approximately 54% are validly named species, 14% are unnamed (but cultivated) and 32% are known only as uncultivated phylotypes. (Human Oral Microbiome Database, 2016).
The problems in culturing oral bacteria are based on their need for very specific nutrients, in part extreme oxygen sensitivity, and, finally, dependence on other neighboring organisms (Wade, 2013).
As an example Table 1.1 lists only those genera which were found and described since 1990 (from Wade, 2013), Table 1.2 lists the predominant species of bacteria present in oral cavity.

Table 1.1. Recently described bacterial genera with oral representatives (since 1990) (Adapted from Wade, 2013)

Phylum	**Genus**
Actinobacteria	*Actinobaculum, Atopobium, Cryptobacterium, Kocuria, Olsenella, Parascardovia, Scardovia, Slackia, Tropheryma*
Bacteroidetes	*Bergeyella, Prevotella, Tannerella*
Firmicutes	*Abiotrophia, Anaerococcus, Aneroglobus, Bulleidia, Catonella, Dialister, Filifactor, Finegoldia, Granulicatella, Johnsonella, Mogibacterium, Parvimonas, Peptoniphilus, Pseudoramibacter, Schwartzia, Shuttleworthia, Solobacterium*
Proteobacteria	*Lautropia, Suttonella*
Synergistetes	*Jonquetella, Pyramidobacter*

Table 1.2. Predominant species of bacteria found oral cavity (Teti and Mattina, 2002)

Genus	**Species**
Streptococcus	*sanguinis, salivarius, mutans, mitis, mitior, milleri, intermedius, durans, morbillorum*
Actinomyces	*viscosus, naeslundii, israeli, odontolyticus*
Rothia	*Dentocariosa*
Arachnia	*Propionica*
Veillonella	*parvula, alcalescens*
Lactobacillus	*casei, acidophilus, salivarius* and other species
Haemophilus	*segnis* and other species
Bacteroides	*asaccharolyticus, melaninogenicus* ss. *intermedius, melaninogenicus* ss. *melaninogenicus, oralis, capillosus*
Fusobacterium	*nucleatum, russi*
Treponema	*denticola, macrodentium, orale*
Borrelia	Various species
Propionibacterium	*acnes, freudenreichii, jensenii*
Neisseria	*flavescens, mucosa* and other species
Capnocytophaga	*ochracea, gingivalis*
Campylobacter	*Sputorum*
Selenomonas	*Sputigena*
Eikenella	*Corrodens*
Peptostreptococcus	*anaerobius, micron*
Leptotrichia	*Buccalis*
Actinobacillus	*Actinomycetecomitans*

2. The oral Biofilm

Dental plaque is an example of microbial biofilm (Socransky and Haffajee, 2002) characterized to be the result of a complex process, which hesitates in the constitution of a complex three-dimensional structure, ideal habitat for microbial populations. Within the biofilm, microbial populations establish relations of community-based co-existence, governed by a complex system of chemical intercellular bio-signalling (Marsh, 2005).
Dental plaque has a positive role in oral ecosystem due to the fact that the commensal microbial species prevent colonization by other exogenous and generally pathogenic species (Marsh, 2009) However its accumulation represents one of the main causes to develop dental caries and periodontal disease (Sbordone and Bortolaia, 2003).

a. Biofilm formation

i. The acquired salivary pellicle

The process of organization of the dental biofilm begins with the formation of acquired salivary pellicle, which represents the basis for the subsequent bacterial adhesion and colonization of the oral surfaces (Lendenmann et al., 2000). This is defined as the initial "proteinaceous layer", a thin layer of saliva, which covers all the oral surfaces (Tinanoff et al., 1976). It is typically found on the tooth surface, immediately after the execution of oral hygiene, as well as on the surface of the oral mucosa and is devoid of bacterial population (Lendemann et al., 2000). This is the principled difference with biofilm (bacterial plaque) which is characterized by the presence of adherent bacteria, which are distributed within a multi-layers three-dimensionally organized structure (Marsh and Bradshaw, 1995).
Acquired salivary pellicle formation is the result of biopolymer adsorption at the tooth/saliva interface (Hannig, 1999). So-called pellicle precursor proteins (PPPs), phosphoproteins with high affinity to hydroxyapatite are the first to adsorb to the tooth surface. Examples are statherin, histatin and proline-rich proteins. This interaction is conveyed by the ionic calcium and phosphate layer at the enamel surface (Hannig and Joiner, 2006).
The literature reports that the "in two phases adsorption model" is the most common and is characterized by a rather fast first stage and a subsequent slower, but continuous and progressive phase (Hannig and Hannig, 2009).
The adsorption process of PPPs in aqueous solution, depends on: the solid surface, the aqueous medium and the solubilized proteins. In this, the polarity of all the components is crucial for the mutual interaction (Hannig and Hannig, 2009).
The function of the pellicle is ambivalent: on the one hand, it serves as a lubricant, as an anti-erosive barrier and buffer (Hannig et al. 2004), in addition, the antibacterial proteins lactoferrin, cystatins and lysozyme add protective properties (Deimling et al., 2007); on the other hand, the pellicle features some properties facilitating bacterial adhesion. Several pellicle components such as amylase, proline-rich proteins, Mucin MG 2, fibrinogen and lysozyme serve as specific receptors for bacterial adherence (Hannig and Hannig, 2007).

ii. Organization and bacterial composition of the biofilm

The dental biofilm is formed via adhesion of planktonic bacteria to a protein pellicle coating the tooth surfaces immediately after cleaning (Karthikeyan et al., 2011). The saliva represents the "planktonic phase" of the oral microbiota. Similar to bacterial laboratory fluid cultures saliva contains up to 10^9 microorganisms per milliliter, which are swallowed continuously. In this way, about 5 g of bacteria 'disappear' into the stomach daily (Schwiertz, 2016).
These bacteria adheres selectively on oral surfaces by binding to specific molecules contained in the acquired film, thus starting the process of transformation of the latter into dental plaque. Initial bacterial adhesion passes through a phase of weak and reversible binding before an irreversible attachment is established (Marsh and Bradshaw 1995). Reversible initial binding occurs preferentially in the surface irregularities where microorganisms are protected against mechanical shear forces (Tanner et al., 2003).
Bacterial colonization into the acquired pellicle is classified into three groups: initial, middle, and late colonizers (Kolenbrander et al., 2010). After that, the pellicle is termed biofilm. Initial colonizers are for example, *Streptococcus sanguinis*, *Streptococcus oralis, Streptococcus mitits, Actinomyces spp*., *Haemophilus spp., Eikanella corrodens, Veillonella, Campylobacter,* and *Prevotella melaninogenica* (Diaz et al., 2006; Hannig and Joiner, 2006). *Actinomyces naeslundii* represents 27% of the pioneer strains (Li et al., 2004). These early colonisers are able to adapt to extremely fluctuating conditions, which is necessary for a pioneer bacteria strain to become adherent to the dental surfaces (Nyvad and Kilian, 1987). Futhermore, these species grow, modify the environment and make conditions suitable for colonisation by later, more fastidious bacteria, many of which are obligately anaerobic. After the attachment to oral surfaces, the further co-aggregation and co-adhesion of genetically different initial colonizers are promoted by adhesive molecules located at bacterial fimbriae and by polysaccharide sediments onto bacteria (Kolenbrander et al., 2006; Marsh and Bradshaw, 1995). Initial and late colonizers require the so-called co-aggregation of middle colonizers. They are mainly represented by *Fusobacteria* (Kolenbrander and London, 1993; Kolenbrander et al., 2010) such as *Fusobacterium nucleatum* that plays a central role as a bridge bacterium between early and late colonizers (Kolenbrander et al., 1989, 2010). Other species of microorganisms that are present in the oral cavity, such as *Candida albicans* and *Lactobacillus casei*, may also cause pathologies, particularly under specific conditions. Consequently, the final structure of the oral biofilm is composed of different bacterial species and extracellular polymeric substances (EPS). Attached organisms synthesise exopolymers such as glucans, which form the biofilm matrix that acts as a scaffold for the biofilm and is biologically active and able to retain molecules within plaque.

b. Correlation between the biofilm and oral pathology

In regard to microbial settlement, shedding surfaces (mucosal sites) like lips, cheeks, palate and tongue have to be differentiated from non-shedding surfaces , the natural teeth as well as artificial

materials surfaces of fissure sealings, tooth fillings, orthodontic appliances, dentures and also oral implants (Marsh and Martin, 2009; Zaura et al., 2009).
Shedding surfaces, where only monolayers of bacteria originate and which are regularly desquamated (cheek, palate) have to be discriminated from the tongue with its 'stable' multilayers of biofilm-like bacteria. It is estimated that the tongue harbours the majority of the microbial burden of the oral cavity, and supports a higher bacterial density and a more diverse microbiota than the other mucosal surfaces; 30% of the bacterial population detectable by molecular studies were found only on the tongue (Marsh et Martin, 2009).
On any non-shedding surfaces of the oral cavity dental plaque starts to form, which meets all criteria for a microbial biofilm and is subject to the so-called succession. When the sensitive ecosystem turns out of balance either by overload or weak immune system it becomes a challenge for local or systemic health. Therefore, the most common strategy and the golden standard for the prevention of caries, gingivitis and periodontitis is the mechanical removal of this biofilm from teeth, restorations or dental prosthesis by regular toothbrushing.
On any non-shedding surfaces dental biofilm **(**dental plaque) starts to form. Such biofilm formation is found at different locations:
- Fissure biofilm (in cavities inside the teeth, approaching the dental pulp) is dominated by facultative species, especially streptococci, many of which produce extracellular polysaccharides, and there are few Gram-negative or anaerobic organisms (Theilade et al., 1982).These species cause fissure caries and eventually endodontic problems.
- Supragingival biofilm (on the dental enamel adjacent to the gingiva) contains, related to its maturation and thickness, a mixture of facultative and anaerobe species, causing an unspecific gingival inflammation (gingivitis); approximal surfaces have a microbiota that is intermediate in composition between that of fissures and gingival crevices, and also harbors many anaerobic species. These sites have high proportions of Actinomyces spp. (Bowden et al., 1975).
- Only when supragingival plaque lasts for quite a long time harming the gingival crevice, periodontitis may occur due to development of subgingival plaque. This type of biofilm contains mainly anaerobe species.
- Plaque on artificial surfaces (e.g. dental fillings) resembles mainly the supragingival entity. Denture plaque may harbour Candida spp., which may cause 'denture stomatitis'. The microbiota relevant for peri-implant mucositis (analogous to gingivitis) and eventually peri-implantitis (analogous to periodontitis) is not yet well understood (Schwiertz, 2016).
The metabolic processes in such communities of biofilm are highly dynamic. However, when the balance of the oral cavity is disturbed the homeostasis in the biofilm breaks down and results in compositional changes causing caries, periodontal or mucosal disease.
For instance, by frequent carbohydrate exposure and/or decreased salivary clearance of foods, biofilm formation initiates plaque formation and the synthesis of glucan from sucrose by *S. mutans*, which is catalysed by glucosyltransferases (GTFases). In this situation, more aciduric bacteria become dominant, involving not only *Streptococcus mutans* and *Lactobacillus*, but also aciduric strains of non-mutans streptococci (*S. salivarius, S. gordonii*, S. *sanguinis*) (Huang et al., 2012), Actinomyces, Bifidobacteria and yeasts. In the meantime, health-associated bacteria, which prefer a neutral pH, will be inhibit. Dental plaque metabolises the carbohydrates contained in foods, releasing organic acid metabolites that demineralise tooth surfaces, resulting in dental

caries. So, caries can develop supporting the notion that caries aetiology is probably complex and multi-faceted (Takahashi and Nyvad, 2008, 2011; Peterson et al., 2011).
Dental caries (tooth decay) is one of the most prevalent diseases worldwide. Its incidence increased dramatically with the introduction of refined carbohydrates in the diet in the 18th century (Hicks et al., 2003).
This disease is the localized destruction of dental hard tissues and a primary cause of oral pain and tooth loss. It is a common health problem in humans across the entire life span; indeed, untreated caries in permanent teeth was the most prevalent of all diseases evaluated in the Global Burden of Disease (GBD) Study 2010, affecting 2.4 billion people worldwide (Marcenes et al., 2013).
Recently, it has been claimed that dental caries is decreasing in developed countries due to the effects of preventative interventions, including the use of fluoride. However, the prevalence and incidence remained unchanged in all regions of the world over 20 years, and the burden of untreated caries is shifting from children to adults (Kassebaum et al., 2015).
Other microbial situation occurs in the case of periodontal disease in which excessive plaque accumulation is found around the gingival margin. If the host is unable to control this microbial insult, an inflammatory response can be shown. The gingival crevicular fluid increases adding further components of the host response and also molecules such as haemoglobin, haptoglobin and transferrin which select for proteolytic bacteria. These proteolytic bacteria can also degrade host molecules that regulate inflammation, resulting in an exaggerated and inappropriate inflammatory response that can be severe enough to cause bystander damage to host tissues.
The host response may cause gingival inflammation, which can progress to irreversible gingival recession, alveolar bone destruction and tooth loss.
Periodontal disease is a multifactorial disease of the oral cavity affecting the majority of the population. Periodontitis is a ubiquitous disease with high prevalence in adults. In particular, the WHO sponsored Global Burden of Diseases study indicates that 11.2% of adults worldwide experience severe periodontal disease (Marcenes et al., 2013).
It is a health concern because it affects the majority of the population and has a negative impact on oral health, ability to chew, appearance, quality of life, dental care costs and can lead to tooth loss. Dental plaque is necessary but not sufficient for the development of periodontitis and clinical management of periodontitis involves mechanical removal of plaque from the tooth surface.
Culture studies of biofilms from periodontal pockets have shown an increase in biomass and higher proportions of Gram- negative, obligately anaerobic, proteolytic bacteria (Marsh, 2010; Socransky and Haffajee, 2005). Some of the bacteria which emerged as putative periodontal pathogens from cultural investigations of the subgingival microbiota are *Porphyromonas gingivalis, Prevotella intermedia, Aggregatibacter actinomycetemcomitans* (Lang and Lindhe, 2015).
As regard the developing of oral mucosal disease, yeasts play an important role. This is the case of xerostomia that is a common complication particularly in a consequence of radiotherapy for head and neck cancer (HNC) (Pinna et al., 2015). Radiotherapy causes xerostomia in up 100% of the patients suffering from HNC and can be develop during and after the therapy as a consequence of the irradiation of salivary glands. The reduced flow compromises lubrification of the oral mucosa that becomes dry and burning (Randal et al., 2013). Also, saliva reduces the buffering capacity in irradiated patients due to a reduction of bicarbonate concentration in parotid saliva. Saliva

becomes highly viscous and reduces its pH from about 7.0 to 5.0 with a slow recovery to the neutral pH in dental plaque after a sugar rinse (Lingström and Birkhed, 1993).
The reduced saliva flow, cause a different oral microflora growth with a prevalence of acidogenic and cariogenic micro-organisms which prefer a low pH. Saliva also reduces the buffering capacity in irradiated patients due to a reduction of bicarbonate concentration in parotid saliva. Together with *Streptococcus mutans* and *Lactobacillus spp*., a marked increases of *Candida spp*. become prevalent in the plaque of irradiated patients (Almståhl and Wikström, 1998), Particularly *Candida albicans* infection is an important issue during xerostomia more compromising the quality of life of these patients (Pinna et al., 2015).
C. albicans interacts with commensal (viridans) streptococci and forms biofilms on acrylic/mucosal surfaces (Diaz et al., 2012) to cause oral mucosal infections (Xu et al. 2014). However, physical coadhesion of *S. mutans* and *C. albicans* is drastically enhanced in the presence of sucrose; these conditions also promote biofilm formation (Falsetta et al., 2014).
Thus, the inhibition of plaque biofilm formation is the key to successful control and prevent oral disease.

c. Oral biofilm isolation and characterization methods

Bacteria are the most numerous group in the oral cavity and initially were characterized using cultural approaches. Over time, it became clear that there was a discrepancy between the number of bacteria in a sample that could be grown by these conventional approaches and those that were observed directly by microscopy (Moter et al., 2006). It is estimated that <50% of the resident oral microflora can currently be cultivated in pure culture in the laboratory (Wade, 2002). Recent application of culture-independent molecular approaches allowed us in the understanding of the richness and variety of the resident oral microflora. Numerous studies of various surfaces and sites based on amplification, cloning and sequencing of the 16S rRNA gene have contributed to increase the knowledge in hundreds of species in the mouth (Marsh et al., 2011).
Biofilm is formed by the different interacting bacteria living in harmony with the host (microbial homeostasis). The resident microflora contributes to the health of the host due to a prevention of exogenous and potentially pathogenic micro-organisms from becoming established in the mouth ("colonisation resistance"), and by regulating the inflammatory host response to oral commensal bacteria (Marsh and Bradshaw, 1999).
Interference in the resident oral microflora can result in overgrowth by previously minor components of the biofilm and such a disruption can cause disease.
Concerning methods in biofilm research there are some crucial prerequisites when evaluating oral (plaque) biofilms: intraoral splint systems, which enable the undisturbed accumulation of dental biofilms on the surface(s) of native enamel slabs (Auschill et al., 2004; Arweiler et al., 2004) or dental materials (Auschill et al., 2002); including the concomitant formation of a native pellicle (Hannig, 1997, 1999).
Traditionally, the oral tooth-related microbiota was and still is assessed either by conventional microbiological methods (cultivation) (Mikkelsen, 1993) or by electron microscopy (TEM and SEM) (Saxton 1973; for review cf. Newman and Wilson, 1999). Furthermore, vital (fluorescence) staining

techniques were used to elucidate the portion of vital or dead bacteria in the dental biofilm (Netuschil et al., 2014), which can also visualize the effect of antibacterial agents by confocal laser scanning microscopy (CLSM). More recently the FISH technology (Fluorescence in situ hybridization) was introduced to plot specific bacterial species and to depict the distribution of them in a biofilm network (Al-Ahmad et al., 2007). Thus, different "visualizing" methods were combined with CLSM to reveal the three-dimensional architecture of oral biofilms (Netuschil et al. 2014; Auschill et al., 2002, 2004; Arweiler et al. 2004, 2013 ; Al-Ahmad et al. 2007).

3. Antimicrobial agents

a. Introduction

Dental diseases can be controlled by meticulous mechanical oral hygiene. However, most individuals have difficulty in maintaining the necessary standards of plaque control for prolonged periods. Additional approaches are being developed that are less dependent on the dexterity of the patient, and which augment conventional oral hygiene methods and keep plaque at levels compatible with oral health. Consequently, many oral care products are now formulated to contain proven antiplaque and antimicrobial agents to help achieve this goal (Scheie and Petersen, 2008; Brading and Marsh, 2003).

The purpose of antimicrobial agents is to control the bacterial plaque in the mouth. The intention of the antiplaque agents is: 1) to prevent the formation of the biofilm, and/or 2) remove established biofilm, in order to have 3) clinical and microbiological benefit, while at the same time 4) not altering the natural microbial ecology of the mouth, which might cause the overgrowth of some opportunistic pathogens (*e.g.* yeasts) or exogenous micro-organisms.

In contrast, the mode of action of antimicrobial agents involves inhibiting the growth or killing of the target bacteria, expressed in terms of their Minimum Inhibitory Concentration (MIC) or Minimum Bactericidal Concentration (MBC), respectively. It is known that the MIC/MBC of an agent is determined in the laboratory on liquid grown (planktonic) cells in tests where the agent is in contact with a pure culture of the organism for prolonged periods (24−48+ hours). However, results change when bacteria are growing on a surface as a biofilm, due to the increased resistance to antimicrobial agents, particularly in the case of older (more mature) biofilms. This can be explained by reduced penetration of the agent due to binding to the biofilm matrix or quenching of the agent at the surface of the biofilm, novel phenotypes expressed by bacteria when growing on a surface, and the slow growth rates of attached bacteria within biofilms (Marsh, 2005).Moreover, the maximum length of time recommended for people to brush their teeth or rinse with a mouthwash is in the order of two minutes. Therefore, an additional requirement of the formulation is the ability to deliver a sufficient concentration of the inhibitor in those two minutes on dental and mucosal surfaces so that the active components can be released over time at levels that will still deliver biological activity (Marsh, 2010).

A representation of an antimicrobial flux curve in the mouth is shown in Figure 3.1. (Marsh, 2010) . This capacity of product retention is termed substantivity and varies among the agents used as antimicrobial products.

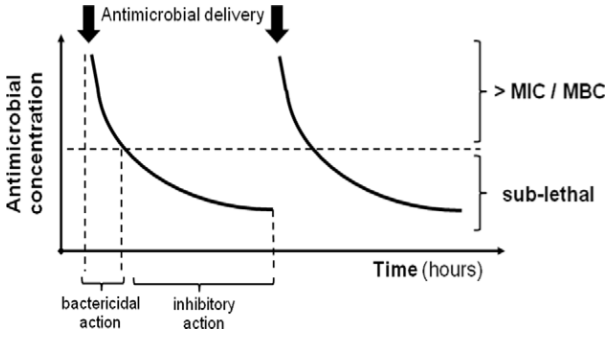


Figure 3.1. Pharmacokinetics of an antimicrobial agents delivered to the mouth. A schematic representation of antimicrobial agent concentration over time following an oral delivery of a care product at different times. The agent is present above its MIC/MBC level for a relatively short period (bactericidal action) while, it keeps at a sub-lethal concentration (inhibition action) for much longer. Other than bactericidal action, agents may still exert beneficial effects by inhibiting traits associated with bacterial pathogenicity. The dynamics of the curve vary for each antimicrobial agent.

b. Classes of inhibitors used as antiplaque/antimicrobial agents

A wide range of agents have been formulated into oral care products in order to enhance their plaque control potential (Scheie and Petersen, 2008; Baehni and Takeuchi, 2003) (Table 3.1).
They are fluorides, alcohols, and synthetic antimicrobials used in tooth pastes and mouth rinses include povidone iodine products, chlorhexidine, cetylpyridinium chloride, triclosan and zinc citrate.

Table 6. Classes and examples of inhibitors used as antiplaque or antimicrobial agentsin mouthwashes and toothpastes (Marsh 2010)

Class of inhibitor	Examples
Bisbiguanide	chlorhexidine
Enzymes	mutanase, glucanase; amyloglucosidase-glucose oxidase
'Essential oils'	menthol, thymol, eucalyptol
Metal ions	copper, zinc, stannous
Natural molecules	plant extracts (apigenin, tt-farnesol)
Phenols	Triclosan
Quaternary ammonium compounds	Cetyl pyridinium chloride
Surfactants	Sodium lauryl sulphate, delmopinol

Fluoride can inhibit bacterial enzymes in addition to its effects on enamel biochemistry (Marsh et al., 2011).
Tooth enamel is composed of crystals of hydroxyapatite, a mineral form of calcium apatite ($Ca_{10}(PO_4)_6(OH)_2$). Free fluoride ions can adsorb to hydroxyapatite crystals, inhibiting

demineralisation during acid challenge and enhancing remineralisation when pH levels subsequently rise (Ten Cate, 2013).
Fluoride may also reduce acid production by inhibiting bacterial glucose metabolism and thus reducing acidogenesis and the associated enrichment of aciduric species in plaque (Bradshaw et al., 2002).
Fluorides are available as a mouth rinse or toothpaste preparations and are usually formulated with sodium, stannous or amine groups as a cation. Amine fluorides (AmF) were developed by the end of 1950. AmF is an organic fluoride with a special molecular structure. It consists of a hydrophilic and fluoride binding amine groups attached to a hydrophobic hydrocarbon chain. The hydrophilic part is aligned toward the tooth surface providing the fluoride to the tooth surface while the hydrophobic part is towards the oral cavity. For this reason, they act like surfactants, reducing the surface tension of saliva, and forming a homogeneous film on teeth and all oral surfaces. This continuous film prevents rapid rinsing off by the saliva. The AmFs are thus available for longer periods of time.
Amine residues of AmF possess their own antibacterial property due to the positively charged amine part. They also inhibit the metabolic activity of bacteria and reduce acid production. AmF has shown stronger antivital and antiadhesive effect on the initial biofilms and demonstrated a maximum antibacterial effect under the experimental conditions (Priya and Galgali, 2015). The primary effect of AmF is anticariogenic while there is some evidence of antiplaque and antigingivitis response with stannous fluoride or AmF/stannous fluoride formulations. (Zimmermann et al., 1993; Hoffmann et al., 2001).
There are only limited studies on AmF alone as an antiplaque and antigingivitis agent, there have been no studies evaluating mouth rinse containing only AmF as an active ingredient on plaque and gingivitis.(Priya and Galgali, 2015).
Chlorhexidine digluconate (CHX) has been used in dentistry for around 40 years (Varoni et al., 2012). Chlorhexidine demonstrated good substantivity, with approximately 30% of chlorhexidine dosed from a mouthwash retained in the mouth. Chlorhexidine has a broad spectrum of activity against Gram-positive and Gram-negative bacteria, and yeasts, and can reduce plaque, caries and gingivitis.
CHX is a positively charged bisbiguanide, which can adsorb to different negatively charged sites including mucous membranes, salivary pellicle on teeth and titanium implant surfaces as well as several components of the biofilm on the tooth surfaces such as bacteria, extracellular polysaccharides and glycoproteins (Kozlovsky et al., 2006).
Chlorhexidine is largely used as bactericidal agent due to the lethal damage to the bacterial membrane at hight concentration. At low sub-lethal concentrations, chlorhexidine can interfere in the oral bacteria metabolism by inhibiting sugar transport and acid production in cariogenic streptococci, various membrane functions in streptococci, including inhibiting enzymes responsible for maintaining an appropriate intracellular pH, and a major protease (gingipain) in the periodontal pathogen, Porphyromonas gingivalis (Marsh, 2010).
Following use of CHX-containing mouthwash, about a third of the active ingredient remains on the teeth, pellicle, oral mucosa, tongue and in salivary proteins (Varoni et al., 2012) providing sustained antibacterial properties for 8–12 h. These actions, especially when combined with tooth brushing, can lead to break up of existing plaque, reduction of plaque re-growth and inhibition of

the development of gingivitis (Van Strydonck et al., 2012). CHX mouthwashes may be recommended by dental professionals for use over a period of a few weeks or months in those for whom gingivitis is problematic (Van Strydonck et al., 2012).
Chlorhexidine mouth rinse (0.2%), among a myriad of oral hygiene products, has acquired an eponym of "gold standard against oral infections" owing to its dramatic therapeutic effect (Autio-Gold, 2008), but is also accompanied by some disquieting characteristics such as taste alteration, increase of supragingival calculus formation, burning of the mouth, more rarely desquamation of oral mucosa and parotid swelling (Jones, 1997; McBain et al., 2003; Guimarães et al., 2006).
Its long-term daily use is not recommended because it has been associated with a number of local side effects such as brownish discoloration of the teeth, restorative materials and the dorsum of the tongue (Leard and Addy, 1997).
In fact cationic antiseptics, such as chlorhexidine, may activate anionic chromatic particles contained in some food and drinks, causing interaction with tooth surfaces (Addy et al., 1995). It causes extrinsic staining by attaching to the polyphenolic and tannin group of beverages such as tea and coffee (Addy et al., 1979). In vitro, these colored particles can produce identically colored complexes such as those caused by chlorhexidine and observed clinically in individuals who drink tea, coffee or red wine compared with those who do not ingest these drinks (Leard and Addy, 1997).
Enzymes such as dextranases and glucanases have been used as antibacterial in order to distroy the structure of the biofilm by destroying the plaque matrix. Metal salts (copper, zinc, stannous) have been showed active against Gram-positive and Gram-negative bacteria and posses also a sub-lethal activity; for example, zinc can inhibit sugar transport, acid production and protease activity (Brading and Marsh, 2003).
Phenols, like as Triclosan are used in several oral care products, owing the broad antimicrobial spectrum and inflammatory properties (Brading and Marsh, 2003). Furthermore, the activity of Triclosan involves the inibition of acid production by oral streptococci and protease activity by *P. gingivalis*. Triclosan was far more active against the Gram-negative anaerobes implicated in gingivitis and periodontal disease, whereas the Gram-positive species associated with oral health, and which had a similar or lower MIC than the periodontopathogens, were relatively unaffected (Bradshaw et al., 1993). Additive anti-plaque and anti-gingivitis effects were reported when triclosan was combined with a complementary antimicrobial agent such as zinc (Brading and Marsh, 2003). The half-life for clearance of bound triclosan is ca. 20 minutes, compared to ca. 45 minutes for zinc, although triclosan can be detected in plaque for at least eight hours after toothbrushing (Cummins, 1992).
Quaternary ammonium compounds such as cetyl pyridinium chloride have been also largely used in mouthrinses. Cetylpyridinium chloride (CPC), has been demonstrated in clinical and in vitro studies to inactivate oral bacteria, reducing plaque and gingivitis. (He et al., 2011; Schaeffer et al., 2011).
Detergents surfactants are introduced in most toothpastes. At high concentrations, the surfactant activity of sodium lauryl sulfate can disrupt biofilm structure, damage cell membranes and kill bacteria; at lower concentrations sodium lauryl sulfate inhibits enzymes (Scheie and Petersen 2008) .
Nevertheless, many of these substances can cause unwarranted undesirable effects like vomiting,

diarrhoea and tooth staining.
So plant extracts have been studied as alternative compounds to chemotherapeutic agents to control localized oral diseases (Mandel, 1988; Baehni and Takeuchi, 2003).
Among the benefit, plant extracts may antagonize moderate or severe local infections and have the capacity to inhibit the cariogenic traits of mutans streptococci (Scalbert et al., 2005; Waikedrea et al., 2010; Risitano et al., 2014; Marsh, 2010).

c. Plant extracts

The increase of bacterial resistance against most of the employed antibiotics and antimicrobials in general, poses serious problems for the coming years (Ventola, 2015). This issue is the outcome of a lasting selective pressure acted on microbial population with a loss of the natural equilibrium among the biota colonizing human body. The permanent use and/or abuse of a narrow number of compounds with similar modes of action have promoted the increase of resistant strains (Norrby et al., 2005). Although increasing concentrations of drugs were used to overcome resistance, not worth results were obtained and often they were associated to unhealthy effects (Rybak et al., 2009). A further problem that jeopardizes the efficacy of antimicrobials is that many human bacterial infections are reinforced by the presence of a strong biofilm. Biofilms are adherent to tissue surfaces providing a protective coating, which is impermeable to the antimicrobial agents (Vieira et al., 2015). This trend has increased researches focusing on new therapeutic strategies based on novel substances, among which natural extracts from terrestrial and marine plants (Al-Haj et al., 2010). In this contest, ethnobotanical reports have evidenced that some plants provide bioactive molecules with antimicrobial activity (Verkaik et al., 2011). Thus, the control of pathogenic bacteria by natural, non-toxic molecules should be an ideal target in biomedical as well as in agrifood applications. Plant metabolites may provide a safe protection against moderate or severe local infections (Scalbert et al., 2005; Waikedrea et al., 2010; Risitano et al., 2014). However, independently by the field of application, a high volatility, instability and low water-solubility limit the efficacy and use of several plants exacts (Bonifácio et al., 2015). Micro and nano-encapsulation of plant metabolites is an innovative strategy to increase activity and persistence of extracts. Nano-encapulation leads to a reduction of the volatilization and degradation of active molecules with an increase of local bioavailability (Yang et al., 2009). Among the different carriers, phospholipid vesicles-like liposomes are widely recognized as a system to improve the therapeutic efficacy of compounds in biomedical and agrifood crops (Caddeo et al., 2013; Manca et al., 2014). The vesicles protect the loaded molecules from light and other degradation processes allowing, at the same time, the transport through biological barriers (Castangia et al., 2014, 2015a). Moreover, a great capacity in carrying drugs was reported by the use of innovative liposome-like structures deriving from partially modified liposomes by the addition of lipid components and water cosolvents (e.g., ethosomes, transfersomes, penetration enhancer containing vesicles, glycerosomes and hyalurosomes). The modified-liposomes (MLs) exhibited a better capacity of transport in comparison to the non-modified liposomes. Moreover, such MLs are able to control the release and cellular uptake of the encapsulated material so acting, not only as penetration enhancers, but also as an effective delivery system of natural

compounds and phytocomplexes (Moulaoui et., al 2015; Manca et al., 2015, 2016; Castangia et al., 2015a, b; Manconi et al., 2016). Furthermore, due to the versatility and absence of toxicity, liposome-like vesicles may be used as potential carriers to increase the efficacy of plant-derived antibacterial phytocomplexes, resulting in a possible useful way to formulate oral antimicrobial products. This should replay to the need of adding antimicrobials in the daily oral hygiene. In fact, oral antimicrobial agents aim to control the oral microbial homeostasis in the mouth. Bacteria either live in (more or less) planktonic form (in saliva) or as a biofilm on the tooth surfaces. Particularly, antimicrobials antagonize the stratification of the dental plaque, which is formed progressively by the colonization of initial, middle, and later bacteria colonizers (Kolenbrander et al., 2010). Preventing stratification of dental plaque, antimicrobials antagonize a breakdown in the biofilm (Marsh 2009) and the consequent development of oral disease (Costerton et al., 1999; Barnet et al., 2003; Marsh, 2005). Particularly, an oral antimicrobial may posses the capacity to oppose the growth of cariogenic bacteria, manly focusing on *Streptococcus mutans* and *Lactobacillum acidofilum* (Takahashi and Nyvad, 2008, 2011; Peterson et al., 2011). Also, antimicrobials may act as host defence in response to accumulation of plaque at the gingival margin, so opposing to periodontal disease (Socransky et al., 2005; Marsh 2010). Furthermore, antimicrobial agents should be useful in the acidic environment of xerostomia, in which the low salivary pH not only causes a prevalence of acidogenic micro-organisms but also plays a marked increase of *Candida albicans* (Almståhl and Wikström 1998; Pinna et al., 2015).

Sardinia (Italy) is a Mediterranean island rich in plant biodiversity and recent archeobotanic findings have evidenced that already in the Bronze Age, the first Paleolithic communities largely employed aromatic plants and started the domestication of fruit trees used for aromatic reasons as well as for popular medicine and/or alimentary purposes (Bozorgi, et al., 2013). This is the case of *Citrus limon. cv.* var. *pompia* Camarda (CLP), Thymus *herba-barona* Loisel (THB), and *Pistacia lentiscus* L. (lentisc) (PL) which are endemic in Sardinia. Also, *Vitis vinifera* L. *cv* Cannonau red grape (VVC) that is an autochthonous variety of Sardinia and is largely known for the health-beneficial effects of polyphenols.

Pompia, belonging to the genus *Citrus* and *Rutaceae* family is an endemic Sardinian's fruit, recently classified as an hybrid between lemon and citron (Camarda et al. 2013), which grows in central-east Sardinia, Italy. The fruit is a large, oblate fruit (500-600 gr) with a thick yellow rind at fully maturity, a small endocarp and a lemon-like taste. Chemical parameters are very similar to those of lemon grown in similar environmental conditions (D'Aquino et al., 1998, 2002). In addition, Pompia is largely used for culinary purpose in Sardinia. Nevertheless, due to a low availability in cultivation, only few papers reported the chemical composition of pompia and its potential application (D'Aquino et al., 1998, 2002; Manconi et al., 2016) and any studies didn't test the antibacterial activity of CLP.

Thymus herba-barona Loisel (THB) (Atzei, 2009) is an old, endemic plant with characters of creeping, woody-based perennial. It grows up to 10 to 25 cm, spreading out across the ground to a width of 30 cm. THB is largely used in Sardinia for culinary purpose due to its intense and aromatic smell. It also has larvicidal and mosquito-repellent effects. Furthermore, THB essential oil has high antiseptic, deodorant and disinfectant properties (Juliano et al., 2000). These characteristics have allowed THB to be used as anti-inflammatory agent in popular medicine in the ages.

Pistacia lentiscus (PL) is an evergreen endemic shrub, belonging to the Anacardiacea family. It is a small tree growing up to 1-8 m, well adapted to harsh conditions of growth due to the high resistance. So, it is largely distributed in dry and warm areas of Sardinia. Interestingly, PL plays a key role in phyto-stabilization of contaminated mines sites with heavy metal (Cd, Pb and Zn) (Bacchetta et al., 2015). A number of studies have shown beneficial effects of different parts of PL. The fatty oil, the so called "oil of the poor" in Sardinia, has characteristics of fragrance, anti-bacterial properties (Mezni et al., 2015), and anti-lipid properties at least in reducing the total cholesterol and triglycerides (Djerrou et al., 2014). Chio mastic derived from *P. lentiscus* may be considered as a conglomeration of effective anti-cancer drug attributed to the ability to inhibit cell proliferation through extrinsic and intrinsic apoptosis signaling pathways (Giaginis et al., 2011).
Vitis vinifera L cv. Cannonau (VVC), is a typical Sardinian red grape that has attracted scientific attention due to a possible relation with its high content of resveratrol, a phytoalexin antioxidant agent, and the longevity of Sardinian people. *Trans*-resveratrol and its oligomers (e.g., dimers called viniferins) can be produced in grapevine tissues as an active defense strategy against diseases. In fact, "inducible" viniferins are hardly detectable in healthy leaves, but their increase in infected leaves was observed as a resistance markers for disease (Bavaresco et al., 2016).
The high amount of resveratrol in the red grape is attributed to the grape variety (Bavaresco et al., 2007; Gatto et al., 2008), the clone (Gatti et al., 2014), the meteorological conditions (Bavaresco et al., 2007), the soil type (Bavaresco et al., 2005) and cultural practices (Bavaresco et al., 2001; Gebbia et al., 2003; Gatti et al., 2011). The low levels of fining agents usually added to stabilize red wines do not significantly reduce the level of *trans*-resveratrol (Threlfall and al., 1999), and it is a relatively stable compound that can remain for years in properly stored wines (*i.e.*, avoiding exposure to heat and assuring the presence of normal levels of exogenous antioxidants such as sulfur dioxide) (Mattivi and Nicolini, 1993). The ability of the grapevine to activate defense mechanisms against some pathogens has been shown and has been linked to the synthesis of resveratrol and other stilbenes by the plant (inducible viniferins) (Bavaresco et al., 2016).

4. Aim

In view of the increasing interest in developing antibacterials of natural origin, this study aims at characterizing chemically and evaluate the activity of CLP and VVC extracts and of the essential oils (EOs) of THB and PL against oral commensal and pathogenic bacteria in comparison to that of the same agents incapsulated in MLs**.** With this intent, we selected *Streptococcus sanguis* as one of initial colonizer of the dental plaque; *Fusobacterium nucleatum* as middle colonizer and *Enterococcus faecalis* as later colonizer. In addition, the antimicrobial activity against *Streptococcus mutans* and *Lactobacillus acidophilu* was tested. These two bacteria are the most common species involved in dental caries. Furthermore, we choose *Candida albicans* to test the antimicrobial activity of the agents against the most common pathogen during xerostomia.

5. Material and Methods

This study was conducted using the extracts of CLP and VVC, and the EOs of THB and PL (group A) and the extracts of CLP and VVC, and the EOs of THB and PL inglobated in MLs (group B).

1. Plant collection and extracts preparation

'Pompia' (*Citrus limon* L. Camarda) (CLP) fruits (Fig.5.1.) were collected in January 2016, near the Cedrino river (Orosei, Sardinia), in the experimental fields of Erbosard Srl (Nuoro, Italy), cold stored at 5 °C until arrival at the laboratory and subsequent rind removal. In October 2016, 'Canonau' (Vitis vinifera L.) (VVC) pomacee (Fig.5.2), without seed, was collected immediately after pressing from a wine factory in the central-East mountain region of Sardinia (Gostolai, Oliena - NU), and cold stored at 5 °C until extraction. Wild thyme (*Thymus herba-barona*) (THB) aerial parts (flowers, leaves and stems) (Fig.5.3) were collected from 3 different areas of Sardinia (Italy): Gennargentu, Limbara and Marghine-Goceano. Harvest took place in July 2016 when the plant earns its "balsamic time", samples were authenticated by Prof. Giovanni Bacchetta and voucher specimens of the plants were deposited in the Herbarium CAG of the University of Cagliari, Italy. Lentisc (Pistacia lentiscus L.) (PL) (fig.5.4), leaves were collected in the Mount Arcosu area (South Sardinia), in Dicember and as for THB the samples were authenticated by Prof. Giovanni Bacchetta and voucher specimens of the plant were deposited in the Herbarium CAG of the University of Cagliari, Italy.

FIGURE 5.1. *Citrus limon* L. cv. *pompia* Camarda

FIGURE 5.2. *Vitis vinifera* L. *cv.* Cannonau

FIGURE 5.3. ***Thymus herba-barona*** **Loisel**

FIGURE 5.4. ***Pistacia lentiscus*** **L.**

1.a. Plant tissue extraction

The rind (flavedo and albedo) of the 'Pompia' fruit was removed within 48 h after harvest and subject to extraction as follows: the rind (1 kg) was minced with a knife (5x5 cm) and then homogenized (Waring Commercial Blender, 06790 Tonington Connecticut USA) adding a cold water/ethanol (60:40 V/V) solution at a 1:2 ratio (W:V). Homogenization took place for 10 min and the homogenate was then centrifuged (Sorvall Super T21, Dupont de Nemours, MI, Italy) at 4000 g and 2 °C for 15 min, the supernatant was collected and the same extraction protocol was performed twice with the pellet. Finally, 5.8 L of extract was yielded, ethanol was removed with a rotovapor (Buchi rotovapor R 300, Buchi-Italia - 20010 Cornaredo, Italy) operating at 45 °C while, water was removed by ice-drying the frozen water-extract. In this way, 72 g of a fine yellow powder was obtained (7.2% yield) and stored under vacuum until use. To perform the chemical characterization of the extract 0.5 g of the powder was dissolving in methanol (1:100; W/V), filtered through a 0.20µm PVDF filter (Whatmann International Ltd., UK) and 5 mL were injected into the analytical system.
The pomace of 'Cannonau' wine grape, without seeds, at arrival in the laboratory was put into a heat (30 °C) ventilated armoire (Memmert 260, Spinea-Venezia, Italy) until dry (aw 0.2). Then the pomace was powdered by milling the matrix with a ball-mill (Retsh Emax, Retsch GmbH, 42781 Haan, Germany) for 20 min. The extraction was performed using 50 g of the fine powder dissolved in water/ethanol (1:1 V/V), and the suspension was then continuously stirred at 25 °C for 24 h. At scheduled time intervals (1, 2, 4, 6, 8 and 24 h) the dispersion was sonicated for 1000 s (200 cycles, 5 on, 5 off, 15 microns of probe amplitude), with a high intensity ultrasonic disintegrator (Soniprep 150, MSE Crowley, London, UK). After that, the suspension was centrifuged (40 min, 1500 g), and ethanol was removed by means of a rotovapor at 30 °C while, water was by ice-drying the frozen water fraction. This protocol provided a dark-red powder that was stored under vacuum until use. To perform the chemical characterization the sample was processed as the 'Pompia' extract.
The fresh material from thyme, used for the extraction of essential oil (EOs), was air-dried with the same device and protocol adopted for 'Cannonau' pomace. Then, 150 grams of the dry areal parts of the plant were steam-distilled using a circulatory Clevenger-type apparatus according to the European Pharmacopoeia for 4 h (European Pharmacopoeia, 2002). The distillate was then dried over anhydrous sodium sulphate and the attained EOs stored at low temperature until analysis and use.
Fresh-cut branches of P. lentiscus were delivered to the laboratory and leaves removed by means of scissors, then a mild drying occurred in a ventilated system kept a 25 °C for 12 h. Following drying, leaves were steam-distilled and the distillate was dried as described for THB.

1.b. Extract and essential oils characterization (Group A)

The main components of the extracts of CLP were separated and identified by liquid chromatograph-mass spectrometer using a Flexar UHPLC AS system (Perkin-Elmer, USA) equipped with a degasser, Flexar FX-10 pump, auto sampler and PE 200 column oven interfaced to an AB Sciex API4000 Q-Trap instrument (Foster City, CA, USA). The mass spectrometer worked with a triple quadrupole analyser in Multiple Reaction Monitoring (MRM) mode. Chromatographic separation was carried out using a XSelect HSS T3 column (Waters, Milford, MA) (100 x 2.1 mm i.d., 2.5 µm d) and a mobile phases containing water and acetonitrile.
While VVC extract was analysed by a reverse phase liquid chromatography (RPLC) employing a High Pressure Liquid Chromatography system consisted of an Hewlett-Packard series 1100 L equipped with a Diode Array Detector (DAD) operated by a HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA). The HPLC-MS system was equiped HPLC-DAD instrument coupled to a quadripole mass spectrometer HP 1100 MSD electrospray interface (ESI) (Agilent Technologies, Palo Alto, CA, USA). In order to obtain the maximum sensitivity of ESI and to use similar analytical conditions to those of the HPLC-DAD protocol, an orthogonal geometry position of the the capilary inlet with respect to nebulizer was used. Separation occurred on a reverse-phase Waters Nova-Pak C18 column [150 mm x 3.9 mm, 4 µm] for anthocyanins analysis and a reverse-phase Waters Nova-Pak C18 column [300 mm x 3.9 mm, 4 µm] for non-anthocyanin phenols both kept at 26°C with a pre-column of the same phase. DAD-detection for anthocyanins was performed from 260 to 600 nm and ESI-MS parameters were: drying gas (N2) at 350°C with a 10 L/min flow; nebulizer pressure, 380 Pa (55 psi), and capillary voltage, 4000 V. The ESI was used in a positive way scanning the mass from m/z 100 to 1500 employing a fragmentator voltage gradient of 100 V from 0 to 17 min and 120 V from 17 to 55 min (Dobes et al. 2013). DAD-detection for non-anthocyanin phenols was performed from 220 to 380 nm and ESI-MS parameters were: drying gas (N2) at 350°C with a 10 L/min flow; nebulizer pressure, 380 Pa (55 psi), and capillary voltage, 4000 V. The ESI was used in a negative way scanning from m/z 100 to 3000 employing a fragmentator voltage gradient of 100 V from 0 to 200 m/z and 200 V from 200 to 3000 m/z. Elution of anthocyanins was performed at a 0.8 mL/min gradient flow of: A) water/formic acid, (90:10, v/v) and B) water/methanol/formic acid, (45:45:10, v/v/v). Formic acid was emploied as a pH modulating agent in order to optimize the anthocyanins detection by maximizing the absorption in the λ 520 nm region. Elution of non-anthocyanin phenols was performed at a 0.7 mL/min gradient flow of: A) water/acetic acid, (98:2, v/v) and B) water/acetonitrile/acetic acid, (78:20:2, v/v/v). The column was washed with MeOH and re-equilibrated from 90 to 120 min. The volume of injected leave extract was 15 µL. Identification of anthocyanins was performed by comparing the results of commercial standards (Sigma-Aldrich) with the positive ion mass spectra achieved from the ESI-MS (retention time (tR), UV λmax and MSn) of the extract or by comparing the ESI-MS attained results with those available in the literature. The identifiction of flavonols under ESI-MS was achieved according to the molecular and fragment ions [M-H-162] and [M-H-176]. In addition, the identity of all other constituents was validated by comparing the attained retention times (tR), UV λmax and MSn of peaks from the leave extract with those reported in the literature. External standard calibration curves were performed in duplicate by analysing five dilutions of stock standards and buildingthe calibration

curve of each compound by linear regression of standards peak area against their known concentrations (R2 between 0.98, 0.97). The result represented the average of 3 runs each concentration.
The identification of THB and PL EOs was carried out by gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS). GC-MS analysis using a gas chromatograph (Agilent, Model 6890N, Palo Alto, USA).
The total phenolic content of CLP and VVC extracts were determined according to the Folin-Ciocalteu colorimetric assay using a UV spectrophotometer (Lambda 25, Perkin Elmer, Monza, Italy). Briefly, an aliquot of the extract in water, an aliquot of the Folin-Ciocalteu reagent and aqueous solution of 20% (w/v) Na_2CO_3 were mixed and the absorbance was read at 765 nm after 30 min of incubation in the dark, at room temperature. The total phenolic content was calculated by means of a calibration curve obtained using gallic acid as a reference, at different concentrations (0-0.125 mg/mL). Results, were expressed as mg of gallic acid equivalents per g of dry extract (mg GAE/g), and data reported are means of six independent determinations.

1.c. Vesicles preparation and characterization (Group B)

The extract of CLP and VVC as well as the THB and PL EOs were incorporated into innovative phospholipid vesicles. The new phospholipid vesicles were obtained by the addition of appropriate water co-solvents (glycerol, lecetin) or polymers in order to improve cell-absorption and avoid the leakage of bioactive molecules in the saliva. The innovative phospholipid incorporating phytocomplexes were prepared using environmentally-friendly techniques (e.g., without organic solvents) as follow: the CLP extract and EOs were dispersed in water or water containing co-solvents or natural polymers with the phospholipids. The dispersions was left to swell overnight at room temperature, and then sonicated to produce the new phospholipid vesicles. Separation of non-incorporated CLP, VVC, THB, PL molecules from charged vesicles was performed at room temperature by loading the dispersions (1 mL) into dialysis tubing (Spectra/Por® membranes, 3 nm pore size; Spectrum Laboratories Inc., Rancho Dominguez, USA) and dialysis was performed against water by changing it each hour. After 2-6 h (depending upon the dispersion) a compete removal of non-incorporated molecules was achieved. Before and after dialysis, the vesicles were disrupted with methanol (1/100, V:V), and the phytocomplex concentration was established by using the DPPH assay (antioxidant activity) or by the quantification of their main components by HPLC. The entrapment efficiency (EE%) was expressed as the concentration percentage of phytocomplexes ex-ante dialysis.
The obtained phytonanovesicles were characterized in terms of size distribution, zeta potential, entrapment efficiency, and stability during storage. Vesicle formation and morphology were checked using transmission electron microscopy (TEM). Samples were stained with 1% phosphotungstic acid and examined with a JEM-1010 (Jeol Europe, Paris, France) transmission electron microscope equipped with a digital camera MegaView III and the software “AnalySIS”, at an accelerating voltage of 80 kV. The average diameter, polydispersity index (PI, a measure of the width of size distribution) of vesicles were determined by Dynamic Light Scattering using a Zeta sizer nano-ZS (Malvern Instruments, Worcestershire, UK). The zeta potential was also estimated

using the Zeta sizer nano-ZS, which converts the electrophoretic mobility by means of the Smoluchowski approximation of the Henry equation. Samples were diluted (1:100, V:V) with PBS, or the appropriate water mixture used as hydrating medium, and analysed at 25 °C. The stability of the vesicles was evaluated by measuring vesicle average size, PI and zeta potential over 3-6 mounts when kept at room temperature.

2. Antimicrobial activity

The antimicrobial activity of the extracts of CLP and VVC and the EOs of THB end PL and that of the corresponding phytonanovesicles (EOs and extract incorporated in liposomes) was tested using the following microrganisms:

1) *Streptococcus sanguinis (*private collection*)*
2) *Fusobacterium nucleatum*(ATCC 25586**)**
3) *Streptococcus mutans* (ATCC 35668)
4) *Lactobacillus acidophilus* (ATCC 4356)
5) *Enterococcus faecalis* (ATCC 29212)
6) *Candida albicans* (ATCC 10231)

2.a. Preparation of Bacterial Inocula

Stock culture of each of the tested bacteria was maintained at −20 °C and was recovered by subculturing using Mueller Hinton Agar nutrient (MHA) for *Enterococcus faecalis* and the same medium with the addition of 5% sheep blood was used for *Steptococcus sanguinis*, *Streptococcus mutans* and *Lactobacillus acidophilus*. The Schaedler Agar with vitamin K1 and 5% sheep blood was employed for *Fusobacterium nucleatum* while, Sabouraud Destrose Agar was used for *Candida albicans* according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2015). Once colony growth became evident, one or two pure colonies have been selected from each culture plate and aseptically transferred by mean of a sterile loop into test tubes containing 2 mL of sterile saline solution. McFarland standard was used (0.5) as a reference to adjust the turbidity of bacterial suspensions to the required range for bioassays (1-2 x10^8 UFC/ml).

2.b. Assessment of the Antibacterial Activity

Antibacterial activity of the extracts of CLP and RG and the EOs of THB end PL and that of the corresponding phytonanovesicles was carried out using the disc diffusion method also known as Kirby-Bauer antimicrobial susceptibility test method (Bauer and Kirby 1966). Sterile Whatman filter paper disks of 6 mm of diameter were impregnated with 15 µl of each agent in a sterile biological safety cabinet. The discs were then aseptically placed in the center of inoculated Petri plates (9 cm in diameter) containing an overnight culture of an uniformly spread with 0.1 ml of the McFarland standard bacterial suspension of each microorganism. Gentamycin (10 mg/disk; Gibco) and Ketoconazole (10 mg/disk; Janssen Pharmaceutics) were used as negative control for bacterial and fungal strains respectively and plates inoculated with only bacterial suspension were used as strains vitality positive controls (Gakuubi et al., 2016). The plates were refrigerated at 4°C for 2 hours to allow the essential oils to diffuse into the agar medium and incubated upside down at 37°C for 48 hours. The tests were conducted in triplicate and the measure of the inhibition zones was read after 24 and 48 hours.

The sensitivity of individual bacteria to the agents was ranked based on the inhibition zone values expressed in millimeters (mm) as follows: not sensitive (−) for total zone diameters of ≤12 mm; sensitive (+) for diameters ranging between 12 and 19 mm; extremely sensitive (+++) for zone diameters of ≥20mm. The bioassays were conducted in a biological safety cabinet and in accordance with the protocols of Clinical and Laboratory Standards Institute (CLSI) formerly National Committee for Clinical Laboratory Standards (NCCLS). (NCCSL, 1997, 1999).

6. Results

a. Extract and essential oils characterization

The 13 main components of the ethanol-water CLP rind extract are represented in Table 6.1.

Table 6.1. Main components of the *pompia* rind extract.

Compound	µg/mg*
Eriocitrin	0.09
Ferulic acid	1.03
Isoquercetin	0.4
Isorhamnetin rutinoside	0.63
Myricitrin	0.5
Naringin	23.77
Neoeriocitrin	46.53
Neohesperidin	44.57
Phlorizin	0.01
Quinic acid	219.67
Robinin	1.08
Rutin	8.61
Sinapic acid	30.13

* Related to fresh weight

VVC pomace ethanol/water extract allowed the identification 12 main compounds (Table 6. 2).

Table 6.2 Main compounds characterized in the pomace extract of *Vitis vinifera* L *cv* Cannonau

Compound	µg/mg*
Gallic acid	12.96
Ethyl gallate	0.04
Methyl gallate	0.25
Catechin	227.50
Epicatechin	174.97
Epicatechin gallate	36.62
Procyanidin B1	176.60
Procyanidin B2	74.97
Rutin	0.24
Quercetin-3-O-glucoside	2.25
Quercetin-3-O-rhamnoside	0.20
Isorhamnetin-3-O-rutinoside	0.01

* Related to fresh weight

The GC analysis of the essential oil extracted from THB aerial parts allowed the identification of 21 main compounds (Table 6.3)

Table 6.3. Main compounds characterized in *Thymus herba-barona* essential oil

Compounds	Percentage (%)*
Heptanone	4.5
α-Thujene	0.5
α-Pinene	0.3
Camphene	0.3
Octanone	5.5
β-Myrcene	0.4
Octanol	2.6
α-Terpinene	0.5
p-Cymene	5.6
Limonene	0.2
γ-Terpinene	4.8
Z-Sabinene hydrate	0.4
Nonanone	0.7
Linalool	4.5
Borneol	30.5
Terpinen-4-ol	2.1
Thymol	34.2
Carvacrol	15.7
Carvacrol acetate	0.2
β-Caryophyllene	0.5
Caryophyllene oxide	0.5

* Percentage related to fresh weight

The GC analysis of the essential oil extracted from PL aerial parts allowed the identification of 17 main compounds (Table 6.4).

Table 6.4. Chemical composition of *Pistacia lentiscus* L. essential oil (%)

Compounds	Percentage (%)*
α-pinene	10.8
Camphene	1.3
Sabinene	0.3
β-pinene	5.4
Myrcene	1.4
α-phellandrene	4.3
α-terpinen	1.3
p-cymene	6.2
Limonene	6.0
β-phellandrene	8.6
γ-terpinene	4.3
α-terpinolene	2.0
terpinen-4-ol	11.1
α-terpineol	4.2
β-caryophyllene	3.7
germacrene D	1.8
δ-cadinene	2.4

* Percentage related to fresh weight

b. Antimicrobial activity

Kirby-bauer disk diffusion susceptibility test showing on the plates the sensitivity of individual bacteria to the agents. (Figure 6.1, 6.2, 6.3, 6.4, 6.5, 6.6).
In Kirby–Bauer test, discs containing the antimicrobial agents that we are testing are placed on agar where bacteria are growing, and the agents diffuse out into the agar. If the agent stops the bacteria from growing, circular areas are shown around the disk where bacteria have not grown.
Gentamycin and ketoconazole used as negative test control for bacterial and fungal strains, respectively showed inhibition halo of 25-30 mm while the positive test control didn't show any halo formation.

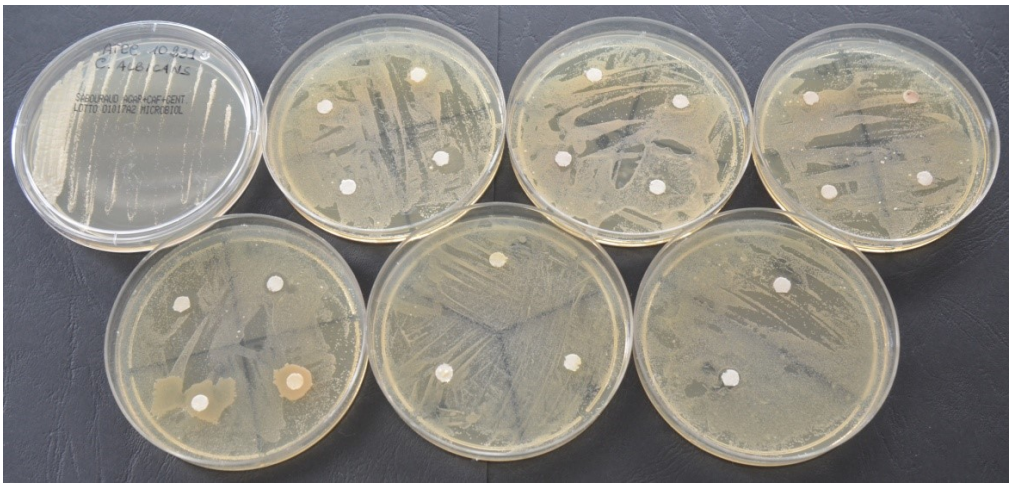


Fig. 6.1. Kirby-bauer test on *C. Albicans*

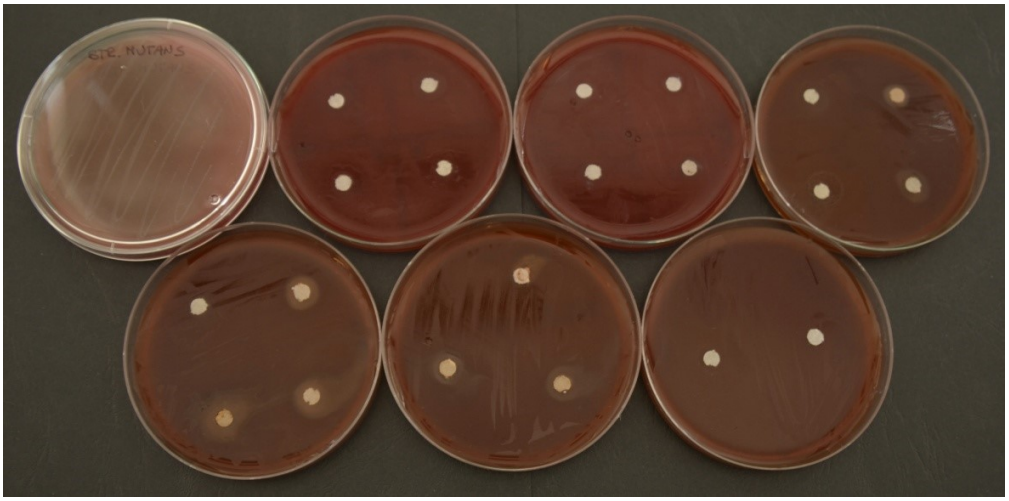


Fig. 6.2. Kirby-bauer test on *S. Mutans*

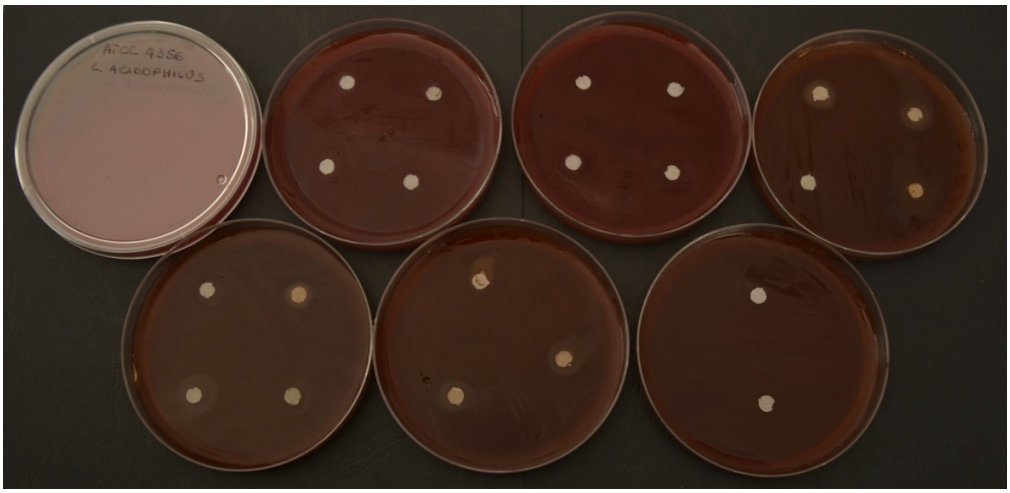


Fig. 6.3. Kirby-bauer test on *L. Acidophilus*

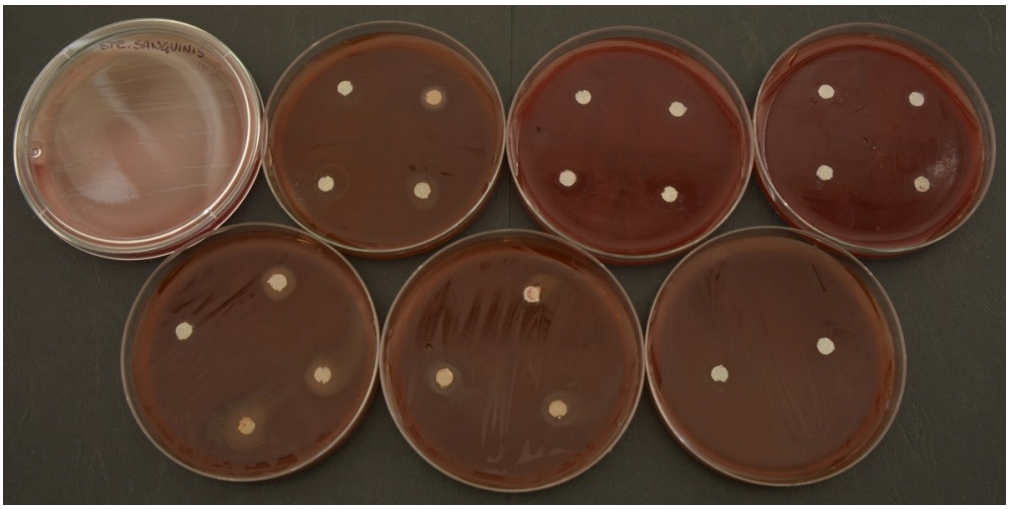


Fig. 6.4. Kirby-bauer test on *S. Sanguinis*

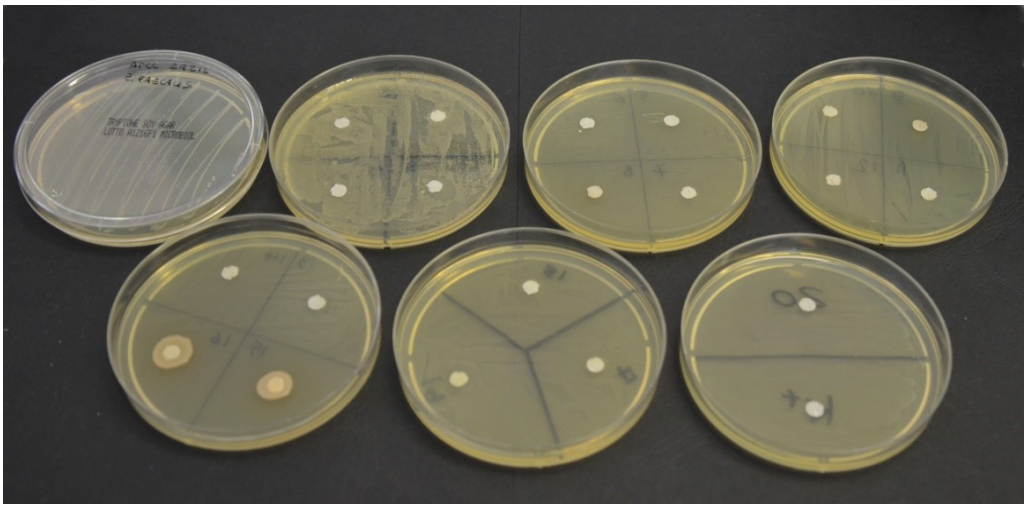


Fig. 6.5. Kirby-bauer test on *E. Faecalis*

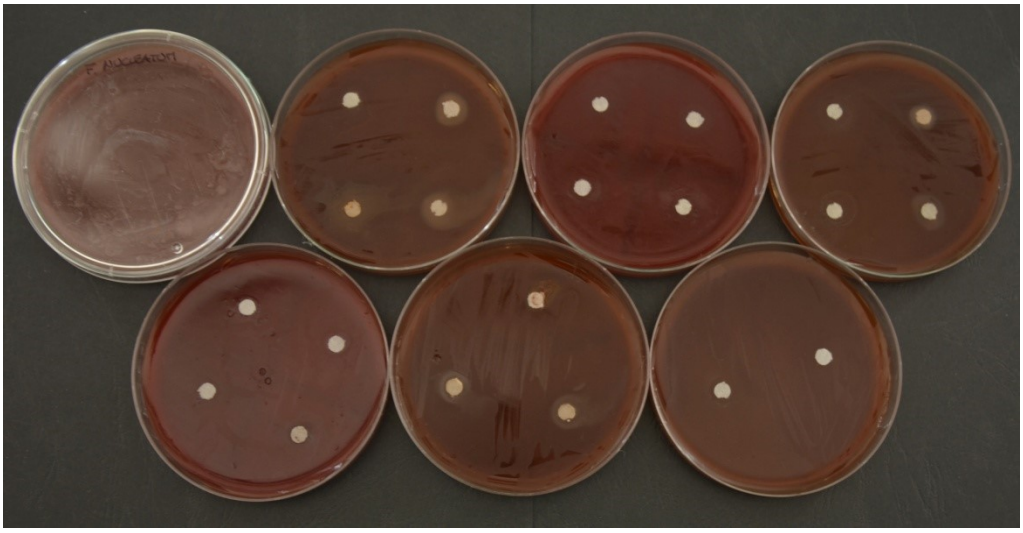


Fig. 6.6. Kirby-bauer test on *F. Nucleatum*

The results indicated in Table 6.5 represent the zone (mm) of inhibition including the diameter (6 mm) of the paper disk in group A and B. The scale of measurement was the following (disk diameter included): >=20 mm is strongly inhibitory, 19-12 mm zone of inhibition is moderately inhibitory, and < 12 mm is no inhibitory. The Chart 6.1 represent sensitivity of individual bacteria to the agents of Group A (EOs and pure extracts) and the Chart 6.2 sensitivity of individual bacteria to the agents of Group B (Agents when encapsulated in MLs).

Table 6.5.

Formulates	*C.albicans*	*S.mutans*	*L.acidophilus*	*S.sanguinis*	*E.faecalis*	*F.nucleatum*
CLP (A)	0	0	0	11	0	0
CLP (B)	0	12	13	12	0	10
VVC (A)	0	10	10	11	0	0
VVC (B)	0	14	12	13	0	0
THB (A)	13	15	13	14	12	0
THB (B)	7	11	11	11	10	0
PL (A)	0	0	0	0	0	0
PL (B)	0	14	12	12	12	11

The zone of inhibition of .*S. mutans*, *S.sanguinis* and *L. acidophilus* appeared wider when CLP, VVC e PL phytocomplex were incorporated in MLs. Instead, they didn't show any activity against *C. albicans.* Otherwise the zone of inhibition of the cariogenic bacteria apperead wider when THB was tested as Eos showing activity also against *C.albicans.*

Chart 6.1.

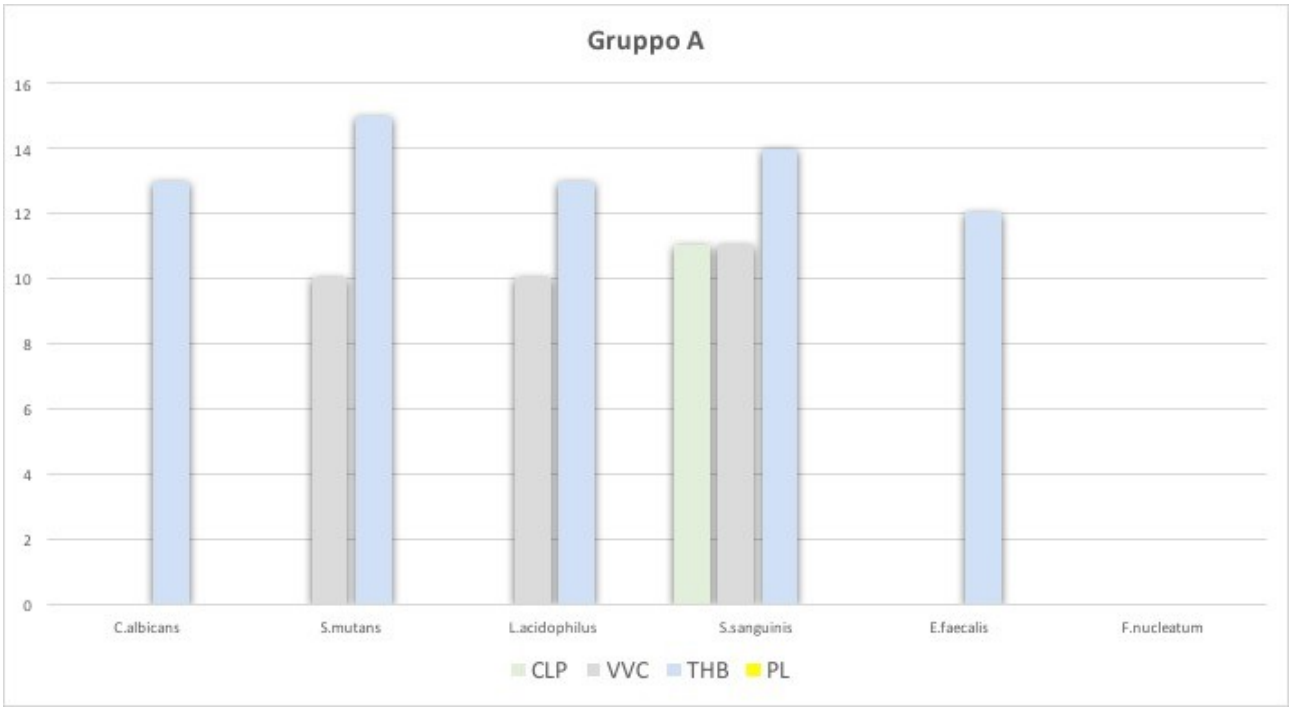
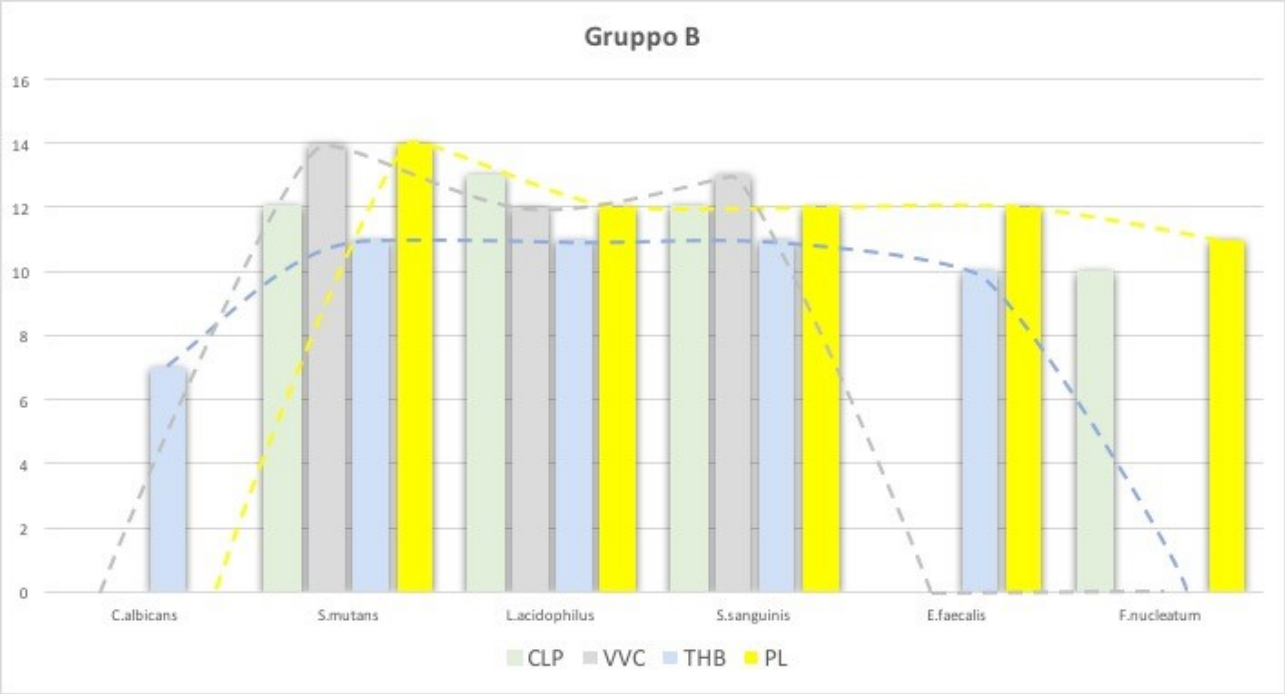


Chart 6.2.


Gruppo B
16
14
12
10
8
6
4
2
0
C.albicans
S.mutans
L.acidophilus
S.sanguinis
E.faecalis
F.nucleatum
CLP
VVC
THB
PL

7. Discussion

Researches on plants derived molecules as antimicrobials has been encouraged in the latest decades based on the view that natural agents should be better for health and environment than synthetic molecules (Ríos et al., 2005) and because of the incresing bacterial resistance against antibiotics and antimicrobials (Ventola, 2015). Many side effects have been reported by the use of pharmaceuticals (Norrby et al., 2005) and alternative, safe compounds obtained from plants would be hopeful.

Oral investigations have pointed on analysing plant extracts as effective antiplaque mean (Quintas et al., 2015) comparing their activity to chemicals with *in vitro* and *in vivo* studies (Charles et al., 2004; Ríos et al., 2015; Kazhila et al., 2016). Moreover, recent papers focused attention on the bactericidal and anti-inflammatory effects of different variety of thyme (Marchese et al., 2016) and *Pistacia Lentiscus* (Iauk et al., 1996; Aksoy et al., 2006). A great amount of research shared antioxidant capacities in *Citrus* and *Vitis Vinifera* as well as abilities in activating host defence against some pathogens (Bavaresco et al., 2016). However, efficacy of plant metabolites depends on different issues, among which the environment in which plants grow (Dell'Agli et al., 2012; Barra et al., 2007). In fact, environmental conditions such as soil, weather, natural *versus* cultivated crops may turn up characteristics and concentration of bioactive metabolites even in the same plants (Barra et al., 2007; Bavaresco et al., 2016; Nassiri-Asl and Hosseinzadeh, 2016). In addition, efficiency of plant derived bioactive metabolites may be affected by a high volatility, instability and low water-solubility (Bonifácio et al., 2015). To overcome this matter, nano-encapulation of phytocomplexes has been introduced in the laboratory practice generally permitting better efficiency to the drug delivery in comparison to non-encapsulated compounds (Yang et al. 2009, Caddeo et al., 2013, Manca et al., 2014, Castangia et al., 2015a, b; Manconi et al., 2016).

This is the first study evaluating phytocomplexes of autochthonous Sardinian plants as oral antibacterial agents. The phytocomplex ability was evaluated as the efficacy of pure extracts and EOs, in group A, in comparison to that of the same agents encapsulated in MLs, group B. A high ratio of extract or oil/phospholipid was used to make MLs in addition to glycerol and sodium hyaluronate with the purpose to preserve activity and allow contact to the bacteria cell. In order to gain knowledge on the antimicrobial ability of CLP, THB and PL and VVC, this first study was conducted using planktonic cells culture of oral commensal, cariogenic and pathogenic bacteria, using the inhibition halo test, according to the NCCSL (1997, and 1999). In addition, chromatographic analysis of the plants was carried out in order to better understand the chemical constituents of the phytocomplex which were involved in the interaction with bacteria.

Data obtained in this study showed that the agents, when in groups A or in B, generally produced different sensitivity in the same bacteria, which may be attributed to the chemical composition of the phytocomplex and the availability of the drug to interact with microorganisms.

The phytochemical characterization of CLP showed that it was rich in phenolic and flavonoids compounds. A high quantity of phenols is a common report in *Citrus* genus and supports beneficial health effects in many disease, like cardiovascular, neurodegenerative and cancer disease (Scalbert et al., 2005). Moreover, a linear relationship between the anti-oxidant capacity of CLP and the anti-inflammatory abilities was reported along with a host defence regulation capacity

(Abirami et al 2014, Manconi et al 2016).
Quinic acid, an oxygenated terpenoid, was the most rappresentative compound of the ethanol/water extract. It was reported that quinic acid has great antimicrobial activity inducing characteristic and distinct antimicrobial patterns (Zengin and Baysal, 2014). Moreover, synergism or antagonism may be observed in the case of terpenes combination (Aslani et al., 2013, Ahameethunisa and Hopper, 2012). The outer membrane or bacteria cell wall is most likely to be the cellular target for terpenes inducing pores and perforations in the membrane. Cell death may occur as a result of an extensive loss of the cell contents, including critical molecules and ions, or the initiation of autolytic processes (Ultee et al 2000). Moreover, differences in cell wall composition (lipid ratio) between different bacterial species, may partially accounts for a different susceptibility to terpenes.
In group A, CLP extract in ethanol/water, displayed to be effective against the Gram-positive, facultative anaerobiotic *Streptococcus sanguinis.* Any other bacteria, neither the oral commensal nor the cariogenic bacteria and *Candida albicans* didn't show sensitivity to CLP. Conversely, when CLP was formulated in MLs, group B, the efficiency significantly rose in comparison to the group A.
The inhibition halo was reported for all the Gram-positive batteria, including the cariogenic *Steptococcus mutans* and *Lactobacillus acidophilus*, *Streptococcus faecalis* and an increase of sensitivity of *Streptococcus sanguinis*. However, the Gram-negative *Fusobacterium nucleatum* and the pathogen *Candida albicans* remained not sensitive to CLP. Thus, we can presume that the encapsulation in MLs had increased the biovailability of the drug against bacterial membrane determining amplification of the antibacterial effects which involved all the type of the Gram-positive bacteria but not Gran-negative and Candida albicans. Our results are in accordance with previous reports which outlined the inefficiency of terpens on Gram-negatives. Differences in the permeability, composition, and charge of the outer membrane of the microorganisms should be determinant in the sensitivity to terpenes (Zengin and Baysal, 2014).
VVC phytochemical characterization demonstrated an abundant phenolic fraction, in particular phenolic acids and flavonoids. Among the phenolic components, gallic acid, the two flavanols cathechin and epicatechin, and the flavonol quercetin, which are widely recognized as anti-oxidant and anti-inflammatory agents (Biasi et al., 2013). Antioxidant and antibacterial activities in polyphenols showed a positive correlation. However, although a broad spectrum of activity was evidenced against Gram-positive, polyphenols had little to no antibacterial efficacy against Gram-negative bacteria (Changmou et al., 2014). Furthermore, studies demonstrated that phytochemicals in raisins were effective against the growth of oral microorganisms associated with dental diseases (Rivero-Cruz et al., 2008, Biasi et al., 2013).
VVC extract, group A, was not effective as antibacterial agent. In fact, the inhibition halos reported, in the case of cariogenic bacteria and *Streptococcus sanguinis*, ranged between 10 to 11mm respectively. Moreover, any halo wasn't formed in the case of the other microorganisms. Conversely, in group B, VVC demonstrated a broad spectrum of effectiveness toward the Gram-positive bacteria showing the formation of halos of sensitivity for cariogenic bacteria and *Streptococcus sanguinis.* However, any sensitivity wasn't confirmed in the case of the Gram-positive *Streptococcus faecalis* and the Gram-negative *Fusobacterium nucleatum* as well as *Candida albicans.* Then, we can say that, as it was in the case of CLP, the encapsulation in MLs had benefices for VVC improving the therapeutic efficacy and turning in effective the activity of VVC

phytochemicals to the Gram-positive bacteria. Moreover, our results confirm previous data reporting the incapacity of poliphenols derived from *Vitis Vinifera* to interact with Gram-negative (Changmou et al., 2014). As regard to *Candida albicans* any benefical effect of VVC extract wasn't show neither in group A nor in B.

THB phytochemical characterization demonstrated that tymol and Borneol (5-isopropyl-2-methylphenol) were predominant followed carvacrol, 3-Octanone, *p*-cymene and linalool. These compounds are aromatic monoterpenes, phenol derivatives that have powerful anti-bacterial and anti-fungal properties (Zengin and Baysal, 2014; Nabavi et al., 2015; Marchese et al 2016). Due to the absence of cytotoxicity and the broad spectrum of activity, carvacrol, α-pinene, *p*-cymene, thymol and limonene have been allowed as flavourings in foodstuffs by the European Commission. Moreover, thymol has some health-beneficial biological functions such as anti-mutagenic and anti-cancer activity as well as antioxidant and anti-inflammatory property (Kazhila, 2016).

THB oil demonstrated the greater spectrum of antimicrobial activity in comparison to the other phytochemicals evaluated in group A. Sensitivity involved any Gram-positives as well as *Candida albicans*. Particularly, the inhibition produced to *Steptococcus mutans* was the strongest of both the groups of this study. Any activity however, wasn't showed in the case of the Gram-negative *Fusobacterium nucleatum.* The combinations of THB terpenes, such carvacrol, linalool and α-pinene, may have had a synergistic interaction managing the better antimicrobial activity (Zengin and Baysal, 2014). Yet, THB in MLs, group B, decreased the activity versus all the microorganisms up to becoming not-effective. This was true particularly in the case of *Candida albicans* that reduced the halo of sensitivity to THB from 13 up to 7 mm. Nanoincapsulation of *thyme* was suggested to enhance lipophilicity of thymol in the hydrophobic domain of the bacterial membrane (Marchese et al., 2016). However, it was not effective for THB probably due to interferences between the drugs and the carrier's chemical characteristics which, in the same way, would have interfered in delivering compounds in contact to the bacterial membrane. The inefficiency of the thyme phytocomplex when delivered by vesicles is in accordance with previous studies (Coimbra et al. 2011).

PL was characterized by the presence of four main terpene-based components: α-pinene, *p*-cymene, terpinen-4-ol, and sabinene. Studies reported the great antimicrobial, antiinflammatory, and antioxidant activities of these compounds (Griffin et al., 1999; Panizzi et al., 1993; Grassmann et al., 2000; Alma et al., 2004). PL had inhibitory effects against Gram-positive, particularly in the case of *Streptococcus mutans,* as well as Gram-negative bacteria strain. Moreover, the anticancer capacity of PL it was reported due to inhibition of cells proliferation by intrinsic and extrinsic apoptosis signalling pathway (Aiche et al., 2015).

PL oil, in group A, was not effective as oral antimicrobial agent with any activity toward the tested microorganisms. However, when PL was in MLs, group B, efficiency against all the strain of Gram-bacteria became high including the *Fusobacterium nucleatum.* The greater capacity of inhibition was seen in *Steptococcus mutans* whose value was the higher in comparison to the other agents in group B.

8. Conclusions

Based on the results obtained in this study, we can argue that the antimicrobial efficiency of the natural drugs used in this first experimentation can be useful as oral antimicrobial agents. Despite the fact that liposomal encapsulation is suggested in delivering effective plant compounds, our results should say that it depends on the phytocomplex and on the chemical characteristics of the carrier. Also, it would be supposed that association of natural drugs may led to better results in activity against oral commensal, cariogenic bacteria and in the case of infection by *Candida albicans*. Further studies are needed to characterize and increase the mechanisms of delivering of antimicrobials in planktonic cell culture and in structured oral biofilm.

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